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THE EFFECTS OF DIETARY β -GUANIDINOPROPIONIC ACID ON GROWTH AND MUSCLE FIBER DEVELOPMENT IN JUVENILE RED PORGY, *PAGRUS PAGRUS*

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Abstract

β -guanidinopropionic acid (β -GPA) has been used in mammalian models to reduce intracellular phosphocreatine (PCr) concentration, which in turn lowers the energetic state of cells. This leads to changes in signaling pathways that attempt to re-establish energetic homeostasis. Changes in those pathways elicit effects similar to those of exercise such as changes in body and muscle growth, metabolism, endurance and health. Generally, exercise effects are beneficial to fish health and aquaculture, but inducing exercise in fishes can be impractical. Therefore, this study evaluated the potential use of supplemental β -GPA to induce exercise-like effects in a rapidly growing juvenile teleost, the red porgy (*Pagrus pagrus*). We demonstrate for the first time that β -GPA can be transported into teleost muscle fibers and is phosphorylated, and that this perturbs the intracellular energetic state of the cells, although to a lesser degree than typically seen in mammals. β -GPA did not affect whole animal growth, nor did it influence skeletal muscle fiber size or myonuclear recruitment. There was, however, an increase in mitochondrial volume within myofibers in treated fish. GC/MS metabolomic analysis revealed shifts in amino acid composition of the musculature, putatively reflecting increases in connective tissue and decreases in protein synthesis that are associated with β -GPA treatment. These results suggest that β -GPA modestly affects fish muscle in a manner similar to that observed in mammals, and that β -GPA may have application to aquaculture by providing a more practical means of generating some of the beneficial effects of exercise in fishes.

Keywords

β -GUANIDINOPROPIONIC ACID; SKELETAL MUSCLE; GROWTH; RED PORGY; CREATINE

1. INTRODUCTION

Early growth rate and muscle quality are key considerations in aquaculture of fishes, and it is important to understand the interaction of growth rate with the qualities that enhance muscle as a product. It is generally established that moderate exercise in fishes can enhance muscle quality (Bugeon et al. 2003; Rasmussen et al. 2011), though it can be logistically difficult to implement. While some aspects of whole body and muscle cell growth differs between mammals and fishes, their regulatory pathways are largely homologous, and exercise has been shown to induce similar effects in both. Therefore, the aims of this study were to investigate (1) if an often used supplement that targets pathways associated with exercise in mammals (β -guanidinopropionic acid or β -GPA) can also be taken up in muscle fibers of a marine fish, and (2) whether β -GPA induces beneficial exercise-like effects in fish muscle that are similar to those found in mammals.

β -GPA is a creatine (Cr) analog that acts as a competitive agonist to Cr for binding to the creatine transporter (Cr_T) (Daly and Seifter 1980; Clark et al. 1994). Cr is a non-essential dietary molecule that is consumed in the diet and synthesized in the liver and pancreas (reviewed by Balsom et al. 1994). It must be transported into skeletal muscle cells via the Cr_T (Snow and Murphy 2001). In resting muscle, ATP and Cr are used by creatine kinase (CK) to produce phosphocreatine (PCr) and ADP (Wallimann et al. 1992). During the elevated energetic demand associated with exercise, ATP is consumed, and the phosphate on PCr is transferred to ADP by CK in order to regenerate ATP (Wallimann et al. 1998). Due to β -GPA's competitive antagonistic relationship with Cr for the Cr_T , chronic exposure causes increased intracellular [β -GPA] while [Cr] and [PCr] decrease (Clark et al. 1994). Within the cell, β -GPA, like Cr, is phosphorylated (P-GPA) by creatine kinase (CK) (Unitt et al. 1993). In this capacity P-GPA acts to replace the PCr pool. However, P-GPA donates its phosphate group to ADP at a rate 100 times slower than PCr, compromising the cell's capacity to replenish ATP during rapid changes in ATP demand (Chevli and Fitch 1979). The impairment of the Cr system's ability to temporally buffer ATP in fast-twitch glycolytic skeletal muscle promotes increased AMP concentration, which is a cellular signal of energy stress that leads to many effects in muscle, such as an increase in aerobic capacity (Freyssen et al. 1995; Bergeron et al. 2001; Zong et al. 2002; Fediuc et al. 2006). Thus, while the mechanisms are different, both exercise and β -GPA treatment lead to elevations in AMP concentration, which induces training-like effects in skeletal muscle that may be useful in fish aquaculture.

Fish muscle fiber density appears to be positively correlated with subjective indicators of texture quality such as firmness and cohesiveness due to a higher surface area to volume ratio and an increase in collagenous connective tissues (Penago et al. 2005). In fishes, muscle mass is increased during growth by both fiber hypertrophy (increases in the size of existing fibers) and hyperplasia (increases in the number of fibers) until the fish reaches about half of the maximal body length, after which fibers grow largely by hypertrophy (Alami-Durante et al. 1997; Johnston et al. 2009). Exercise not only enhances textural qualities (Bugeon et al. 2003) and lipid profiles (Rasmussen et al. 2011), but also increases growth rate, growth period, survival rates, immune status, reproductive control and general health by reducing stress levels (Palstra and Planas 2011). Growth hormone expression,

growth rate, and conversion efficiency have been shown to be increased by chronic exercise at intermediate swimming speeds (Davison and Goldspink 1977; Walker and Emerson 1978; East and Magnan 1987; Christiansen and Jobling 1990). In addition, intermittent and burst swimming exercise in the natural environment has been found to cause white fibers to undergo hyperplasia sooner leading to an increase in cellular density (Johnston and Moon 1980). In mammals, exercise has been shown to increase polyunsaturated fatty acids, as well as the unsaturated fatty acid:saturated fatty acid ratio (Mataix et al. 1998; Andersson et al. 2000; Dobrzyń and Górski 2002).

