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2   Title

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4   Full:

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6   Gluten-free Fish? Marine Carnivores Cobia (*Rachycentron canadum*) and European Sea Bass

7   (*Dicentrarchus labrax*) Have Different Tolerances to Dietary Wheat Gluten

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9   Short:

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15   aquaculture feed

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Abstract

In developing more sustainable fishmeal-free diets for a broad range of fish species, a "one-size-fits-all" approach should not be presumed. The production of more ecologically sustainable aquaculture diets has increased the incorporation of plant-based protein sources such as wheat gluten. Here we show that wheat gluten at even less than 4% inclusion in a compound feed has a negative impact on growth and survivorship in juvenile cobia (*Rachycentron canadum*). In addition, plasma factors capable of binding wheat gluten were detected in the plasma of cobia fed diets containing this ingredient but not in wild cobia with no exposure to dietary wheat gluten. Furthermore, there is evidence that supplementary taurine partially mitigates the deleterious effects provoked by wheat gluten. Based on these results, we propose that wheat gluten should be added with caution to aquaculture diets intended for juvenile cobia and potentially other marine carnivores. After observing that dietary wheat gluten can cause deleterious effects in cobia, we sought to evaluate a possible effect in European sea bass (*Dicentrarchus labrax*), another large, carnivorous, marine species. There were no major effects in terms of growth rate, plasma biochemical parameters, or detectable induction of plasma IgM, IgT, or factors capable of binding gliadin in response to 4% dietary wheat gluten. However, plasma levels of taurine doubled and there were considerable changes to the intestinal microbiome. There was increased diversity of predominant taxonomic orders in the pyloric caeca, anterior, middle, and posterior intestinal sections of fish consuming wheat gluten. Despite these measurable changes, the data suggest that dietary inclusion of 4% wheat gluten is well tolerated by European sea bass in feed formulations. Together these findings underscore the need to evaluate tolerance to ingredients in aquaculture formulations on a species by species basis.

3

59

60 Introduction

61   Reducing fishmeal inclusion in favor of more sustainable and cost-effective protein

62 sources is a high priority for the aquaculture industry (1). Aquaculture diets that incorporate low-

63 fat, high-protein concentrates derived from plants such as soy or wheat (including processed

64 wheat gluten) have been described for many species (2)(3). Feed formulations often incorporate

65 wheat gluten as a protein source and for binding and adding a desirable chewy texture to pellets.

66 Wheat flour is processed to remove soluble fibers and starches, and the vital wheat gluten

67 product that remains contains two fractions: soluble gliadins and insoluble glutenins (4)(5). Vital

68 wheat gluten, marketed as a dry powder, regains its elastic properties when rehydrated (6). In

69 addition to being a low-cost protein source, it is useful for the binding together of feed

70 ingredients into granules and pellets. Gliadins are known to trigger an immune response in

71 susceptible people, such as those with celiac disease (7–9).

72   When plant proteins replace fishmeal, essential amino acids (e.g. lysine, methionine,

73 threonine), as well as various vitamins and minerals, often need to be supplemented to

74 counteract deficiencies in the protein source (10–12). One such necessary nutritional constituent

75 found in fishmeal but is absent in plants is taurine. Some fish species are inadequate synthesizers

76 of taurine and require dietary intake (13). Previous work by our laboratory showed that cobia is

77 one such species, and it is essential to add taurine to a plant-based feed formulation intended

78 for their consumption (14). Findings presented in this work suggest that taurine may help to

79 mitigate some of the deleterious effects prompted by dietary wheat gluten in cobia.

80   The original intent of the cobia study described here was to test various plant-based diets

4

81   formulated from available and cost-effective ingredients that had been previously found to be

82   highly digestible by cobia and to be effective fishmeal replacements in rainbow trout

83   (*Oncorhyncus mykiss*) and other species (14). Commercial feed formulations tend to vary based

84   on the availability and cost of ingredients, with different batches potentially containing

85   significantly different proportions and quality of ingredients, or even different ingredients

86   altogether. The assessment of multiple components and combinations is necessary in the

87   development of optimized fishmeal replacement diets. It is also necessary to identify which

88   components might need to be supplemented for particular species when plant proteins replace

89   fishmeal in a formulation.

90   In a previous study, when a plant protein-based diet lacking taurine was fed to cobia,

91   poor growth and palatability were observed. However, when taurine was added to a similar plant

92   protein-based diet, growth performance was as good if not better than a fishmeal-based

93   commercial diet (14). It is likely that taurine supplementation greatly contributed to the

94   improved performance of the diet. However, two of the plant protein sources were also replaced:

95   barley meal (10.4%) and wheat gluten (8.2%) in exchange for solvent-extracted soybean meal

96   (12.1%) and wheat flour (22.7%). The barley meal was replaced because of poor digestibility (<

97   60%), and the wheat gluten was replaced with wheat flour as part of a slight reformulation

98   unrelated to specific concerns regarding wheat gluten. In the current study, which was not

99   originally designed to evaluate wheat gluten as an ingredient, we prepared plant-based diets for

100   cobia with varying concentrations of taurine (0 to 5%) at a fixed vital wheat gluten level of 2.2%.

101   Wheat flour in the formulations also contributed a small amount of wheat gluten. Dietary wheat

102   gluten at less than 4% inclusion appeared to be poorly tolerated, causing poor growth and even

103   mortalities in juvenile cobia. We also detected the presence of a plasma factor(s) capable of

104   binding gliadin that was present only in cobia exposed to dietary wheat gluten. Our laboratory

105   wished to expand on this study to further delineate the impacts of wheat gluten and taurine but

106   were unable to procure another cohort of cobia. We decided to investigate the impact of dietary

107   wheat gluten in European sea bass (*Dicentrarchus labrax*), another marine carnivore.

108   European sea bass are a globally important aquaculture fish, with approximately 60,000

109   tons of fish produced each year as part of a greater than 300 million dollar market. They can be

110   found on restaurant menus as branzino or branzini (15). A study in European sea bass suggests

111   that they perform well on a diet containing wheat gluten (~25% of formulation) when fishmeal is

112   also included in the formulation (16). Another study included 20% gluten in an entirely plant-

113   based formulation and noted differences in growth rates between the fish consuming that diet

114   as compared to a fishmeal-based diet (17). It is important to note than in the dietary formulations

115   for the study, the added wheat flour contributes a small amount of gluten. However, results from

116   cobia suggest that wheat flour does not elicit the same negative effects as the processed vital

117   wheat gluten. Being uncertain as to the taurine requirements of European sea bass, we

118   supplemented the diets in the study with 1.5% taurine to ensure that taurine deficiency would

119   not be a confounding factor in evaluating the effects of wheat gluten in European sea bass.

120   In the current study, we sought to determine if the inclusion of 4% wheat gluten into the

121   diet of European sea bass impacted overall growth, health and immune status, and the intestinal

122   microbiome. This entailed tracking growth rates, assaying plasma parameters and tissue weights,

123   detecting adaptive immune factors IgM and IgT, and evaluating differences in the intestinal

124   microbiome. European sea bass, like other teleost fish, have both innate and adaptive immunity.

6

125   European sea bass and other teleosts have three types of immunoglobulins: IgM, IgT, and IgD

126   (18,19). The functions of the first two types have been well described. The role of IgM in fish is

127   similar to its role in other organisms. It is the principal immunoglobulin found in teleost plasma

128   and though its primary role is in systemic immunity, a role in mucosal immunity has also been

129   described. IgT is an integral part of mucosal immunity and functions similarly to IgA in mammals.

130   Like IgM, it is detectable in plasma, though levels tend to be lower (20).

131   Regarding the gut microbiome, it is known that predominant intestinal microbes in fish

132   by diet, including with the addition of plant-based protein sources such as soybean meal (21–23).

133   A study of European sea bass fed combined fish- and plant-based protein sources reported the

134   main gut genera to be *Lactobacillus*, *Pseudomonas*, *Vibrio*, and *Burkholderia* (24). In this study,

135   we wished to characterize both the microbiome of European sea bass fed a plant-based diet

136   and the contribution of wheat gluten to the microbial landscape.

137   European sea bass fed the 0 and 4% wheat gluten diets had similar growth rates and levels

138   of common plasma parameter, and neither group had detectable induction of plasma IgM, IgT,

139   or factors capable of binding gliadin. Notable differences between the groups included twice the

140   plasma levels of taurine in the fish fed 4% wheat gluten and considerable differences in the

141   predominant taxonomic orders of the intestinal microbiome. Despite these measurable changes,

142   the data suggest that dietary inclusion of 4% wheat gluten is well tolerated by European sea bass

143   in an aquaculture feed formulation.

