Title

Full:

Gluten-free Fish? Marine Carnivores Cobia (*Rachycentron canadum*) and European Sea Bass (*Dicentrarchus labrax*) Have Different Tolerances to Dietary Wheat Gluten

Short:

Gluten-free Fish? Marine Carnivores Have Different Tolerances to Dietary Wheat Gluten

Key words: wheat gluten, taurine, microbiome, cobia, European sea bass, aquaculture, 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.

Introduction

Reducing fishmeal inclusion in favor of more sustainable and cost-effective protein sources is a high priority for the aquaculture industry (1). Aquaculture diets that incorporate low-fat, high-protein concentrates derived from plants such as soy or wheat (including processed wheat gluten) have been described for many species (2)(3). Feed formulations often incorporate wheat gluten as a protein source and for binding and adding a desirable chewy texture to pellets. Wheat flour is processed to remove soluble fibers and starches, and the vital wheat gluten product that remains contains two fractions: soluble gliadins and insoluble glutenins (4)(5). Vital wheat gluten, marketed as a dry powder, regains its elastic properties when rehydrated (6). In addition to being a low-cost protein source, it is useful for the binding together of feed ingredients into granules and pellets. Gliadins are known to trigger an immune response in susceptible people, such as those with celiac disease (7–9).

When plant proteins replace fishmeal, essential amino acids (e.g. lysine, methionine, threonine), as well as various vitamins and minerals, often need to be supplemented to counteract deficiencies in the protein source (10–12). One such necessary nutritional constituent found in fishmeal but is absent in plants is taurine. Some fish species are inadequate synthesizers of taurine and require dietary intake (13). Previous work by our laboratory showed that cobia is one such species, and it is essential to add taurine to a plant-based feed formulation intended for their consumption (14). Findings presented in this work suggest that taurine may help to mitigate some of the deleterious effects prompted by dietary wheat gluten in cobia.

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

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.

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*

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

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.

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

were also taken for histology. Blood plasma was separated by centrifugation (16,000 x g for 20 min at 4 °C) and plasma osmolality measured in triplicate (10 µl) on a Vapro™ Model 5520 vapor pressure osmometer (Wescor, Logan, UT, USA). Plasma samples from three fish per dietary treatment were sent to the Pathology and Laboratory Medicine Services department at the University of California at Los Angeles for constituent analysis. Remaining plasma, fillet, and liver samples were frozen and stored at -80 °C and portions of each were lyophilized to constant weight for water and taurine content analysis. Triplicate samples of each liver (~10 mg), fillet (~50 mg), plasma (~10 µL), and diet (~50 mg) sample were used for taurine extractions based on Chaimbault et al., with samples homogenized in cold 70% EtOH, sonicated for 20 min, dried, and resuspended in 1 ml H_2O prior to injection into the LC-MS (27).

European sea bass

At the conclusion of the 6-month trial, food was withheld for 24 hours and 10-12 fish from each diet were sedated with 25 mg/L MS-222 (Syndel, Ferndale, WA, USA) buffered with 50 mg/L sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA) and exsanguinated via the caudal vein to collect blood for plasma analysis. Following blood collection, the spinal cord was severed, and tissues were harvested for analysis.

Approximately 1 ml blood from the caudal vein was put into a tube containing 20 µL of 1000 units/ml heparin and gently inverted to mix. Samples were centrifuged at 2000 x g for 15 minutes at 4 degrees C and the plasma fraction retained. This processing was sufficient for plasma chemistry and immunoblotting. In preparation for taurine analysis by HPLC, 10 µL plasma was mixed with 90 µL (1:10) 70% ethanol containing 0.154 mM D-norleucine. The solution was

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™ 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™ Ultra Eluent (C) (Waters Corporation, Milford, MA, USA),

ultra-pure water (A) and acetonitrile (B) according to the following gradient: Initial, 98.0% C/2.0% 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% C/9.0% B; 38 min, 40.0% A/60.0% B; 43 min, 98.0% C/2.0% B. Derivatized amino acids were detected at 260 nm using a photo diode array detector. Signals were referenced to AABA (alpha-Aminobutyric acid), D-norleucine, and standard hydrolysate amino acids.

SDS-PAGE and western blotting

A solution of gliadin (Sigma, St. Louis, MO, USA) was made to an original concentration of 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, all in Laemmli sample buffer. All samples were heated to 95 °C for 3 min and centrifuged at 10,000 x g for 1 min prior to electrophoresis. Recombinant *Amphidinium carterae* eIF4A-1A (obtained from Grant Jones at IMET, 7/28/14) was diluted into Laemmli sample buffer to a concentration of 57.5 ng/µl. 15 µl of a 1:1 volume ratio of gliadin and eIF4A was loaded into each lane of a Novex NuPAGE 4-12% Bis-Tris gel and electrophoresed in a Bolt® Mini Gel Tank at 165 V for 1 h with MOPS SDS running buffer (Life Technologies, Frederick, MD, USA).

PVDF membrane was activated by a brief dip in 100% methanol and equilibrated for 5 min in Novex™ NuPAGE® transfer buffer. The gel was electroblotted onto a prepared PVDF in a Bolt Mini Blot Module at 30 V for 1 h in Novex™ NuPAGE® transfer buffer (Life Technologies, Frederick, MD, USA). Following transfer, the membrane was washed in ddH_20 for 5 min. Duplicate transferred lanes were divided and designated as “No Plasma Block” or “With Plasma Block” for subsequent incubation procedures.