Many of these effects of exercise and β -GPA treatment can be linked to two opposing regulatory proteins: AMP-activated kinase (AMPK) and the mechanistic target of rapamycin complex 1 (mTORC1), both of which are well studied in mammals. AMPK has a clearly defined role of re-establishing energetic homeostasis during energetic stresses. It is activated when the energetic demand of muscle is elevated, such as during exercise (reviewed by Young 2009). In mammalian muscle, AMPK activation leads to mitochondrial proliferation (Wu et al. 1999; Terada et al. 2002). It also stimulates catabolic processes leading to glucose, amino acid, and lipid degradation (Yamauchi et al. 2002; Rutter et al. 2003; Nakashima and Yakabe 2007). AMPK simultaneously restricts anabolic processes (Yamauchi et al. 2002; Bolster et al. 2002; Krawiec et al. 2007; Henin et al. 1995), resulting in muscle atrophy after sustained periods of activation (Tong et al. 2009). The strong inhibitory effect of AMPK on protein synthesis is primarily mediated through deactivation of mTORC1 (Garami et al. 2003; Aguilar et al. 2007; Tong et al. 2009). When active, mTORC1 strongly promotes muscle growth and hypertrophy (Bodine et al. 2001) as well as lipid biosynthesis (reviewed by Laplante and Sabatini 2009). Thus, maximal AMPK activation might be expected to alter muscle quality by making it more aerobic, while inhibiting growth through effects on mTORC1 and altering the intracellular metabolite concentrations.

During hypertrophic growth, such as when mTORC1 is activated, skeletal muscle relies on the fusion of mono-nucleated satellite cells found in the extracellular matrix with existing muscle fibers (Moss and Leblond 1970). This leads to multinucleated fibers where each nucleus serves a volume of cell known as the myonuclear domain (MND) (Landing et al. 1974; Hall and Ralston 1989; Jimenez et al. 2012), and it is generally accepted that myonuclear content increases during hypertrophy (Enesco and Puddy 1964; Adams et al. 1998; Roy et al. 1999; Bruusgaard et al. 2010; van der Meer et al. 2011; Jimenez et al. 2012). Changes in myonuclear content have been shown to be influenced by both mechanical loading (McCall et al. 1998; Adams et al. 1999; Roy et al. 1999; Bruusgaard et al. 2010) and neural stimulation (Ohira 1989; Schmalbruch et al. 2000; Kawano et al. 2007; van der Meer et al. 2011), suggesting that exercise can alter satellite cell fusion. However, there is some evidence that in cultured mammalian muscle cells, AMPK activation limits satellite cell activity and therefore nuclear accretion during growth (Williamson et al. 2009; Miyake et al. 2012).

AMPK activation in mammals due to either metabolite intervention (e.g. β -GPA, AICAR) or exercise has also been shown to cause metabolic shifts such as decreased glycolytic enzyme activities, increased mitochondrial enzymes, and increased lipid oxidation (Merrill et al. 1997; Winder 1998; Rasmussen et al. 1999; Peterson et al. 2012; Oudman et al. 2013),

whereas some studies of exercise effects have shown increased citric acid cycle intermediates (reviewed by Bowtell et al. 2007) and branched-chain amino acid oxidation (Knapik et al. 1991; reviewed by Rennie and Tipton 2000). Therefore, chronic AMPK activation in fish due to β -GPA supplementation may be expected to alter the metabolome as well.

In the present study we examined the effects of β -GPA treatment on whole-body growth and skeletal muscle growth and metabolism in red porgy, *Pagrus pagrus*. We measured the effects of β -GPA on the energy state and metabolome of skeletal muscle, as well as muscle fiber growth, mitochondrial density, and MND. Because it is suitable for human consumption, β -GPA is an inexpensive dietary supplement, making it a potential alternative to exercise as a means of improving muscle quality in a teleost.

2. METHODS

2.1 Animal maintenance

Forty juvenile red porgy (*Pagrus pagrus*; ~20 grams, 65mm) of a single cohort were acquired from the University of North Carolina Wilmington Aquaculture facility (Wilmington, NC). All study procedures adhered to the UNCW Institutional Animal Care and Use guidelines. Fish were separated into 4 groups of 10, each in a 125-gallon tank. Tanks were exposed to a 10-hour light:14-hour dark photoperiod and temperature was maintained at 22–25°C. Temperature and water quality were measured every other day. The fish were given an initial two-week period to acclimate to their new conditions, and during this time all fish were given a standard control diet that contained 5% α -cellulose by weight (Table 1). At day zero, two groups were randomly selected and continued on the control diet while the other two began the treatment diet that contained 5% β -GPA by weight that was substituted for α -cellulose. Fish were fed twice daily, morning and afternoon, until satiated. For each group, food was weighed before and after each feeding to provide consumption rates and any excess food left in the tanks was siphoned off, blot-dried, weighed, and subtracted from the amount of food fed. Feed conversion efficiencies were calculated as a group-wide average of the ratio of total grams gained to the total grams fed, expressed as a percent (gained/fed*100). Two of the tanks were designated for a 4-week experiment, and two of the tanks were used for a 12-week experiment. Studies on the effects of β -GPA in mammalian models have focused on rats and mice, using 1–2.5% β -GPA dietary supplementation for an average of 6 weeks (Oudman et al. 2013). However, due to the low metabolic rates and low perfusion of fish white muscle (Mosse et al. 1978), we chose a higher dosage and a longer duration than is typical for mammalian studies.

2.2 Sampling

At day 0 and week 6 (for the 12-week treatment), fish were starved for 24 h, anesthetized with a sub-lethal dose (0.1g/L) of MS-222 (tricaine methanesulfonate, Argent Laboratories, Redmond, WA, USA), which was administered without disturbing the fish, and the fish were weighed. At week 4 or 12, on the night prior to sampling, fish were retrieved from their tanks and put into individual small, dark, enclosed containers with aerated seawater overnight to recover from handling stress. The next day, fish were again anesthetized and

final wet weight and length were measured, after which fish were placed in a small tank containing a lethal dose (0.5g/L) of MS-222. It was necessary to sacrifice all 10 of the fish in two of the tanks at 4 weeks, rather than subsampling 5 fish from all 4 tanks at this time point in order to maintain adequate school size for these highly social juvenile fish. This resulted in only two tanks (control and treatment) remaining for the 12-week experiment, which introduces the potential for tank effects. However, as discussed below, the results of this study suggest that there were no tank effects that confounded our findings. Once euthanized, rectangular epaxial muscle sections were removed parallel to the long axis of the fibers. Samples harvested from the right side were placed in 4% formaldehyde in Sörensen's phosphate buffered saline (PBS, pH 7.38) for later microscopic analysis. Samples harvested from the left side were immediately placed in a cryovial and frozen in liquid nitrogen, then stored at -80°C for metabolomics analysis. A portion (~ 0.1 g) of left or right epaxial muscle for five fish of both groups was used for nuclear magnetic resonance (NMR) spectroscopy.