144

145   Materials and Methods

146   *Fish System Maintenance and Care*

7

147   *Cobia*

148   This study was carried out in accordance with the guidelines of the International Animal

149   Care and Use Committee of the University of Maryland Medical School (IACUC protocol

150   #0610015). Approximately 500 juvenile (~2 g) cobia were obtained from the Virginia Agricultural

151   Experiment Station, Virginia Tech, Hampton, VA, USA, for the first and second trials and

152   approximately 500 juveniles (~2 g) were obtained from the University of Miami in Miami, FL, USA,

153   for the third trial. Juveniles were housed at the Institute of Marine and Environmental

154   Technology's Aquaculture Research Center in Baltimore, MD. Fish for the first and second trials

155   (PP1-PP4) were maintained on a fishmeal diet until they reached an average weight of ~10 g (Trial

156   1) or ~120 g (Trial 2), at which point 18 fish were stocked into each of 12 identical tanks and

157   randomly assigned one of the four experimental diets using three replicate tanks per dietary

158   treatment. Six 340-liter tanks connected to bubble-bead and biological filtration as well as

159   protein skimmers constituted the recirculating systems. Four replicate systems were occupied

160   simultaneously during trials with the photoperiod maintained at 14 hours light, 10 hours dark

161   throughout the trials. The first trial was conducted for 8 weeks, with tank weights recorded and

162   feeding rates adjusted weekly to 5% bw day^{-1}. The second trial commencing with ~120 g fish was

163   conducted for 8 weeks, with tank weights recorded weekly and feeding rates adjusted from 3.5%

164   bw day^{-1} to 2.5% bw day^{-1}, with a bi-weekly 0.25% bw day^{-1} reduction throughout the trial.

165   The third trial (EPP3 diet) was initiated with ~18 g average weight individuals, with 12 fish

166   stocked per tank. The third trial was conducted for 12 weeks, with tank weights recorded and

167   feeding rates adjusted weekly to 5% bw day^{-1} for the first 6 weeks, reduced to 3.5 % from 6 weeks

168   through 10 weeks, and 3.0% for the final 2 weeks of the trial as feed conversion ratio gradually

8

169   increased. Fish received the daily ration by hand over the course of 4 feedings.

170

171   *European sea bass*

172   This study was carried out in accordance with the guidelines of the International Animal

173   Care and Use Committee of the University of Maryland Medical School (IACUC protocol

174   #0616014). European sea bass were obtained from the laboratory of Yonathan Zohar, PhD, and

175   maintained in the Aquaculture Research Center at the Institute of Marine and Environmental

176   Technology. Starting at ~25 g of body weight, fish were fed diets containing either 0 or 4% wheat

177   gluten (Zeigler Bros., Gardners, PA, USA), starting with 75 fish per diet. Fish were maintained on

178   these diets for 6 months, at which time the average weight was 250 g. Fish were fed 3.5% of their

179   body weight per day over 3-4 feedings.

180   Temperature was maintained at 27 degrees C and salinity was 25 ppt. Fish were divided

181   by diet and housed in one of 2 eight-foot diameter, four cubic meter, recirculating systems

182   sharing mechanical and bio-filtration as well as life support systems. The recirculating system has

183   a filtration system which including protein skimming, ozonation, mechanical filtration in the form

184   of bubble-bead filters, and biological filtration. Water samples were tested 2-3 times per week

185   and analyzed by the National Aquarium water quality lab at IMET. Water quality was not

186   significantly different between systems utilized (ANOVA, p>0.05) during the study and overall

187   parameters were: dissolved oxygen, 5.69 ± 1.62 mg/L ; temperature, 26.85 ± 1.77 degrees C; pH

188   (4,500-H$^+$), 7.61 ± 0.27; total ammonia nitrogen (4,500-NH$_3$), 0.06 ± 0.06 mg/L ; nitrite (4,500-

189   NO$_2^-$), 0.12 ± 0.08 mg/L ; nitrate (4,500-NO$_3^-$), 49.28 ± 8.87 mg/L, alkalinity (2,320), 95.77 ± 23.11

190   meq/L ; and salinity (2,510) 24.91 ± 1.65 ppt.

9

191

192   *Diet preparation*

193   *Cobia*

194   Formulations of the five plant protein (PP) diets used in the feed trials are shown in S1

195   Table: Feed formulations for the cobia dietary study. For all diets, ingredients were ground using

196   an air-swept pulverizer (Model 18H, Jacobsen, Minneapolis, MN) to a particle size of < 200 μm.

197   All ingredients for PP1, PP2, PP3, and PP4 were mixed prior to extrusion, while EPP3 was top-

198   coated with the oil ingredient after extrusion. Pellets were prepared with a twin-screw cooking

199   extruder (DNDL-44, Buhler AG, Uzwil, Switzerland) with an 18-second exposure to 127 °C in the

200   extruder barrel. Pressure at the diet head was approximately 26 bar, and a die head temperature

201   of 71 °C was used. The pellets were dried for approximately 15 minutes to a final exit air

202   temperature of 102 °C using a pulse bed drier (Buhler AG, Uzwil, Switzerland) followed by a 30

203   min cooling period to product temperature less than 25 °C. Final moisture levels were less than

204   10% for each diet. Diets were stored in plastic lined paper bags at room temperature and were

205   fed within six months of manufacture. Portions of each diet were analyzed by New Jersey Feed

206   Labs, Inc. (Trenton, NJ, USA) for proximate composition (S2 Table: Proximate composition and

207   measured taurine values of the cobia diets). Calculations of feed gluten content are based on

208   wheat flour containing 8% gluten plus any added vital wheat gluten (25)

209

210   *European sea bass*

211   The formulations for the 0 and 4% wheat gluten diets are shown in S3 Table: Feed

212   formulations for the wheat gluten European sea bass dietary study (Zeigler Bros., Gardners, PA,

213   USA). The proximate composition of the feeds in shown in S4 Table: Proximate composition and

214   measure taurine values of the European sea bass diets. The analysis was performed by New

215   Jersey Feed Laboratory, Inc. (Ewing Township, NJ, USA).

216

217   *Blood and Tissue Sampling and Analysis*

218   *Cobia*

219   At the conclusion of the first trial, two individuals from each tank were sacrificed for

220   intestinal analysis. Portions of the anterior intestine were preserved in 4% paraformaldehyde and

221   dehydrated from 70% to 90% EtOH in 10% increments over eight hours. Dehydrated samples

222   were sent to AML Laboratories (Baltimore, MD) for sectioning, mounting, and H&E staining.

223   Slides were analyzed for pathologies and abnormalities with the aid of the acknowledged

224   pathologist, Dr. Renate Reimschuessel, VMD, PhD, (FDA in Laurel, MD). Gall bladders were

225   removed and bile was extracted and stored at -20 °C prior to bile salt analysis. Total bile salts

226   were assayed with 3 α-hydroxysteroid dehydrogenase (26). Blood samples were taken from the

227   caudal vein with heparinized needles, plasma was separated by centrifugation (16,000 x g for 20

228   min), and total plasma protein was quantified after a 1:600 dilution utilizing a Micro BCA™ Protein

229   Assay Kit (ThermoFisher Scientific, Waltham, MA, USA).

230   At the conclusion of the second trial, two fish from each tank (total of six per dietary

231   treatment), were randomly selected for sampling. Fish were anesthetized with Tricaine

232   methanosulfonate (MS-222, 70 mg L^{-1}, Finquel, Redmond, WA, USA), blood samples were taken

233   from the caudal vein with heparinized needles, after which fish were euthanized with MS-222

234   (150 mg L^{-1}) and gall bladders were removed for bile analysis as in Trial 1. Liver and fillet samples

1

235 were also taken for histology. Blood plasma was separated by centrifugation (16,000 x g for 20

236 min at 4 °C) and plasma osmolality measured in triplicate (10 µl) on a Vapro™ Model 5520 vapor

237 pressure osmometer (Wescor, Logan, UT, USA). Plasma samples from three fish per dietary

238 treatment were sent to the Pathology and Laboratory Medicine Services department at the

239 University of California at Los Angeles for constituent analysis. Remaining plasma, fillet, and liver

240 samples were frozen and stored at -80 °C and portions of each were lyophilized to constant

241 weight for water and taurine content analysis. Triplicate samples of each liver (~10 mg), fillet (~50

242 mg), plasma (~10 µL), and diet (~50 mg) sample were used for taurine extractions based on

243 Chaimbault et al., with samples homogenized in cold 70% EtOH, sonicated for 20 min, dried, and

244 resuspended in 1 ml H_2O prior to injection into the LC-MS (27).

245

246 *European sea bass*

247 At the conclusion of the 6-month trial, food was withheld for 24 hours and 10-12 fish from

248 each diet were sedated with 25 mg/L MS-222 (Syndel, Ferndale, WA, USA) buffered with 50 mg/L

249 sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA) and exsanguinated via the caudal vein to

250 collect blood for plasma analysis. Following blood collection, the spinal cord was severed, and

251 tissues were harvested for analysis.

252 Approximately 1 ml blood from the caudal vein was put into a tube containing 20 µL of

253 1000 units/ml heparin and gently inverted to mix. Samples were centrifuged at 2000 x g for 15

254 minutes at 4 degrees C and the plasma fraction retained. This processing was sufficient for plasma

255 chemistry and immunoblotting. In preparation for taurine analysis by HPLC, 10 µL plasma was

256 mixed with 90 µL (1:10) 70% ethanol containing 0.154 mM D-norleucine. The solution was

1

257   vortexed and centrifuged at 2000 x g for 5 min. 50 µL supernatant was retained and dried down

258   at 70 degrees C overnight.