Tissues were harvested for molecular and microscopic analyses at both the 4- and 12-week end points. However, as described below there was no effect of β -GPA on growth or conversion efficiency after 4, 6 or 12 weeks, and there was very limited loading of β -GPA into skeletal muscle tissue after 12 weeks. For these reasons, we did not perform the costly and time consuming molecular and microscopic analyses on the tissues harvested at 4 weeks, since our findings at 12 weeks suggest that this would not have provided additional insights to this study. We do, however, report the growth and conversion efficiency data for the 4-week time point.

2.3 Nuclear magnetic resonance

^{31}P -NMR was used to evaluate whether β -GPA can be transported into fish muscle fibers and be phosphorylated, and to examine the effect that phosphorylated β -GPA has on the intracellular high energy phosphates (HEPs). To do this, 5 fish from each group were selected randomly and ~ 0.1 g of muscle tissue was excised immediately postmortem, freeze clamped in liquid N_2 , and weighed. Sample preparation followed that of Ross et al. (2017). Once prepared, the supernatant was immediately analyzed by ^{31}P -NMR spectroscopy, since HEPs can be degraded over time. Supernatants were transferred to a 5 mm NMR tube and ^{31}P -NMR spectra were collected at 162 MHz on a Bruker 400 MHz DMX spectrometer. 750 scans were collected for each spectrum using a 45° excitation pulse with a 0.6 s relaxation delay, resulting in a total acquisition time of 7.5 min. The relative peak areas of α -, β -, and γ -ATP, PCr, inorganic P (P_i), and P-GPA were integrated using Xwin-NMR software (Fig. 1B). Fractional peak area for each compound was calculated as a percent of the total peak area for each specimen. A one-tail Student's t -test was used to test for a significantly reduced fractional peak area of HEPs, as well as a significantly increased fractional peak area of P-GPA in treatment groups.

2.4 Metabolomic analysis

For polar metabolite extraction, 200 mg of muscle tissue from each animal was placed in a microcentrifuge tube with 400 μL of methanol and thoroughly homogenized and sonicated. Samples were agitated for 1 h at room temperature then centrifuged at $13,000\times g$ for 10 min

at room temperature. Supernatants were collected and added to new 2 mL microcentrifuge tubes. The supernatants were vacuum centrifuged at 65°C for 105 min until dry. The remaining pellet was stored at -80°C until further analysis. Immediately prior to analysis, the pellets were reconstituted in 30 μ L anhydrous pyridine (Sigma, cat. 270970) and derivatized with 40 μ L N-Methyl-N- (trimethylsilyl) trifluoroacetamide (MSTFA) (Regis, cat. 270590) for 1 h at 60°C. Analysis was performed using a Pegasus 4D gas chromatography/gas chromatography time-of-flight mass spectrometer (GC $\times$ GC/TOF-MS, LECO Corporation, St. Joseph, MI). The GC column was a non-polar DB-5 capillary column (J&W Scientific, 30 m x 0.25 mm x 0.25 μ m). High purity helium was used for the carrier gas (1.2 ml/min). An injection volume of 2 μ L was used, with a 20:1 split ratio. The temperature program began at 80°C with a hold time of 0.5 min and then increased at a rate of 10°C/min to 335°C with a hold time of 5 min. The injection inlet temperature was 280°C and mass spectrometer transfer line was held at 250°C. The electron impact (EI) ion source was held at 200°C, with a filament bias of -70 V. Mass spectra were collected from 50 to 600 m/z at 30 spectra/sec. LECO Corporation ChromaTOF software was used to compare MS spectra of eluted metabolites to that of the NIST MS database to identify compounds of each peak. Similarity values (SV) were given between 0 and 999 based on the sample peak spectrum compared to that of the NIST database hit. Those identified with a SV of $\geq$ 700 were taken as correctly identified and named with the corresponding library metabolite. Final metabolite concentrations were normalized with respect to the weight (g) of tissue used for each sample.

Standard curves for creatine were generated using seven concentrations in the range of 4 – 900 μ g/mL. It was observed that the derivatization process converts creatine to creatinine consistent with previous reports (Shoemaker and Elliott 1991). Therefore, the concentration of creatine was derived from the summation of both the creatine and creatinine peaks. The creatinine derivative peak had a retention time of 593 seconds, and was quantified using the fragment mass at 115 Da. Standard curves for β -GPA were generated using seven concentrations in the range of 4 – 900 μ g/mL. The β -GPA derivative peak had a retention time of 313 seconds, and was quantified using the fragment mass at 102 Da.

In total, 284 metabolites were detected in polar extracts. Of these, 124 were unidentified and excluded from further analysis. Metabolites detected in <4 individuals in either group were removed as well. The final dataset consisted of 83 metabolites. Of these remaining metabolites, missing values constituted $\sim$ 12%. Random Forest technique was utilized to impute missing values as suggested by Stekhoven and Buhlmann (2012) and Gromski et al. (2014) using the “missForest” package under CRAN in R-studio. This method utilizes the observed data to predict the missing values using a bootstrapped sampling method, and has been shown to be superior to other types of missing value substitutes such as replacement with zero, replacement with the detection limit, or estimations with k -nearest neighbors (see Gromski et al. 2014). Bray-Curtis similarity measures were used to calculate a resemblance matrix of the metabolite profiles in PRIMER 7.0 (Primer-E, Auckland, New Zealand) and a permutational multivariate analysis of variance (PERMANOVA+) was used to test whether metabolite arrays were significantly different between control and β -GPA-treated groups. PERMANOVA+ was calculated using Type III SS, with the treatment as a fixed effect, and the model was based on a Monte Carlo approach. Non-metric multidimensional scaling

(nMDS) was used to visualize individuals in 2-D space. Vectors (corresponding to variables) explaining the most variability in the data (correlation >0.2) were identified and plotted.