259

260   Plasma Analysis

261   Plasma analysis was performed by Jill Arnold of the National Aquarium in Baltimore, MD.

262   Samples were processed using standard procedures for biochemistry analytes (S5 Table:

263   Common plasma analytes measured) using the ChemWell-T analyzer (CataChem, Oxford, CT,

264   USA). Calibration and quality control materials were used per manufacturer's instructions

265   (Catacal and Catatrol control level 1 and 2, CataChem, Oxford, CT, USA). Osmolality was

266   measured using the Wescor Osmometer (Wescor, Inc., Logan, UT, USA) after calibration with two

267   levels of standards (290 and 1000 mmol/kg, OPTIMOLE, ELITechGroup Biomedical Systems,

268   Logan, UT, USA).

269

270   Plasma taurine analysis by HPLC

271   Dry samples were resuspended in 300 µL 0.1 N HCl and filtered through 0.45 micron filters

272   (EMD Millipore, Billerica, MA, USA). 5 µL of the filtered extracts was derivatized according to the

273   AccQTag^{TM} Ultra Derivitization Kit protocol (Waters Corporation, Milford, MA, USA). Amino acids

274   were analyzed using an Agilent 1260 Infinity High Performance Liquid Chromatography System

275   equipped with ChemStation (Agilent Technologies, Santa Clara, CA, USA) by injecting 5 µL of the

276   derivatization mix onto an AccQTag™ Amino Acid Analysis C18 (Waters, Milford, MA, USA)

277   4.0 um, 3.9 x 150 mm column heated to 37 °C. Amino acids were eluted at 1.0 mL min^{-}1 flow

278   with a mix of 10-fold diluted AccQTag^{TM} Ultra Eluent (C) (Waters Corporation, Milford, MA, USA),

279   ultra-pure water (A) and acetonitrile (B) according to the following gradient: Initial, 98.0% C/2.0%

280   B; 2.0 min, 97.5% C/2.5% B; 25.0 min, 95.0% C/5.0% B; 30.5 min, 94.9% C/5.1% B; 33.0 min, 91.0%

281   C/9.0% B; 38 min, 40.0% A/60.0% B; 43 min, 98.0% C/2.0% B. Derivatized amino acids were

282   detected at 260 nm using a photo diode array detector. Signals were referenced to AABA (alpha-

283   Aminobutyric acid), D-norleucine, and standard hydrolysate amino acids.

284

285   *SDS-PAGE and western blotting*

286   A solution of gliadin (Sigma, St. Louis, MO, USA) was made to an original concentration of

287   2 mg/ml and subsequently diluted 2-fold to 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.125 mg/ml,

288   all in Laemmli sample buffer. All samples were heated to 95 °C for 3 min and centrifuged at

289   10,000 x g for 1 min prior to electrophoresis. Recombinant *Amphidinium carterae* eIF4A-1A

290   (obtained from Grant Jones at IMET, 7/28/14) was diluted into Laemmli sample buffer to a

291   concentration of 57.5 ng/µl. 15 µl of a 1:1 volume ratio of gliadin and eIF4A was loaded into each

292   lane of a Novex NuPAGE 4-12% Bis-Tris gel and electrophoresed in a Bolt® Mini Gel Tank at 165 V

293   for 1 h with MOPS SDS running buffer (Life Technologies, Frederick, MD, USA).

294   PVDF membrane was activated by a brief dip in 100% methanol and equilibrated for 5

295   min in Novex™ NuPAGE® transfer buffer. The gel was electroblotted onto a prepared PVDF in a

296   Bolt Mini Blot Module at 30 V for 1 h in Novex™ NuPAGE® transfer buffer (Life Technologies,

297   Frederick, MD, USA). Following transfer, the membrane was washed in ddH$_2$0 for 5 min. Duplicate

298   transferred lanes were divided and designated as "No Plasma Block" or "With Plasma Block" for

299   subsequent incubation procedures.

300   For the "No Plasma Block" sections, the membrane was incubated at room T for 1 h

301 followed by overnight at 4 °C with 5 ml 5% nonfat milk in TBS-T. For cobia, the "With Plasma

302 Block" membrane was incubated at room T for 1 h followed by overnight at 4 °C with either mixed

303 plasma from a total of 7 fish from Trial 2 fed diets containing 3.2-3.6% gluten (PP1-PP4) or mixed

304 plasma from two wild-caught cobia diluted 1:10 into TBS-T containing 5% nonfat milk. For

305 European sea bass, the "0 Wheat Gluten Plasma" and "4% Wheat Gluten Plasma" membranes

306 were incubated at room T for 1 h followed by overnight at 4 °C with mixed plasma from a total

307 of 3 fish fed diets containing either 0 wheat gluten or 4% wheat gluten, respectively, diluted 1:12

308 into TBS-T containing 5% nonfat milk. The following day, blots were washed 4 times for 10 min

309 each time with TBS-T. Both blots were incubated with polyclonal anti-gliadin antibody (Biorbyt,

310 Cambridge, UK) and rabbit anti-*A. carterae* eIF4E-1A (GenScript, Piscataway, NJ, USA) diluted

311 1:500 and 1:2000, respectively, into TBS-T containing 5% nonfat milk at room temperature for 1

312 h. The blots were again washed 4 times for 10 min each time with TBS-T. Both blots were

313 incubated with goat anti-rabbit IgG-HRP conjugate (Bio-Rad, Hercules, CA, USA) diluted 1:2500

314 into TBS-T containing 5% nonfat milk at room temperature for 1 h followed by four 10-min

315 washes with TBS-T. The HRP signal from bound antibody was visualized using Clarity™ Western

316 ECL Substrate (Bio-Rad, Hercules, CA, USA). Imaging was performed in a Flourchem™, and the

317 AlphaView program was used to analyze densitometry (ProteinSimple, San Jose, CA, USA).

318

319 *IgM and IgT immunoblotting in European sea bass*

320   Plasma was diluted 1:40 in 1x SDS-PAGE sample buffer. Samples were heated for 3

321 minutes at 95 degrees C and centrifuged for one minute at 10,000 x g. 13 µL of each sample was

322 electrophoresed on a 4%–12% Bis-Tris protein gel (NuPAGE Novex, (ThermoFisher Scientific,

1

323   Waltham, MA, USA) for 35 minutes at 200 V using MOPS buffer in a PowerPac™ HC Power Supply

324   (Bio-Rad, Hercules, CA, USA. Proteins were transferred to a PVDF membrane for 14 minutes on

325   the high molecular weight setting (25 V) in the Trans-Blot® Turbo™ Transfer System (Bio-Rad,

326   Hercules, CA, USA). Immunoblotting was performed in the iBind™ Western System

327   (ThermoFisher Scientific, Waltham, MA, USA).

328   For IgT detection, an anti-European sea bass IgT polyclonal antibody (rabbit IgG "RAIgT1,"

329   kindly provided by Giuseppe Scapigliati, Tuscia University, Italy) was used at a dilution of 1:1000

330   as the primary antibody, and goat anti-rabbit IgG H&L HRP conjugate at a dilution of 1:2000 (Bio-

331   Rad, Hercules, CA, USA) was used as the secondary antibody. For IgM detection, the Magic™ anti-

332   European sea bass IgM monoclonal antibody (mouse IgG) (Creative Diagnostics, Shirley, NY, USA)

333   was used at a dilution of 1:1000 as the primary antibody, and goat anti-mouse IgG H&L HRP

334   conjugate (Bio-Rad, Hercules, CA, USA) was used as the secondary antibody at a dilution of

335   1:2000. A chemiluminescent signal was generated with addition of Clarity™ Western ECL

336   substrate and imaged in a ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA, USA). Image

337   Lab software (Version 5.2.1, Bio-Rad, Hercules, CA, USA) was used to visualize immunoblots and

338   analyze protein molecular weights.

339

340   *Microbiome analysis in European sea bass*

341   DNA Extraction

342   For sampling of tank water, 1 L of water was filtered through a pore size of 0.2 microns.

343   For the feed, 0.127 g (3 pellets) of each diet was used. For intestinal samples (pyloric caeca,

344   anterior intestine, mid-intestine, and posterior intestine), ~0.25 g tissue was collected. DNA

1

345   extraction was performed using the Qiagen DNeasy® Powerlyzer® Powersoil® kit (Qiagen,

346   Germantown, MD, USA). Samples were manually processed through the beat beating step 6 m/s

347   for 30 sec, repeated once (FastPrep®-24, MP Biomedicals, Santa Ana, CA, USA) and centrifugation

348   procedure for two minutes at 10,000 x g (Eppendorf 5415 D, Sigma-Aldrich, St. Louis, MO, USA).

349   In the case of the water analysis, the filter was treated as the sample for processing. Following

350   Step 5 in the manufacturer's protocol, DNA extracted was completed in a Qiacube® (Qiagen,

351   Germantown, MD, USA) according to manufacturer's instructions for DNA extraction including

352   the optional PCR inhibitor removal. DNA was stored at -20 degrees C.

353

354   PCR and Gel Electrophoresis

355   PCR was performed to confirm the presence of amplifiable DNA for 16s rRNA sequencing.

356   One μL of extracted DNA was used in a 25 μL reaction volume with Promega™ PCR Master Mix

357   (Thermo Fisher Scientific, Waltham, MA) and amplified in a DNA Engine Dyad (MJ Research,

358   Quebec, Canada) using primers 16S_27F 5' AGAGTTTGATCMTGGCTCAG 3'

359   and 16S_1492R 5' TACGGYTACCTTGTTACGACTT 3' with the following reaction conditions:

360   95 degrees C for 5 min, 92 degrees C for 30 sec, 50 degrees C for 2 min, 72 degrees C for 1 min,

361   30 sec, cycle to step 2 for 39 more times, incubate at 72 degrees C for 5 minutes, hold at 4 degrees

362   C. Agarose gel electrophoresis was performed on each PCR product to detect the presence of a

363   1465 bp DNA band spanning bacterial rRNA variable regions 1-9. Samples were electrophoresed

364   in a 1% agarose gel containing 0.5 μg/mL ethidium bromide at 150V for 40 minutes and imaged

365   in a ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA, USA).

366

1

367 Sequencing

368   Sequencing was performed at the BioAnalytical Services Laboratory (BAS Lab) at IMET on

369 an Illumina MiSeq™ (San Diego, CA, USA) using 5 ng DNA from each sample. Primers

370 complementary to the V3-V4 hypervariable region of the bacterial 16s rRNA gene were designed

371 based on those characterized by Klindworth et al.: S-D-Bact-0341-b-S-17 5'-

372 CCTACGGGNGGCWGCAG-3' and S-D-Bact-0785-a-A-21 5'-GACTACHVGGGTATCTAATCC-3' (28).

373 Including the Illumina adaptor sequences, the full-length primers were F 5'

374 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and R 5'

375 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. CLC Genomics

376 Workbench 8 (Version 8.5.1, Qiagen, Germantown, MD, USA) was used to trim, pair, and merge

377 sequences (default parameters used for all functions). They were exported as a merged FASTA

378 file and imported into the Quantitative Insights into Microbial Ecology (QIIME) program (Version

379 1.9.1) (29) for open reference operational taxonomic unit (OTU) and taxonomic classification

380 using the Silva 128 reference database (30). Rarefaction curves were generated based on

381 observed OTUs. Identify threshold was set to 97%. Representative sequence alignments for each

382 OTU were generated using Python Nearest Alignment Space Termination (PyNAST) (31), and R

383 (Version 3.4.3) (32) was used to generate a bar graph of the bacterial orders, as well as a PCA

384 plot. QIIME was used to generation the rarefaction plot.

385

386 *Statistics*

387   Statistical significance was evaluated using analysis of variance (ANOVA) and Student's t-

388 test (2-tailed) with a 95% confidence interval.

1

389

Results

Cobia Trials

392   Diets 1-4 for both PP (plant protein) and FM (fishmeal) were formulated to contain graded

393   levels of taurine of approximately 0, 0.5%, 1.5% and 5%. PP1-4 contain approximately 3.6%, 3.6%,

394   3.5%, and 3.2% total wheat gluten, respectively. This calculation includes any wheat gluten

395   present in the wheat flour, estimated to be ~8% of the total weight. The FM1-4 diets contain an

396   average of 1.8% total wheat gluten. These diets are part of a previously published study

397   performed by our laboratory (33). The EPP3 diet contains approximately 1.2% wheat gluten and

398   1.5% taurine.

399

Growth Data

401   Growth data for Trial 1 of diets PP1-PP4 are displayed in Figure 1 (A). For this trial, the

402   average initial fish weight was ~10 g, and fish were maintained on the diets for 8 weeks. Data

403   from Trial 3 with fish of average initial weight ~18 g fed the EPP3 diet are also included on this

404   graph, as well as averaged growth data for fish fed various fishmeal diets (FM1-FM4). Growth

405   data from Trial 2 (~120 g initial weight) are shown in Figure 1 (B). The trial continued for 8 weeks

406   and as with the graph for the first trial, data are included for comparison from the EPP3 and FM1-

407   FM4 studies. For both trials, the fishmeal and EPP3 diets trend higher in terms of growth. PP1

408   appears to be the least favorable diet in terms of growth. It contains the greatest amount of

409   wheat gluten but also contains no taurine.

410

411 **Figure 1: Growth data from 8-week dietary trials in juvenile cobia.**
412 Plot A presents data from the first growth trial starting with ~10 g average weight juveniles for
413 four plant protein based diets (PP1, PP2, PP3, and PP4) with graded levels of supplemental
414 taurine and the average of four fishmeal-based diets (FM, data from Watson et al. 2014) (33).
415 Plot B presents data from the second growth trial starting with ~120 g average weight juveniles.
416 Included are the growth data for the trial with diet EPP3 starting with ~18 g average weight
417 juveniles. Plotted are the average tank weights from 3 replicates ± standard deviation.
418
419 *Survival and performance characteristics*

420 Performance characteristics for fish fed PP1-PP4 are shown in Table 1. Differences in

421 survival are substantial, with PP4 containing the lowest amount of wheat gluten and highest

422 amount of taurine being the best performer. There were no significant differences among

423 surviving individuals in percent weight gain, feed conversion ratio (FCR), specific growth rate

424 (SGR), total plasma protein concentration, or total bile salt concentration (ANOVA, P>0.05). Due

425 to low survival in several replicates of PP1 and PP2 resulting in 0 individuals in some tanks, these

426 diets were not included in statistical analyses other than survival for the first trial.

427

428 **Table 1: Performance characteristics from Trial 1 with cobia (~10 g initial weight).**
429 Within a row, values that share common superscripts are not significantly different from one
430 another (P>0.05).
431

Characteristic	PP1 (0.02% T)	PP2 (0.39% T)	PP3 (1.35% T)	PP4 (4.08% T)
Survival (%)	1.96 ± 1.96[a]	9.80 ± 5.18[a,b]	9.83 ± 1.97[a,b]	19.6 ± 7.06[b]
Weight Gain (%)	315.55 [3]	1194.73 ± 21.22 [3]	1155.92 ± 410.70	1200.68 ± 214.22
FCR[1]	1.93 [3]	0.96 ± 0.02 [3]	1.17 ± 0.44	0.95 ± 0.11
SGR[2]	2.54 [3]	4.57 ± 0.03 [3]	4.26 ± 0.72	4.53 ± 0.31
Total Plasma Protein (g dL^{-1})	3.49[3]	3.40 ± 0.17[3]	3.35 ± 0.17	3.48 ± 0.23
Total Bile Salts (mM)	40.84[3]	42.17 ± 0.46[3]	41.37 ± 1.99	41.50 ± 1.36

432
433
434 T=Taurine
435 [1] FCR =feed conversion ratio = (g fed/g gained)

2

436   [2] SGR=specific growth rate = $((\ln BW_f - \ln BW_i) * (\text{days of growth trial}^{-1})) * 100$

437   [3] Not included in statistical analyses due to lack of replicates (survivors)

438

439   Performance characteristics from the second trial (~120 g initial weight) are shown in

440   Table 2. Percent weight gain was significantly lower in diets PP1 and PP2 compared to the other

441   four fishmeal-based diets (p<0.05), but not significantly different from diets PP3 and PP4

442   (p>0.05). Diet PP1 resulted in significantly lower FCR and SGR than the other diets (p<0.05). Diets

443   PP2, PP3, and PP4 did not result in significantly different SGRs than one another (p>0.05). The

444   compared outcomes from diets PP1 and PP2 containing roughly the same amount of wheat

445   gluten with and without taurine supplementation reinforce the importance of the amino acid for

446   promoting growth and suggest that it may partially alleviate adverse effects resulting from wheat

447   gluten. Total bile salt concentration was not significantly different between any of the 4 dietary

448   treatments (p>0.05).

449

450   **Table 2: Performance characteristics from Trial 2 with cobia (~120 g initial weight).**
451   Within a row, values that share common superscripts are not significantly different from one
452   another (P>0.05).

453

454

Characteristic	PP1 (0.02% T)	PP2 (0.39% T)	PP3 (1.35% T)	PP4 (4.08% T)
Weight Gain (%)	23.35 ± 22.82[a]	80.57 ± 60.24[a]	130.87 ± 25.20[b]	133.82 ± 10.83[b]
FCR	6.38 ± 1.49[a]	2.97 ± 1.17[b]	1.98 ± 0.19[b]	2.12 ± 0.23[b]
SGR	0.57 ± 0.12[a]	1.31 ± 0.26[b]	1.47 ± 0.19[b]	1.51 ± 0.09[b]
Total Bile Salts (mM)	38.76 ± 7.40	28.37 ± 1.94	36.30 ± 5.69	28.75 ± 3.22

455
456

457   T=Taurine

458   [1] FCR =feed conversion ratio = (g fed/g gained)

459   [2] SGR=specific growth rate = $((\ln BW_f - \ln BW_i) * (\text{days of growth trial}^{-1})) * 100$

460

461   For the EPP3 diet in the third trial (initial weight ~18 g), weight gain, FCR, and SGR were

462   much improved from the first two trials, and were an improvement over all previous plant protein

463   diets tested in our laboratory. Performance characteristics included weight gain of 1673.52% ±

464   192.75, FCR of 1.22 ± 0.04, and SGR of 3.42 ± 0.13. The EPP3 diet contains the lowest gluten

465   content of all diets tested in this study (1.2%).

466

467   Wheat gluten: Survival and pathology

468   Figure 2 presents survival curves based on percent wheat gluten for fish fed diets PP1-

469   PP4 (Trial 1), EPP3 (Trial 3), or fishmeal-based diets FM1-FM4 (averaged) from a previous study

470   (33). These data suggest that wheat gluten inclusion may be deleterious to cobia at this early

471   stage of development, though it may be tolerated at later stages. Histological analysis of the

472   proximal intestinal epithelium showed no distinct differences for fish fed the PP1-PP4 and FM1-

473   FM4 diets. However, pathological effects in the distal intestinal region cannot be ruled out. No

474   histological analysis was performed on the EPP3 fish. Survival was approximately 100% for fish in

475   Trial 2 fed the PP1-PP4 diets and in Trial 3 with the EPP3 diet. Cannibalism was not observed to

476   be a contributing factor to the low survival in Trial 1, and dead individuals were promptly

477   removed from the tanks so as to not be a nutritional/taurine source for remaining fish.