2.5 Confocal microscopy

Six size matched specimens from each group were used to determine fiber size. Laser scanning confocal microscopy was used to image muscle fibers following the methods described by Hardy et al. (2009) and Priester et al. (2011). Alexa Fluor 594-labeled wheat germ agglutinin (WGA; Life Technologies, Grand Island, NY, USA) was used to identify cell membranes, and 4'-6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR, USA) to label nuclei. Sections were mounted on slides using a 1:9 solution of tris:glycerol and coverslipped.

Fiber cross-sections were viewed on an Olympus FV1000 laser scanning confocal microscope (Olympus, Center Valley, PA, USA). Images were collected under a 20× objective lens using three channels: differential interference contrast (DIC), 594 nm wavelength (WGA-Alexa Fluor 594; fiber periphery), and 405 nm wavelength (DAPI; nuclei). Ten-μm thick stacks were collected via 10 1μm thick optical slices and viewed using Olympus Fluoview v2.0 software. Approximately 150 skeletal muscle cells were analyzed for each specimen ($n = 117 - 209$) (Ceglia et al. 2013). To determine the area and mean diameter of each muscle fiber, an Intuos 3 tablet (Wacom Co., Ltd, Vancouver, WA, USA) was used to trace the outline of each fiber in Adobe Photoshop v. 7.0 (Adobe Systems Inc., San Jose, CA, USA). From the outlined fibers, ImagePro Plus v. 6.1.0.346 (MediaCybernetics Inc., Bethesda, MD, USA) was used to calculate the dimensions of each muscle fiber.

Cells that were measured for fiber area and diameter were also analyzed for the nuclear density. Muscle fibers are multi-nucleated cells and the number of nuclei increases with fiber growth and reflects past satellite cell fusion events. Nuclei near each cell were categorized as either extracellular or intracellular based on their position relative to the WGA stained fiber periphery, and intracellular nuclei were evaluated. The nuclear density (# nuclei/mm fiber) for each group was non-normally distributed (right-skewed; Shapiro-Wilk, $p < 0.01$). Therefore, the number of nuclei/mm fiber between the groups was tested with a Mann-Whitney U -test.

Myonuclear domain (MND), which is the volume of cell serviced by each nucleus, was calculated using the formula from Schmalbruch and Hellhammer (1977) as previously described by Priester et al. (2011). The optical thickness was 1 μm with an estimated nuclear length of 9.0 μm, which was found to be the average nuclear length of 17 teleosts in a study from our lab by Jimenez et al. (2012; nuclear length data unpublished).

The effect of treatment on mean fiber size and MND was tested using a full factorial design analysis of covariance (ANCOVA) using body mass and fiber size as covariates, respectively, since both fiber size and MND are altered during animal growth. Since there was no significant interactions for treatment and body mass for fiber size or treatment and fiber size for MND, the interaction term was removed for the final analysis.

2.6 Transmission electron microscopy

Mitochondrial volume density was assessed for three specimens per group, chosen randomly. Fibers from the right side of the fish were removed from 4% formaldehyde and placed in 2.5% glutaraldehyde for >24hr. These samples were then cut and mounted on a single Formvar-coated high-transmission copper grid using the protocols described by Johnson et al. (2004) and Nyack et al. (2007). Five 90-nm sections were placed on each grid, then 1 μm of tissue was skipped to ensure a new population of mitochondria, and the process was repeated four more times, yielding five grids per specimen. Grids were stained with 2% uranyl acetate in 50% ethyl alcohol followed by Reynolds' lead citrate. Grids for each specimen were randomly chosen and examined on a Tecnai G2 Spirit BioTWIN TEM. A starting point was randomly chosen at low magnification for each grid. Magnification was increased to 1200 $\times$ and any area of muscle sample found in the field of view was imaged. To create the images, a montage of the sample found within the field of view at 1200 $\times$ was made by imaging the area at 5000 $\times$ with 15% overlap per micrograph, resulting in a maximum of 25 micrographs per montage. After collecting a montage, or if no muscle sample was found in the field of view, a systematic random technique was followed until a section of muscle was found. This was done for three montages per grid, then another grid was randomly selected and the process was repeated, resulting in six montages with high resolution comprised of a total of ~100–120 smaller micrographs per specimen (Fig. 5).

Intermyofibrillar (IM) mitochondrial volume density, which is determined for mitochondria that are not associated with the fiber sarcolemmal membrane, was estimated by the point-counting method described previously (Howard and Reed 1998; Nyack et al. 2007) using ImagePro Plus v.6.1.0.346 (Media Cybernetics Inc., Bethesda, MD, USA). Subsarcolemmal (SS) mitochondrial volume density, which is determined for mitochondria clustered at the fiber periphery, was determined by measuring the length of the muscle fiber sarcolemma in each micrograph, and then outlining all SS mitochondria found along the intact sarcolemma in Adobe Photoshop v.7.0 (Adobe Systems, Inc., San Jose, CA, USA). SS mitochondria were then analyzed for area in ImagePro Plus. The resulting SS mitochondrial volume (μm^3) per sarcolemmal surface area (μm^2) was multiplied by the average surface area (μm^2) per fiber volume (μm^3) of the specimen found through light microscopy (see above) to yield SS mitochondrial volume density. Micrographs were considered as pseudoreplicates and were therefore averaged to obtain a mean value for each of the three specimens for both groups. These values were then compared between control and treated groups using a Student's t -test ($n=3$).

3. RESULTS

3.1 β -GPA effects on muscle energy state

GC $\times$ GC/ToF-MS detected Cr and β -GPA, providing evidence that β -GPA is transported into the skeletal muscle cells of a teleost fish (Fig. 1A). After 12 weeks of β -GPA supplementation, cellular Cr concentrations were reduced by 30% compared to control levels, while β -GPA was at a concentration that was 31% of the control group's mean Cr concentration. This resulted in the sum of the total concentration of Cr + β -GPA in treated fish changing by only +1% compared to the total Cr concentration in controls, and suggests

that β -GPA functions as a competitive agonist for the Cr_T in fishes, as is the case in mammals.

^{31}P -NMR spectroscopy also confirmed that β -GPA was found in skeletal muscle cells of red porgy and is phosphorylated by a teleost form of CK to form P-GPA (Fig. 1B). This evidence supports the notion that β -GPA has an affinity for the Cr_T and is phosphorylated by CK in fishes as it is in mammals. The P-GPA to ATP- β ratio in the five treated specimens ranged from 0 to 1.11 (there was no detectable P-GPA in one treated specimen), while no P-GPA peak was found in control animals. The fractional PCr and ATP- β (i.e. the PCr peak area/total peak area and ATP- β peak area/total peak area) were significantly lower by 27% ($n=5$, $p=0.01$) and 26% ($n=5$, $p=0.03$) in the treated group (Fig. 1C), indicating that β -GPA leads to lower intracellular high energy phosphate (HEP) levels and therefore a lower cellular energy state. Though one β -GPA treated individual showed no detectable level of intracellular P-GPA, this individual showed unphosphorylated β -GPA within its musculature (GCxGC/TOF-MS) and had relative peak areas of PCr and ATP- β that were in line with other β -GPA treated individuals.

3.2 Metabolomic analysis

Metabolite profiles were significantly different between control and treated groups (PERMANOVA+, Pseudo- $F=4.4$, $p=0.01$). Of the 83 metabolites that were analyzed, eight contributed most strongly to the variation between control and treatment groups, and the control and β -GPA treated groups separated well along MDS1 (Fig. 2). Separation of the β -GPA treated individuals along MDS1 was explained most strongly by higher proline concentration which was negatively correlated with glycine concentration. Higher hydroxyproline and galactose were also associated with higher proline and negatively correlated with alanine and glycine. Individuals also separated well along MDS2, which was largely explained by different concentrations of lysine and urea, which were negatively correlating with lactic acid concentration. Clusters within each treatment (four individuals from the control group, five from the treated) appear to separate along MDS2 as well, however the remaining three individuals do not conform to this pattern and therefore the centroids of each group do not appear to differ along MDS2. The β -GPA treated individual that showed no intracellular P-GPA is found within the cluster of other β -GPA treated individuals in the upper left of Fig. 2, reflecting metabolite changes indicative of β -GPA treatment. Therefore, the reason as to why there was no detectable P-GPA in this individual is unclear.

3.3 Body mass and skeletal fiber size

Body mass was not significantly different between groups at the start of the experiment (ANOVA, $F=0.24$, $p=0.87$). After four weeks of β -GPA treatment, the first groups sampled showed no significant difference in growth (data not shown; Student's T -test, $n=10$, $p=0.77$). Over this period, tank-wide averages of food consumption (0.57 g/fish/day in the control group, 0.56 g/fish/day in the β -GPA treated group) and conversion efficiencies (60.46% in the control group, 60.52% in the β -GPA treated group) were very similar between groups. In the 12-week experiment, consumption per fish was 22% lower in the β -GPA treated group (0.45 g/fish/day) than the control group (0.59 g/fish/day) after the first 6 weeks of β -GPA

treatment but conversion efficiencies were very similar (76.8% in controls versus 78.6% in β -GPA treated fish) and any difference in body mass between the two groups was highly insignificant ($n_{\text{Control}}=10$, $n_{\beta\text{-GPA}}=8$, $p=0.77$). By the end of the 12-week experiment, consumption rates continued on a similar trajectory, with 25% lower consumption in the β -GPA treated group (0.38 g/fish/day) than the control group (0.50 g/fish/day). At this point, though, conversion efficiency could not be accurately calculated due to the sudden death of one treated fish and a partial cannibalistic event, leading to an impossibly high (>100%) conversion efficiency. However, growth was still not significantly different ($n_{\text{Control}}=9$, $n_{\beta\text{-GPA}}=7$, $p=0.84$, Fig. 3).

Representative images of muscle fiber cross-sections collected using confocal light microscopy, which were used to quantify nuclear content and fiber CSA, are presented in Fig. 4. Representative images of muscle fiber cross-sections collected using TEM, which were used to quantify mitochondrial content, are presented in Fig. 5. In initial ANCOVA, there was no significant interaction between body mass and treatment on fiber size ($F=2.7$, $p=0.14$), so the interaction term was not evaluated. ANCOVA determined that there was no significant effect of treatment ($F=2.7$, $p=0.13$; Fig. 6) or body mass ($F=3.8$, $p=0.08$) on fiber diameter, although there was a non-significant increase of fiber size with body mass as would be expected.

3.4 Mitochondrial and nuclear densities

We imaged and evaluated a total of $4418 \mu\text{m}^2 - 7873 \mu\text{m}^2$ of fiber cross sectional area per fish to determine mitochondrial density, an average of 2.3 fiber cross sections. Mean SS mitochondrial volume density was significantly higher by 64% in treated groups compared to controls ($n=3$, $p=0.02$). Mean IM and total mitochondrial volume densities were also higher by 48% and 33% in treated groups, but neither were significantly different ($n=3$, $p=0.40$; $n=3$, $p=0.10$, respectively; Fig. 7A).

There was no significant difference between groups in nuclei/mm of fiber (Mann-Whitney U -test, $n=6$, $\chi^2=0.93$, $p=0.34$, Fig. 7B). ANCOVA revealed that there was not a significant interaction between fiber CSA and treatment on the nuclear density ($F=0.03$, $p=0.86$), so the interaction term was removed. There was also no significant relationship between nuclear content and fiber CSA in either group (data not shown), which is consistent with a significant positive correlation of MND with fiber CSA (Fig. 7C) for both control and treated groups (ANCOVA, $n=12$, $F=18.1$, $p=0.002$). However, ANCOVA revealed that there was no significant effect of treatment on MND, using fiber CSA as a covariate ($F=1.3$, $p=0.27$).