478

479   **Figure 2: Dietary wheat gluten level affects survival of young cobia.**
480   For Trial 1 fish (growth data shown in Figure 1(A)), probability of survival is graphed as a function
481   of dietary gluten. For fish fed PP1-PP4, (> 3% gluten) or FM1-FM4 (< 3% gluten) (33), survival data
482   from the diet subtypes (1,2,3, and 4) are averaged. Also shown is survival data from fish fed EPP3
483   (1.2% gluten).
484
485   Muscle and liver characteristics

2

486   Fillet and liver characteristics from the second and third trials are shown in S6 Table: Fillet

487   and liver characteristics from the second and third cobia growth trials. Fillet water content was

488   highest in fish fed the PP1 diet with a gradual reduction in fillet water content as dietary taurine

489   level increased. Hepatosomatic index show showed a similar trend of reduction as dietary taurine

490   level increased. Fillet yield, liver water, fillet taurine, and liver taurine contents all showed

491   increasing trends with increasing dietary taurine levels.

492

493   Plasma analysis

494   Results of the plasma analysis are shown in Table 3. Plasma water content significantly

495   decreased as dietary taurine level increased (p<0.05). Concentrations of glucose, calcium,

496   phosphorus, and magnesium ions, as well as overall osmolality, are indicators of health status.

497   Total protein levels also often correlate with nutritional status and general health (34)·(35).

498   Triglycerides are an energy substrate, and a fasting study in European sea bass demonstrated

499   their potential as a marker for nutritional condition (36). AST (aspartate aminotransferase) and

500   ALP (alkaline phosphatase) are indicators of liver health and function (37).

501   Plasma taurine levels were significantly lower in diets PP1 and PP2 than the other diets

502   (p<0.05). Plasma cholesterol, phosphorous, and albumin all showed similar trends of significantly

503   increasing with dietary taurine level (p<0.05). Lower creatine kinase levels can be indicative of

504   muscle wasting (38). Though biochemical markers of liver failure are not well characterized in

505   fish, elevated bilirubin is a classic sign (39). Stress on the liver may be a result of insufficient

506   taurine required for bile acid conjugation or a manifestation of gluten-induced enteritis (40). The

507   substantial difference in plasma glucose levels between PP1-PP4 and EPP3 is likely an artifact of

508   MS-222 and not a barometer of health status (41).

509

510   **Table 3: Plasma measures from fish from the second and third cobia growth trials.**

511   Values represent the mean ± standard error for three fish per dietary treatment. Within a row,

512   values that share common superscripts are not significantly different from one another (p

513   >0.05).

514

515

Plasma Component	PP1 (0.02% T)[1]	PP2 (0.39% T)[1]	PP3 (1.35% T)[1]	PP4 (4.08% T)[1]	EPP3 (1.05% T)[2]
Water Content (%)	96.75 ± 0.19[a]	94.75 ± 0.21[b]	95.19 ± 0.29[a,b]	94.73 ± 0.13[b]	94.94 ± 1.21[a,b]
Osmolality (Osm L^{-1})	329.17 ± 5.57	343.00 ± 5.57	325.17 ± 18.94	336.33 ± 4.60	332.84 ± 9.65
Taurine (nmol ml^{-1})	421.25 ± 41.03[a]	565.79 ± 17.91[a]	658.47 ± 50.92[b]	723.86 ± 89.62[b]	601. 29 ± 47.97[a,b]
Albumin (g dL^{-1})	0.53 ± 0.12[a]	0.70 ± 0.06[a,b]	0.80 ± 0.00[a,b]	0.87 ± 0.03[b]	0.52 ± 0.07[a]
Total Bilirubin (mg dL^{-1})	0.40 ± 0.10[a]	0.30 ± 0.00[a]	0.23 ± 0.03[a]	0.23 ± 0.03[a]	0.12 ± 0.00[b]
Calcium (mg dL^{-1})	9.70 ± 0.88	10.90 ± 0.61	11.10 ± 0.10	11.37 ± 0.27	11.69 ± 0.30
Cholesterol (mg dL^{-1})	49.33 ± 12.17[a]	68.33 ± 1.20[a,b]	80.33 ± 3.18[b]	79.33 ± 4.26[a,b]	46.59 ± 11.43[a]
Creatine Kinase (U L^{-1})	96.67 ± 46.77	386.67 ± 276.01	301.33 ± 81.63	552.00 ± 113.15	nd
Creatinine (mg dL^{-1})	0.17 ± 0.03	0.20 ± 0.00	0.17 ± 0.03	0.17 ± 0.03	nd
Glucose (mg dL^{-1})	48.00 ± 4.36[a]	51.33 ± 3.48[a]	48.33 ± 3.92[a]	41.67 ± 3.93[a]	109.13 ± 19.13[b]
Phosphorous (mg dL^{-1})	7.13 ± 1.34[a]	8.37 ± 0.69[a]	8.87 ± 0.38[a]	9.33 ± 0.23[a]	13.82 ± 1.78[b]
Magnesium (mg dL^{-1})	2.37 ± 0.22	1.97 ± 0.07	2.13 ± 0.03	2.23 ± 0.08	2.77 ± 0.18
Triglycerides (mg dL^{-1})	66.67 ± 27.43	112.00 ± 19.67	91.00 ± 36.12	109.67 ± 20.96	75.73 ± 17.35
Sodium (mmol L^{-1})	169.10 ± 3.56	178.67 ± 2.89	177.33 ± 2.32	174.83 ± 3.89	nd
Potassium (mmol L^{-1})	8.78 ± 1.09	8.07 ± 0.36	8.92 ± 0.53	9.35 ± 0.23	nd
Chloride (mmol L^{-1})	169.97 ± 1.93	173.97 ± 2.08	168.83 ± 0.88	169.43 ± 3.85	nd

516
517
518   T=Taurine
519   [1] UCLA DLAM analysis
520   [2] NOAA-NWFSC analysis by A.W. (excluding water content, osmolality, and taurine)
521
522   Liver histology
523   Figure 3 shows H&E-stained liver sections from Trial 2 fish fed PP1 (Panel A) or PP4 (Panel

524   B). Supplementation with taurine prevents pervasive steatosis. This effect was also seen in fish

525   fed FM1 vs. FM4 (data not shown). Taurine supplementation may have therapeutic potential in

526   the treatment of nonalcoholic fatty liver disease, suggesting that supplementation may be of

527   value even in cases where there is not an underlying deficiency (42).

528

529   **Figure 3: Taurine supplementation mediates hepatic steatosis.**
530   Histological sections from fish were stained with H&E and microscopically examined. Panel A
531   presents a representative section from fish fed PP1 (0.02% taurine). Panel B presents a
532   representative section from fish fed PP4 (4.08% taurine). Pervasive lipidosis is apparent in Panel
533   A.
534

535   Detection of immune factors capable of binding to wheat gluten

536   Suspecting that wheat gluten might be a contributing factor to poor growth performance,

537   we assayed in Trial 2 fish plasma an immune response to gliadin, a potentially immunogenic

538   component of wheat gluten. Standard methods for detecting specific antibody responses to

539   wheat gluten in human celiac patients cannot be utilized as they are designed to detect IgA. Cobia

540   and other teleosts have only three classes of immunoglobulins: IgM, IgT, and IgD, and there are

541   currently no reagents to detect these immunoglobulins in cobia (43). It is not known if antibodies

542   designed to detect these adaptive factors have cross-reactivity to these immune components in

543   cobia.

2

544   To detect plasma factors capable of binding to gliadin, varying concentrations of the

545   protein as well as a fixed amount of eIF4E-1A from the dinoflagellate *A. carterae* were subjected

546   to immunoblotting. eIF4E-1A, a translation protein, was used as a control for loading, transfer

547   efficiency, and specificity of binding. After electroblotting, membranes were pre-incubated in

548   blocking buffer only or blocking buffer containing plasma. The 30-40 kDa bands visible on the

549   immunoblots correspond to a range of α/β and γ gliadins (44). These gliadin bands are less visible

550   over the course of 2-fold dilutions (Figures 4 and 5).

551   Pre-incubation with plasma from cobia fed diets containing 3.2-3.6% wheat gluten

552   diminished binding of an anti-gliadin polyclonal antibody, even at the highest concentration of

553   gliadin (Figure 4). A comparison of slopes from linear regressions based on densitometry data for

554   "No Plasma Block" vs. "With Plasma Block" relatively quantifies this difference in signal as

555   approximately 10-fold. A similar dot blot experiment with gliadin but no eIF4E-1A confirmed the

556   results of the western blot (not shown). Similar results were obtained using plasma from fish fed

557   diets containing 1.5-1.9% wheat gluten, suggesting that a small amount is sufficient to mobilize

558   a response (not shown).

559

560   **Figure 4: Factor(s) that specifically bind gliadin are present in plasma of fish fed plant-based**
561   **diets containing 3.2-3.6% wheat gluten.**
562   Immunoblotting of gliadin without pre-incubation of plasma (A) or with pre-incubation with
563   plasma from fish fed diets containing 3.2-3.6% wheat gluten from Trial 2 (B) demonstrates the
564   ability of factor(s) in plasma to bind to gliadin (30-40 kDa) and inhibit binding of anti-gliadin
565   polyclonal antibody. Each lane also contains 431 ng recombinant A. *carterae* protein eIF4E-1A (50
566   kDa).