4. DISCUSSION

4.1 β -GPA was transported into fish muscle tissue and altered the cellular energy state

This study is the first to investigate the effects of β -GPA in a teleost. Through metabolomic analysis we demonstrated for the first time that β -GPA can be transported into teleost muscle fibers. Because delivery was through the diet, this also implies that β -GPA is successfully transported via the teleost Cr_T across the epithelium of the gut, into the blood, and across the

sarcolemma of muscle cells (Boehm et al. 2002; Tosco et al. 2004; Peral et al. 2005). Using ^{31}P -NMR we also showed that β -GPA can be phosphorylated intracellularly, putatively by the teleost CK, and that this reduces the intracellular ATP and PCr concentrations, and therefore lowers the cellular energy state. Though β -GPA supplementation in mammals typically replaces nearly all of the intracellular PCr after 5–7 weeks (reviewed by Oudman et al. 2013), in red porgy it caused a significant but more modest 27% and 26% decrease in PCr and ATP- β , respectively, after 12 weeks (Fig. 1).

The difference in β -GPA influx in fishes may be the result of a number of factors controlling Cr transport in fishes. For instance, fish white muscle has a very low aerobic capacity and is poorly perfused (Nyack et al., 2007), which may limit β -GPA delivery to muscle. There also could be differences in the Cr_T affinity for β -GPA due to non-homology between mammalian models and red porgy, which could reduce the efficiency with which it is transported into the cell. The Cr_T in zebrafish (*Danio rerio*) is distinct from that in mammals, which is highly conserved among species as diverse as rat, mouse, dog, rabbit, cow, and human (Wang et al. 2007). However, there is no direct data on the affinity of β -GPA for the Cr_T in a teleost model.

Another possible reason for relatively low loading of β -GPA in muscle is the expression of the Cr_T in the gut (Tosco et al. 2004; Peral et al. 2005). In rats, the rate of Cr uptake in the ileum and jejunum was shown to vary during ontogeny, with changes seemingly based on both dietary Cr content and genetic programming (Peral et al. 2005). Dietary shifts in red porgy may have implications for creatine transport as well. Elmor (2012) reported that the natural diet of adult red porgy contains 0 – 17.4% fish parts. However, red porgy in a natural setting that were the same size as the ones in this study have a diet containing 0 – 2% fish parts, while the vast majority of the diet was crustaceans, mollusks, polychaetes, and seagrasses. Aside from polychaetes, these prey items utilize the arginine kinase system to buffer intracellular ATP concentration rather than the creatine kinase system (Ellington and Suzuki, 2006), and therefore at this age red porgy may not be naturally consuming large amounts of Cr. If this is the case, these may be genetically programmed to have low Cr_T expression levels in the gut, which would result in reduced β -GPA uptake by the small intestine for this species.

4.2 β -GPA increased fish muscle aerobic capacity and altered the metabolite profile

β -GPA alters many aspects of the metabolism and energetic homeostasis in mammalian muscle cells. A hallmark of β -GPA treatment is mitochondrial proliferation (Bergeron et al. 2001) and enhanced mitochondrial oxidative enzyme expression (Freyssenet et al. 1994; Freyssenet et al. 1995). This is associated with increased glucose transporter (GLUT-4) expression (Pandke et al. 2008) and increased glycogen content (Shoubridge et al. 1985; Ohira et al. 1994), resulting in an increase in glucose tolerance in muscle cells (Ohira et al. 1994). Further, an increase in the fatty acid transporter FAT/CD36 located on the outer mitochondrial membrane has been observed (Pandke et al. 2008; Smith et al. 2011). Thus, chronic treatment with β -GPA causes a shift toward increased oxidative metabolism of both carbohydrates and lipids.

The most notable metabolite differences between the groups in the present study were amino acids proline, alanine, and glycine. There were further differences in hydroxyproline and galactose (Fig. 2). Though exercise can induce changes in key lipids of rainbow trout (Rasmussen et al. 2011), we found no evidence of changes in the lipid profiles within the treated group. This was surprising considering that we observed an increase in mitochondrial volume density, which is an expected response to AMPK activation via β -GPA treatment, and this dramatically alters muscle metabolic profiles in mammals (Martinez-Martin et al. 2012). However, a lack of changes in the lipid profile as well as TCA cycle intermediates is consistent with the very low mitochondrial volume density found in fish white muscle, even in the presence of β -GPA, and suggests that white muscle has a limited capacity for upregulating aerobic capacity (Nyack et al., 2007; Burpee et al., 2010). Fish white muscle may therefore simply lack the developmental plasticity found in mammals, which may limit the capacity for metabolic remodeling.

AMPK activation can also inhibit protein synthesis and enhance protein degradation (Yamauchi et al. 2002; Bolster et al. 2002; Krawiec et al. 2007; Henin et al. 1995), and we therefore predicted a reduction in growth in β -GPA treated individuals, but we did not find evidence of this (Fig. 3). However, alanine, an insulinotropic amino acid (Andoh 2007) that can stimulate protein synthesis and cell growth through the PI3P pathway (Fox et al. 1998; Nobukuni et al. 2005), was found to be higher in control individuals. Therefore, there may still be some inhibition of protein synthesis, but it was not apparent in growth rate or muscle tissue growth. Urea, which was higher in a cluster of β -GPA treated fish than in a cluster of controls, suggests increased protein degradation rates in those individuals. A decrease in protein degradation is again an expected effect of AMPK activation, although this did not clearly separate the treatment groups and so we interpret this with caution.

Glycine was much more prevalent in control groups. While glycine plays many roles in metabolism, such as heme and purine synthesis, in the present context it is likely more important to consider that it is consumed by the L-arginine:glycine aminotransferase (AGAT) in the production of Cr. Therefore, it is feasible that it is being consumed in the attempt to produce more Cr to make up for the deficiency in PCr buffering in treated individuals. While GC $\times$ GC/TOF-MS of skeletal muscle tissue did not show increased [Cr] in treated fish, it may be that β -GPA's agonistic binding to the Cr_T is preventing excess Cr from entering the muscle fibers. However, our methods are not able to distinguish whether there was increased Cr production in the liver of treated individuals.