567

568   Pre-incubation with plasma from wild-caught cobia with no dietary exposure to wheat

569   gluten does not diminish binding of an anti-gliadin polyclonal antibody, even at the lowest

570   concentrations of gliadin (Figure 5). For all experiments, there was no reduction in eIF4E-1A signal

571   as a result of pre-incubation with plasma, demonstrating the specificity of plasma factors for

572   gliadin. There was also no detection of a component capable of binding gliadin found in the

573   plasma of fish fed the FM1-4 diets (data not shown).

574

575   **Figure 5: Factor(s) that bind gliadin are absent in plasma from wild cobia with no dietary gluten**
576   **exposure.**
577   Immunoblotting of gliadin without pre-incubation of plasma (A) or with pre-incubation with
578   plasma (B). No factors capable of binding to gliadin (30-40 kDa) and inhibiting binding of anti-
579   gliadin polyclonal antibody are detectable. Each lane also contains 431 ng recombinant *A.*
580   *carterae* protein eIF4E-1A (50 kDa).

581
582   *European sea bass*

583   Growth data

584   Growth rates were equivalent (p>0.05) for the fish fed plant-based diets with or without

585   added wheat gluten (4%). The study commenced with fish of an average weight of ~25 g and

586   continued for 6 months. The growth curve is shown in Figure 6.

587

588   **Figure 6: Dietary inclusion of 4% wheat gluten does not affect growth of European sea bass.**
589   European sea bass were fed plant-based diets containing 0 or 4% wheat gluten starting at an
590   average weight of ~25 g. The study continued for 6 months, and growth rates were similar
591   throughout the study (p>0.05).

592

593   Plasma analysis

594   As shown in Table 4, analysis of common plasma analytes using a bioanalyzer revealed no

595   differences in levels between the 0 or 4% wheat gluten dietary groups with the exception of

596   calcium and AST (aspartate aminotransferase) (p<0.05). Plasma calcium is higher in the group fed

597   4% wheat gluten, whereas plasma AST levels are lower.

2

598

Table 4: Levels of common plasma parameters for European sea bass fed diets with 0 or 4% wheat gluten are similar in value except for levels of calcium and AST.

Plasma Component	0 Wheat Gluten	4% Wheat Gluten
Glucose (mg/dL)	120.727 ± 27.335	113.874 ± 32.011
Calcium (mg/dL)	***11.045 ± 0.465**	***11.75 ± 0.567**
Magnesium (mg/dL)	3.545 ± 0.398	3.7 ± 0.25
Phosphorus (mg/dL)	7.909 ± 0.76	8.125 ± 0.784
Triglycerides (mg/dL)	410.364 ± 77.747	388 ± 129.836
Cholesterol (mg/dL)	199.545 ± 33.074	206.75 ± 43.702
ALP (U/L)	24.091 ± 1.486	25.125 ± 1.511
AST (U/L)	***47.8 ± 28.558**	***35.25 ± 17.484**
Osmolality (mOsmol/kg)	363 ± 5.773	368.917 ± 6.851
Total Protein (g/dL)	4.291 ± 0.241	4 ± 0.525

603

*Statistically different between diets
ALP=Alkaline phosphatase
AST=Aspartate aminotransferase

604

Body and tissue weights

Whole body and tissue weights are similar between fish fed either 0 or 4% wheat gluten with the exception of the mid-intestine ($p<0.05$). This is not a typical place for major changes to the intestine to manifest, so this is an interesting result. These data are summarized in S7 Table: Body and tissue weights for fish fed 0 or 4% wheat gluten are similar with differences manifesting in the mid-intestine. Hepatosomatic indices were an average of 0.01 for both dietary groups.

Plasma taurine analysis

Though there were minimal differences between the diets for levels of most common plasma analytes, a separate analysis of plasma taurine levels by HPLC showed that levels were

615   approximately twice as high in the fish fed the diet containing wheat gluten (Figure 7). One

616   possible explanation is that the fish are producing or sequestering more taurine to counter some

617   effect induced by the wheat gluten.

618

619   **Figure 7: 4% dietary wheat gluten substantially raises plasma taurine levels in European sea**
620   **bass.**
621   Taurine levels in plasma were measured using HPLC. Average concentrations of plasma taurine
622   for fish fed the 0 or 4% wheat gluten diets are 33.82 ± 7.133 or 62.515 ± 15.719 nmol/mL,
623   respectively.
624

625

626   Gliadin immunoblotting

627   To detect plasma factors capable of binding to gliadin, varying concentrations of the

628   protein as well as a fixed amount of eIF4E-1A from the dinoflagellate *A. carterae* were subjected

629   to immunoblotting. eIF4E-1A, a translation protein, was used as a control for loading, transfer

630   efficiency, and specificity of binding. After immunoblotting, membranes were pre-incubated in

631   blocking buffer only or blocking buffer containing plasma. In the immunoblot, bands are visible

632   in the 30-40 kDa range corresponding to various α/β and γ gliadins (Figure 8) (44). These gliadin

633   bands are less visible over the course of 2-fold dilutions. There does not appear to be a

634   component in European sea bass plasma produced in response to dietary wheat gluten that is

635   capable of binding gliadin. This would be evidenced by decreased binding of the primary

636   antibody, and subsequently the secondary signal antibody. This is in contrast to data presented

637   in Chapter 2 showing that juvenile cobia produce a plasma factor capable of binding gliadin when

638   they are fed a diet containing 3.2-3.6% wheat gluten.

639

640 **Figure 8: Plasma factors capable of binding gliadin are not detectable in European sea bass fed**
641 **4% wheat gluten.**
642 Western blot analysis of gliadin without pre-incubation of plasma (a) or with pre-incubation with
643 plasma (b). No factors capable of binding to gliadin (30-40 kDa) and inhibiting binding of anti-
644 gliadin polyclonal antibody are detectable. Each lane also contains 431 ng recombinant *A.*
645 *carterae* protein eIF4E-1A (50 kDa).
646

647 Ig and IgM

648 We used immunoblotting to assay for IgT and IgM to see if dietary inclusion of wheat

649 gluten alters their levels in plasma. The results shown in Figure 9 suggest that levels do not

650 change in response to dietary wheat gluten. Any immune response mounted against gliadin or

651 some other component of wheat gluten is not likely due to an adaptive response, in this case.

652 The size of ~73 kDa for the heavy chains of the antibodies is similar to the size of ~78 kDa

653 detected by Picchietti et al. (45).

654

655 **Figure 9: Dietary wheat gluten does not induce changes to levels of plasma IgT or IgM.**
656 IgT and IgM were measured using immunoblotting, and their levels do not appear to be altered
657 by 4% dietary wheat gluten.
658

659 Intestinal microbiome analysis

660 To characterize the microbiome of the water, feed, and intestinal sections of the

661 European sea bass, 16S rRNA gene analysis was performed using the MiSeq platform. Figure 10

662 shows a bar graph of order-level taxonomic abundance. Proteobacteria dominate in the tank

663 water. The "cyanobacteria" in feed are most likely chloroplasts from plant ingredients. This is

664 also true for "cyanobacteria" in the pyloric caeca, which likely corresponds to undigested feed

665 despite the fact that food was withheld from these fish for 24 hours before tissue sampling. For

666 fish consuming no dietary gluten (Tank 6-11), the predominant phylum for all intestinal sections

667   analyzed (pyloric caeca, anterior intestine, mid-intestine, and posterior intestine) is

668   Proteobacteria. The same is true for the intestinal sections of the fish fed 4% gluten (Tank 6-12),

669   but there is a greater diversity of predominant orders of Proteobacteria, and Bacteroidetes

670   presents in the mid- and posterior intestines.

671

672   **Figure 10: European sea bass fed 4% wheat gluten as part of a plant-based diet exhibit a**
673   **greater diversity of predominant taxonomic orders across the intestine.**
674   DNA extracted from water, feed, and various sections of the intestine for the two diet groups
675   underwent *16S* rRNA gene analysis using the MiSeq platform to characterize the microbial
676   landscape. The addition of 4% wheat gluten to a plant-based diet dramatically shifts the
677   intestinal microbiome of European sea bass.
678
679   Figure 11 shows an alpha-diversity rarefaction curve based on OTU data. The PCA

680   (principal component analysis) plot in Figure 12 shows that samples cluster by absence or

681   presence of dietary wheat gluten. The ellipse shows the 95% confidence interval.

682

683   **Figure 11: Species richness for each microbiome sample.**
684   Species richness of the microbiome samples is shown in a rarefaction curve of observed OTUs vs.
685   sequences per sample.
686

687   **Figure 12: Samples cluster by absence or presence of dietary wheat gluten in a PCA plot.**
688   Samples cluster by absence or presence of dietary wheat gluten. The ellipse shows the 95%
689   confidence interval.
690

691   **Discussion**

692   Overall, in the cobia study, dietary wheat gluten negatively impacted growth and survival

693   in juvenile cobia, and taurine supplementation appeared to partially ameliorate those effects.

694   Negative effects observed in the first trial are not likely due to a poor cohort as other fish from

695   this brood used for separate feeding trials grew well on both fishmeal-based formulations and

3

696   commercial feeds in the IMET ARC facility. Palatability issues with plant protein diets have been

697   well described for many species, and it is known that taurine has the potential to serve as a feed

698   attractant due to its small nitrogenous structure (46,47). Increased feed palatability rather than

699   solely physiological benefits from taurine supplementation may have contributed to the

700   differences in growth and survival during the first trial. Concerns over palatability and the overall

701   formulation prompted the second trial, which was initiated with a size that readily accepted and

702   performed well on plant protein formulations. Although growth and feed conversion in the

703   second trial were lower than for some of the other plant protein formulations such as EPP3,

704   survival was 100% in all treatments, a significant improvement over the first trial. As with the first

705   trial, there was a significant increase in performance with increasing dietary taurine, indicating

706   that taurine is promoting growth and is possibly remediating the negative impacts of this

707   formulation.

708   The fact that levels of taurine and wheat flour are similar between the high-performing

709   EPP3 diet and the poorer performing PP3 diet suggests that 2.2% added wheat gluten is

710   contributing to a reduction in performance characteristics. There is the possibility that the soy

711   protein concentrate HP300 in EPP3 is greatly enhancing growth characteristics, but trials with

712   another diet, EPP2, point to the gluten as a critical component. EPP2 has an identical formulation

713   to PP3, except EPP3 contains no added wheat gluten with the exception of what is introduced

714   from the wheat flour itself (which is in slightly greater proportion). EPP2 contains roughly 1.8%

715   wheat gluten, and as was the case with EPP3, fish consuming this diet outperformed fish fed the

716   other plant protein-based diets.

717   The identity of the plasma factor(s) capable of binding gliadin cannot be determined

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718   without the necessary reagents to isolate non-specific and specific immune factors in cobia. It is

719   probable that constituents of the cobia innate immune system recognize gliadin, the potentially

720   pathogenic component of wheat. Enzyme-solubilized wheat gluten extracts have been shown to

721   initiate the alternative complement pathway, and it has been suggested that similar to some

722   pathogens, gliadin may be capable of binding to toll-like receptors and initiating an innate

723   immune response (48,49).

724   Wheat gluten has been shown to have negative impacts on humans, often but not always

725   affiliated with celiac disease, and other mammalian models (50,51),(52,53). It is possible that IgT,

726   the primary mucosal antibody in teleosts, might play a similar role to human IgA. In gluten-

727   sensitive humans, IgA mediates the transport of gliadin across the gastrointestinal epithelium

728   and induces an adaptive immune response (54,55). Without purified antibodies against cobia IgM

729   or IgT, the role of adaptive immunity cannot be assessed. However, it is clear that ingestion of

730   wheat gluten triggers the mobilization or production of plasma factors capable of binding gliadin,

731   be they innate or adaptive.

732   The glutamine to glutamate deamidation reaction in gliadin catalyzed by tTG (tissue

733   transglutaminase) is instrumental in gluten-induced pathology. During transamidation, another

734   reaction catalyzed by tTG, the reaction of glutamine with lysine or lysine methyl ester abrogates

735   the T-cell mediated immunotoxic effects of gliadin in the intestine (56). It is possible that the

736   success of wheat gluten incorporation into many fish feeds is due in part to the addition of lysine

737   favoring the transamidation rather than deamidation of gluten catalyzed by tTG. Zebrafish fed a

738   diet containing 60% wheat gluten without adequate lysine supplementation had compromised

739   growth (57). A low pH environment favors deamidation over transamidation, which offers a

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740   possible explanation for why gluten may be less tolerated in the gastrointestinal tract of

741   carnivorous fish compared to omnivorous fish (58,59).

742   Taurine is present in several tissues and possesses powerful anti-inflammatory

743   properties. It mitigates inflammation by scavenging free radicals, ameliorating oxidative injury,

744   and modulating the immune response (60). Intestinal enteritis has been observed in several

745   species with specific plant protein inclusion such as soy ingredients in salmon and carp (61,62).

746   Wheat gluten may have triggered inflammation in the cobia intestinal tract, leading to the

747   proliferation of circulating plasma factor(s) capable of binding gliadin.

748   Previous studies have demonstrated the ability of taurine to mitigate degeneration of the

749   intestinal mucosa and inflammatory bowel disease. It is possible that taurine supplementation of

750   the cobia diet may partially counteract intestinal inflammation caused by dietary ingredients such

751   as wheat gluten (60,63). Taurine may also offer some protection in the gut against deamidated

752   gluten (64). Unfortunately, samples of the distal intestine were not preserved from fish in this

753   study. This would have been useful for histological analysis of any gluten-induced enteritis.

754   Genetic predisposition is the primary risk factor for CD in humans, but there is evidence

755   that early and abrupt introduction of gluten-containing foods into the infant diet alters the

756   microbiome and increases likelihood of developing the disease (65). Several microorganisms are

757   known to uptake taurine via Tau transporters for utilization as sulfur or carbon sources (66).

758   Though no characterization has been performed of the cobia microbiome, such analysis has been

759   performed in cats, another strict carnivore. Dietary studies have revealed that cats, like cobia,

760   require dietary intake of taurine due to insufficient synthesis. By virtue of the fact that they have

761   similar dietary needs (high protein: carbohydrate ratio) and metabolism (can only conjugate bile

762   acids to taurine), there may also be common commensal bacteria. Similar to cats, alterations to

763   populations of intestinal bacteria in cobia caused by intake of fermentable feed ingredients might

764   increase deconjugation of bile salts and contribute to taurine depletion (67). Rapid shifts in the

765   microbiome induced by diet are not isolated to carnivores, as it has also been well documented

766   in humans along with celiac disease-associated dysbiosis (68,69). In the case of juvenile cobia in

767   Trial 1, it is possible that gut immaturity and incomplete establishment of commensal microbiota

768   may have increased injurious effects from gluten. However, alterations to the normal carnivore

769   microbial landscape from ingredients such as gluten could have the potential to affect the overall

770   health of cobia at any stage of development.

771   The most significant physiological impacts of 4% dietary wheat gluten in European sea

772   bass are the substantial increase in plasma taurine and induction of greater diversity of

773   predominant taxonomic orders of intestine microbiota. These changes in no way suggest a

774   poorer fitness outcome, and there were no significant differences in growth between the two

775   groups. Therefore, there is no reason to contraindicate the addition of 4% wheat gluten addition

776   to a completely plant-based diet for European sea bass.

777   The common plasma measures of overall health that differed between the 0 and 4%

778   groups were calcium and AST. Plasma calcium was higher in the group fed 4% wheat gluten,

779   whereas plasma AST levels were lower than they were in the fish fed the diet without wheat

780   gluten. European sea bass stressed by hydrogen peroxide exposure exhibited higher levels of

781   plasma calcium, so it could be indicative of a health effect (70). AST is an indicator of liver health

782   and function, along with ALP (alkaline phosphatase) (71). In a fasting study in European sea bass

783   performed by Peres et al., AST levels increased during the fasting period (36).

784   Our values for all plasma markers measured are similar to those reported by Peres et al.

785   in European sea bass maintained on a fishmeal diet with the exception of cholesterol, ALP, and

786   AST. For ALP and AST, our values were substantially lower, almost half of the values obtained by

787   their group. This may be a function of a plant-based vs. fishmeal-based diet. Changes to

788   cholesterol were not apparent in our study, but they have been in other studies with wheat

789   gluten as a feed component. Plasma cholesterol decreased in European sea bass fed diets with

790   graded levels of wheat gluten partially replacing up to 70% of the fishmeal as a protein source,

791   but that may be due to the greater proportion of plant protein incorporation rather than an effect

792   unique to wheat gluten (72). Interestingly, a partial replacement of fishmeal with corn as a

793   protein source and no added wheat gluten was shown to raise plasma cholesterol and

794   phospholipid levels. The authors attributed this effect to the higher carbohydrate content in the

795   diet containing corn (73). Different plant sources of protein may have a variety of influences on

796   plasma parameters.

797   The only change in measured tissue weights between diets was for the mid-intestine.

798   There have been no reports of major histological changes to only the mid-intestine prompted by

799   dietary ingredients. However, a gene expression study performed in European sea bass

800   suggested that there is functional specialization across the length of the intestinal tract (47).

801   Another study found that mid-intestine lactic acid bacteria (order *Lactobacillales*) are highly

802   modulated by diets in a recirculating aquaculture system (74).

803   There are no studies correlating wheat gluten to higher levels of taurine in plasma.

804   Recently there have been reports of diets for pets containing legumes marketed as "grain-free"

805   causing cardiomyopathies related to taurine deficiency (75). It is possible that a feed ingredient

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806 such as wheat gluten might be influencing sequestration of taurine in plasma. Alternatively,

807 endogenous levels of taurine may be increased as a result of dietary wheat gluten, perhaps to

808 counter some pro-inflammatory effect of the wheat gluten.

809 The lack of detectable induction of higher levels of plasma IgM, and IgT suggest a lack of

810 adaptive immune response. We also attempted to detect TNF-α in the plasma samples but

811 obtained a high standard error suggesting that our antibody was non-specific. There was no

812 detectable plasma factor capable of binding to gliadin. This is in contrast to our data presented

813 in Chapter 2 for cobia consuming a diet containing less than 4% wheat gluten in which a gliadin-

814 binding plasma factor(s) was produced.

815 The dietary inclusion of wheat gluten induced marked changes to the intestinal

816 microbiome. In the intestines of fish fed 4% wheat gluten, there were increased predominant

817 diversities of Proteobacteria as compared to the 0 wheat gluten group as well as the presence of

818 Bacteroidetes in the mid- and posterior intestines. Several studies have linked dietary changes

819 to alterations in the intestinal microbiome, and a small number have probed for this effect

820 specifically with wheat gluten. Human studies in which participants shifted to a gluten-free diet

821 showed variations to the microbiome, though the most significant variation was inter-patient.