Proline and hydroxyproline make up a large portion of the collagenous tissue found in the extracellular matrix. Higher levels of these two amino acids were associated with β -GPA treatment (Fig. 2), suggesting an increase in the production and formation of collagenous extracellular tissue. This result is consistent with exercise-induced increases in connective tissue that improve tissue texture (Bugeon et al. 2003) and suggests, again, that there may be some exercise-like responses to β -GPA treatment in fish. However, glycine is also a major component of collagen, and so the relatively higher glycine levels in control animals is somewhat perplexing.

4.3 β -GPA did not alter fish growth or muscle fiber size

There was not a significant difference in mean body mass or muscle fiber size after 12 weeks of 5% β -GPA dietary supplementation in red porgy. Based on studies in mammalian models, β -GPA supplementation would be expected to reduce body mass over a few weeks (reviewed by Oudman et al. 2013; Baumgarner et al. 2015; Ross et al. 2017). It is possible that no significant difference was detected due to the relatively low level of β -GPA loading in fish muscle tissue compared to that typically seen in mammals. It is also possible that the results are confounded by tank effects, since we only had one control and one treatment tank for the 12-week part of the study. However, we do not believe this to be the case for two reasons. First, we found largely negative results that are consistent with the low β -GPA loading into tissue, which suggests that control and treatment fish did not experience tank-specific differences in environment. Second, in the pair of tanks that were sampled at 4 weeks, we found a lack of effect of β -GPA on either growth or conversion efficiency similar to that seen in the pair of tanks sampled at 12 weeks. Thus, while we cannot rule out a tank effect, we think it is unlikely to be a substantial confounding variable.

Body mass typically correlates with fiber CSA (Jimenez et al. 2013). However, despite a 2.5-fold range in body mass between individuals, there was no significant correlation of body mass with fiber CSA ($p=0.08$, Fig. 6). This implies that muscle growth at this stage may still be largely reliant on hyperplasia. This is in concordance with a study by Johnston et al. (2009), which suggested that early muscle growth is through a combination of hyperplasia and hypertrophy until the fish reaches approximately 50% of its final body length. Further, β -GPA treated fish had larger fibers than control fish, but this was also not significant ($p=0.13$, Fig. 6). The lack of significant effects suggests that there is no effect of β -GPA treatment on either body or muscle fiber size. However, by the end of the study, the largest individuals were approaching 40% of the maximum body length found in a natural population of red porgy off the coast of North Carolina (Klibansky and Scharf 2013). At these larger body sizes, there appears to be more separation between control and β -GPA treated individuals. Potentially, this may suggest that β -GPA results in an earlier reliance on fiber hypertrophy during growth compared to controls (i.e. before 50% of final body size), and that the low but non-significant p -values may therefore reflect a low n and inappropriate range of body sizes to address this question. Further study would be needed to address this potential pattern, but the ability to manipulate muscle growth patterns with β -GPA may have application to aquaculture, particularly since β -GPA did not inhibit growth.

Aside from changes in fiber density, our results also showed differences in mitochondrial density. There was a significantly higher SS mitochondrial volume density in treated fish (Fig. 7), consistent with previous reports from mammals. The mean IM and total mitochondrial volume densities were also higher following β -GPA treatment but were not significantly different. Howald et al. (1985) noted that, in humans, SS mitochondrial content had a larger percent change in response to exercise relative to IM mitochondrial content, but that both are significantly elevated. Therefore, significant increases in SS mitochondrial volume density is consistent with an exercise-like response in fish treated with β -GPA. Insignificant changes in IM mitochondrial volume may be due to either differences in muscle physiology of red porgy compared to humans or a low sample size ($n=3$). The

increase in SS mitochondrial volume density does suggest a direct link between the known effects of β -GPA supplementation on cellular energy state via activation of AMPK (Freyssenet et al. 1995; Bergeron et al. 2001; Zong et al. 2002; Fediuc et al. 2006), although activation of AMPK was not directly studied here.

4.4 β -GPA did not alter nuclear recruitment and myonuclear domain

We predicted an increase in MND in treated individuals because AMPK has been shown to limit satellite cell activity and nuclear accretion (Williamson et al. 2009; Miyake et al. 2012). However, given that we did not see any major effects of β -GPA on fiber size or body mass, it was not surprising to find no significant difference in nuclear content in treated fish. It was interesting, however, that there was no relationship between nuclei/mm fiber and fiber CSA, which implies that there was no detectable satellite cell recruitment during this period of growth. The apparent lack of nuclear recruitment during this phase of hypertrophic growth is consistent with the positive correlation of MND with fiber CSA (Fig. 7C). The results from the ANCOVA showed that there was no difference in the MND sizes between the control and treated groups, suggesting that β -GPA treatment and the approximate 25% decrease in PCr and ATP- β in fishes did not affect satellite cell recruitment.

The lack of differences in nuclear recruitment between the two groups was unexpected since many studies have shown that during exercise in mammals, satellite cell proliferation and recruitment into fibers was required prior to hypertrophy (Tseng et al. 1992; Rosenblatt et al. 1993; Rosenblatt et al. 1994; McCall et al. 1998). This is contradictory to the findings of the present study, as MND increased due to hypertrophy with no detectable satellite cell recruitment. Some recent work has shown that exercise-induced hypertrophy without satellite cell recruitment is possible (Bruusgaard et al. 2010; McCarthy et al. 2011; van der Meer et al. 2011; Bruusgaard et al. 2012). However, the uncoupling of fiber volume and myonuclear recruitment remains atypical during growth (Enesco and Puddy 1964; Bruusgaard et al. 2006; Mantilla et al. 2008). These results suggest that, as in those exercise-induced studies, the MND has not reached a size where nuclear function becomes compromised by diffusion constraints. Further, a relatively large MND may be tolerable in fish white muscle due to the relatively low protein turnover rates in fast-twitch fibers (Lewis et al. 1984).

5. CONCLUSIONS

We have demonstrated that β -GPA can be transported into fish skeletal muscle cells and that it can be phosphorylated by a teleost CK, though it is not transported as effectively as in mammals. Regardless, β -GPA supplementation did affect intracellular high energy phosphate concentrations and therefore the cellular energy state. This led to a significant increase in subsarcolemmal mitochondrial density and changes in the metabolite pool that suggest an increase in skeletal muscle collagen content, results that are consistent with some of the changes in fish muscle associated with exercise. In contrast, there were no detectable differences in body mass or skeletal muscle fiber size or myonuclear density, although there were indications that β -GPA treated fish had a stronger reliance on hypertrophy during juvenile growth compared to controls. Overall, β -GPA may have some application in

aquaculture of fishes due to its potential for mimicking some of the beneficial effects of modest exercise without inhibiting growth and development.

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Figure 1. β-GPA uptake and phosphorylation within teleost myofibers

(A) Total concentrations of creatine and β-guanidinopropionic acid (β-GPA) in control ($n=6$) versus β-GPA treated fish ($n=6$) determined via GC×GC/TOF-MS. Creatine and β-GPA concentrations were derived from a standard curve ranging from 4 – 900 μg/mL. Creatine was taken as the sum of both creatine and creatinine peaks. Data are means ± SEM. (B) Representative ³¹P-NMR spectra of a control (left) and β-GPA treated fish muscle tissue (right) with integral symbols bracketing each peak. As expected, the peak areas for adenosine triphosphate-β (ATP-β) and phosphocreatine (PCr), were reduced, while the phospho-guanidinopropionic acid (PGPA) and inorganic phosphate (P_i) peak areas were increased in the treated fish, indicative of a reduction in the cellular energy state. PCr was not completely reduced in treated fish, though it was significantly depressed. (C) High energy phosphate profile within the white muscle of control (black) and β-GPA treated (white, hashed) red porgy ($n=5$). Treated fish showed significantly reduced PCr and ATP-β concentrations, while P-GPA constituted 12% of the total high energy phosphate profile. This suggests that β-GPA competes with creatine (Cr) for the teleost creatine transporter (Cr_T) and alters the intracellular HEP profile and energy state. Data are means ± SEM.

Figure 2. Shifts in metabolite profiles of red porgy in response to β -GPA treatment
 nMDS of metabolite profiles of 12 red Porgy. Individuals were either control (black downward triangles, $n=6$) or treated with 5% β -GPA (gray upward triangles, $n=6$). Metabolite profiles were significantly different between control and β -GPA treated groups (PERMANOVA+, Pseudo- $F=4.4$, $p=0.01$). The 8 metabolites with correlation coefficients within MDS1 or MDS2 that were >0.2 are overlaid with directionality and effect size (direction and line length, respectively). Differences in the control versus treated groups are explained mostly by a lower concentration of L-proline and a higher concentration of L-glycine in the control group.

Figure 3. β -GPA treatment does not affect growth in red porgy

Body mass of control and β -GPA treated groups over the 12-week experiment. Body mass did not differ significantly between the groups at any time point. See results for additional details. Data are means $\pm$ SEM.

Figure 4. Confocal light microscopy of myonuclei within teleost skeletal muscle fibers
Myofibers cut in cross section and stained with DAPI (light blue) and AlexaFluor-594 conjugated WGA (red). (A) Examples of subsarcolemmal (SS) myonuclei are indicated with white arrows; examples of extracellular nuclei that were not counted are shown with white arrowheads. (B) Examples of intermyofibrillar (IM) myonuclei are indicated with block arrows.

Figure 5. Transmission electron micrograph of epaxial muscle

(A) One of six high resolution micrographs per specimen (5000× magnification). (B) Expanded view of the image in (A) showing the edge of a myofiber with a cluster of subsarcolemmal (SS) mitochondria at the fiber periphery, denoted by white arrows. (C) Expanded view of the image in (A) showing intermyofibrillar (IM) mitochondria between myofibrils denoted by white arrows, surrounded by myofibrils.

Figure 6. Average fiber diameter (μm) versus final body mass (g) in β -GPA treated red porgy ANCOVA revealed no significant interaction of treatment and body mass ($F=2.66$, $p=0.14$), so the interaction term was omitted. There was no significant effect of treatment ($F=3.22$, $p=0.11$) or body mass ($F=3.14$, $p=0.11$) Error bars represent the standard error of the estimated mean fiber diameter for each individual.

Figure 7. Response of mitochondria and myonuclei within myofibers of red porgy in response to β -GPA treatment

(A) Percent volume density of subsarcolemmal (SS), intermyofibrillar (IM), and total mitochondria in control versus treated fish. IM and total mitochondrial densities were not significantly different between groups (Student's *T*-tests; $n=3$, $p=0.40$; $n=3$, $p=0.10$, respectively). SS mitochondrial percent volume density was different between control and treated muscle, as noted with an asterisk ($n=3$, $p=0.02$). Data are means $\pm$ SEM. (B) Density of intracellular nuclei in muscle cells in control and treated groups, determined via light microscopy. Data were non-normally distributed (right-skewed; Shapiro-Wilk, $p<0.01$), and therefore a nonparametric Mann-Whitney *U*-test was used to test for differences between means. Nuclear density was not significantly different between control and treated groups ($\chi^2=0.93$, $p=0.34$). (C) Myonuclear domain (MND) as a function of fiber cross sectional area (CSA) by treatment. ANCOVA revealed that there was not a significant interaction between fiber CSA and treatment ($F=0.03$, $p=0.86$), so the interaction term was removed. Treatment did not significantly affect MND ($F=1.3$, $p=0.28$), though there was a strong positive correlation between fiber CSA and MND ($F=18.1$, $p=0.002$). $MND = (\text{fiber CSA } (\mu\text{m}^2) \times 16.56) - 1775 \mu\text{m}^2$.

Table 1
Composition of pelletized fish diets

Dietary constituents of fish food expressed as percent weight (grams per ingredient/total grams*100).

Ingredient	Control Diet (% w/w)	Treated Diet (% w/w)
Solvent-extracted soybean meal	20	20
Menhaden fish meal	32	32
Poultry meal	20	20
Squid meal	5	5
Wheat starch	2	2
Wheat gluten (binder)	4	4
Menhaden fish oil	5	5
Soybean lecithin	1	1
Vitamin mix	3	3
Mineral mix	3	3
α -cellulose	5	0
β -GPA	0	5
Total	100	100