822 One of these studies found a significant decrease in the family *Veillonellaceae* of the *class*

823 *Clostridia* (76)⁻(77). One study in zebrafish in demonstrated that fish fed diets containing wheat

824 gluten (~50% of formulation) had heightened abundances of *Legionellales, Rhizobiaceae,* and

825 *Rhodobacter* over fishmeal-fed fish (78). In another study in zebrafish, fish fed wheat gluten had

826 decreased *Bifidobacterium* relative to fish fed brine shrimp (79). Only an abstract could be

827 located for this study, so the actual percentage of wheat gluten is unknown. In a study of Atlantic

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828   salmon, wheat gluten (~14-20% of formulation) mixed with a legume protein (soybean meal or

829   guar meal) increased abundance of lactic acid bacteria in the gut as compared to the reference

830   fishmeal diet (80). Our data do not appear to correlate with these other studies, but many of the

831   differences are likely attributable to the difference between species and the overall constitution

832   of the diet: Plant-based, fishmeal-based, or a combination. Overall, the study results do not seem

833   to indicate some type of disease state induced by the addition of 4% wheat gluten, as the fish

834   have overall health comparable to that of the fish consuming a dietary containing no wheat

835   gluten.

836   Taken together, these studies suggest that different species and life stages, even among

837   the marine carnivores, may have different reactions to dietary ingredients such as wheat gluten.

838   It is possible that the ability to synthesize taurine is significant in determining tolerance.

839   Preliminary data from another study performed by our laboratory in European sea bass using

840   diets containing 0 or 5% taurine suggest that unlike cobia, European sea bass are adequate

841   endogenous synthesizers of taurine and do not require supplementation. Future studies of the

842   effects of dietary wheat gluten in other marine carnivorous species with various taurine

843   requirements will clarify this connection. Additionally, the observed partial amelioration of the

844   negative effects induced by dietary wheat gluten in cobia should spur future research into

845   taurine's ability to mitigate effects elicited by other general stress factors.

846

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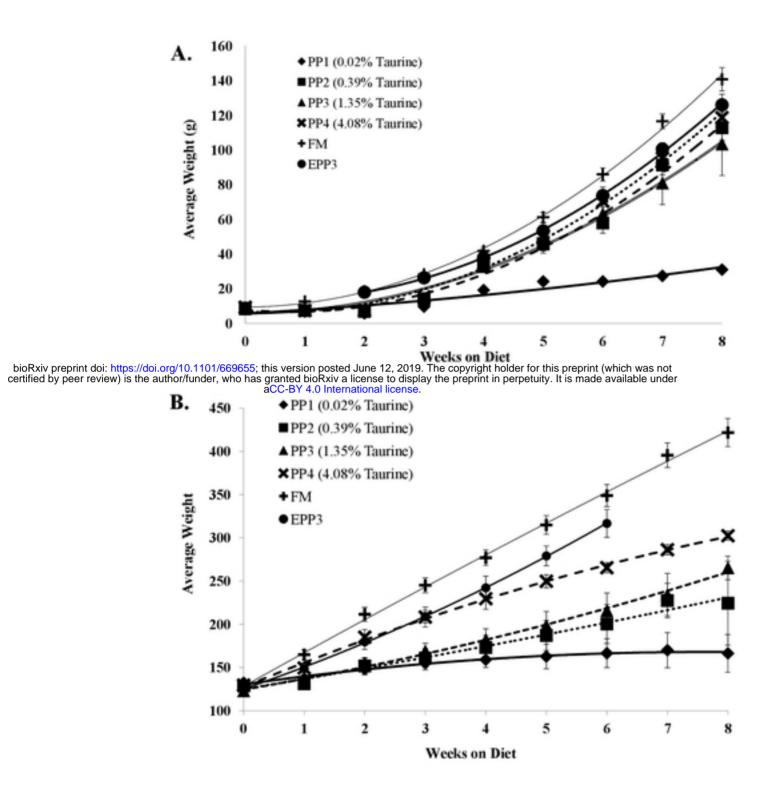890   18.   Danilova N, Bussmann J, Jekosch K, Steiner LA. The immunoglobulin heavy-chain locus in
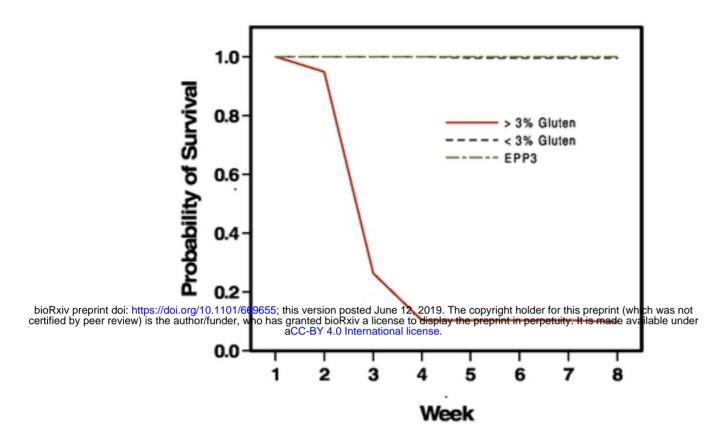
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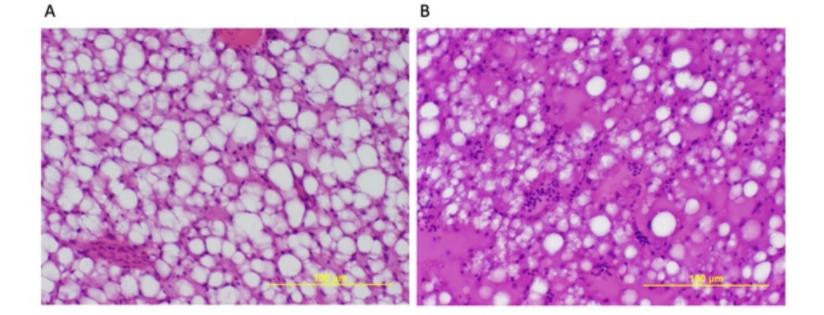978 receptor in celiac disease. J Exp Med. 2008.

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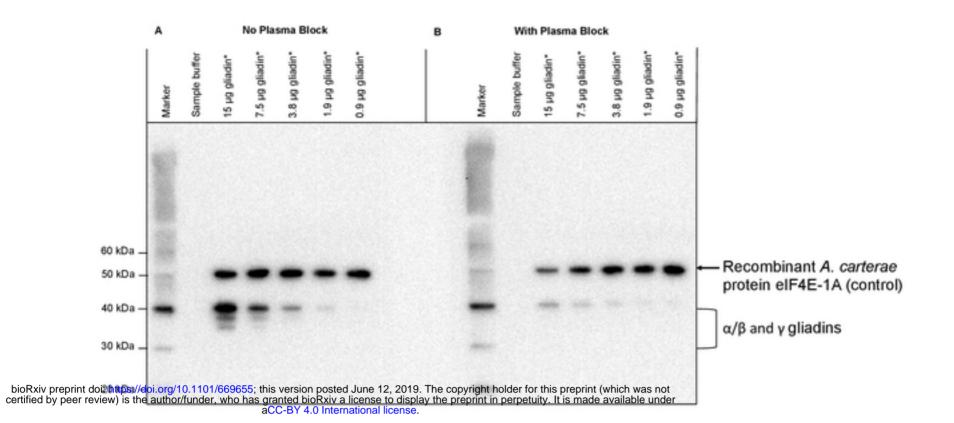
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1043

A.

B.

Figure 1

Figure 2

A   B

Figure 3

A   No Plasma Block   **B**   With Plasma Block

Marker · Sample buffer · 15 µg gliadin* · 7.5 µg gliadin* · 3.8 µg gliadin* · 1.9 µg gliadin* · 0.9 µg gliadin*

60 kDa
50 kDa
40 kDa
30 kDa

→ Recombinant *A. carterae* protein eIF4E-1A (control)

α/β and γ gliadins

Figure 4

Figure 5

Figure 6

KBW: Silverman's -Based h * 0.5

Range of Taurine

No Gluten
Gluten

No Gluten   Gluten

*Statistically different between diets

Figure 7

a 0 Wheat Gluten Plasma Block

b 4% Wheat Gluten Plasma Block

Marker · Sample buffer · 15 µg gliadin* · 7.5 µg gliadin* · 3.8 µg gliadin* · 1.9 µg gliadin* · 0.9 µg gliadin*

50 kDa
40 kDa
30 kDa
20 kDa

← Recombinant *A. carterae* protein eIF4E-1A (control)

α/β and γ gliadins

Figure 8

Figure 9

Order Level Abundance

Figure 10

observed_otus: Name

6-7 anterior intestine fish 4	6-8 anterior intestine fish 8	
6-7 mid intestine fish 4	6-8 anterior intestine fish 9	
6-7 posterior intestine fish 2	6-8 anterior intestine fish 10	Feed from 6-7
6-7 posterior intestine fish 3	6-8 mid intestine fish 8	Feed from 6-8
6-7 posterior intestine fish 4	6-8 mid intestine fish 9	Water from 6-7
6-7 pyloric caeca fish 2	6-8 mid intestine fish 10	Water from 6-8
6-7 pyloric caeca fish 4	6-8 mid intestine fish 8	
	6-8 mid intestine fish 9	
	6-8 posterior intestine fish 10	
	6-8 pyloric caeca fish 8	
	6-8 pyloric caeca fish 9	

Figure 11

Figure 12