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,

Waltham, MA, USA) for 35 minutes at 200 V using MOPS buffer in a PowerPac™ HC Power Supply (Bio-Rad, Hercules, CA, USA. Proteins were transferred to a PVDF membrane for 14 minutes on the high molecular weight setting (25 V) in the Trans-Blot® Turbo™ Transfer System (Bio-Rad, Hercules, CA, USA). Immunoblotting was performed in the iBind™ Western System (ThermoFisher Scientific, Waltham, MA, USA).

For IgT detection, an anti-European sea bass IgT polyclonal antibody (rabbit IgG "RAIgT1," kindly provided by Giuseppe Scapigliati, Tuscia University, Italy) was used at a dilution of 1:1000 as the primary antibody, and goat anti-rabbit IgG H&L HRP conjugate at a dilution of 1:2000 (Bio-Rad, Hercules, CA, USA) was used as the secondary antibody. For IgM detection, the Magic™ anti-European sea bass IgM monoclonal antibody (mouse IgG) (Creative Diagnostics, Shirley, NY, USA) was used at a dilution of 1:1000 as the primary antibody, and goat anti-mouse IgG H&L HRP conjugate (Bio-Rad, Hercules, CA, USA) was used as the secondary antibody at a dilution of 1:2000. A chemiluminescent signal was generated with addition of Clarity™ Western ECL substrate and imaged in a ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA, USA). Image Lab software (Version 5.2.1, Bio-Rad, Hercules, CA, USA) was used to visualize immunoblots and analyze protein molecular weights.

Microbiome analysis in European sea bass

DNA Extraction

For sampling of tank water, 1 L of water was filtered through a pore size of 0.2 microns. For the feed, 0.127 g (3 pellets) of each diet was used. For intestinal samples (pyloric caeca, anterior intestine, mid-intestine, and posterior intestine), ~0.25 g tissue was collected. DNA

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

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.

389

390 Results

391 *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

400 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)

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

For the EPP3 diet in the third trial (initial weight ~18 g), weight gain, FCR, and SGR were much improved from the first two trials, and were an improvement over all previous plant protein diets tested in our laboratory. Performance characteristics included weight gain of 1673.52% ± 192.75, FCR of 1.22 ± 0.04, and SGR of 3.42 ± 0.13. The EPP3 diet contains the lowest gluten content of all diets tested in this study (1.2%).

Wheat gluten: Survival and pathology

Figure 2 presents survival curves based on percent wheat gluten for fish fed diets PP1-PP4 (Trial 1), EPP3 (Trial 3), or fishmeal-based diets FM1-FM4 (averaged) from a previous study (33). These data suggest that wheat gluten inclusion may be deleterious to cobia at this early stage of development, though it may be tolerated at later stages. Histological analysis of the proximal intestinal epithelium showed no distinct differences for fish fed the PP1-PP4 and FM1-FM4 diets. However, pathological effects in the distal intestinal region cannot be ruled out. No histological analysis was performed on the EPP3 fish. Survival was approximately 100% for fish in Trial 2 fed the PP1-PP4 diets and in Trial 3 with the EPP3 diet. Cannibalism was not observed to be a contributing factor to the low survival in Trial 1, and dead individuals were promptly removed from the tanks so as to not be a nutritional/taurine source for remaining fish.

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

Muscle and liver characteristics

Fillet and liver characteristics from the second and third trials are shown in S6 Table: Fillet and liver characteristics from the second and third cobia growth trials. Fillet water content was highest in fish fed the PP1 diet with a gradual reduction in fillet water content as dietary taurine level increased. Hepatosomatic index show showed a similar trend of reduction as dietary taurine level increased. Fillet yield, liver water, fillet taurine, and liver taurine contents all showed increasing trends with increasing dietary taurine levels.

Plasma analysis

Results of the plasma analysis are shown in Table 3. Plasma water content significantly decreased as dietary taurine level increased ($p<0.05$). Concentrations of glucose, calcium, phosphorus, and magnesium ions, as well as overall osmolality, are indicators of health status. Total protein levels also often correlate with nutritional status and general health (34),(35). Triglycerides are an energy substrate, and a fasting study in European sea bass demonstrated their potential as a marker for nutritional condition (36). AST (aspartate aminotransferase) and ALP (alkaline phosphatase) are indicators of liver health and function (37).

Plasma taurine levels were significantly lower in diets PP1 and PP2 than the other diets ($p<0.05$). Plasma cholesterol, phosphorous, and albumin all showed similar trends of significantly increasing with dietary taurine level ($p<0.05$). Lower creatine kinase levels can be indicative of muscle wasting (38). Though biochemical markers of liver failure are not well characterized in fish, elevated bilirubin is a classic sign (39). Stress on the liver may be a result of insufficient taurine required for bile acid conjugation or a manifestation of gluten-induced enteritis (40). The 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

T=Taurine

[1] UCLA DLAM analysis

[2] NOAA-NWFSC analysis by A.W. (excluding water content, osmolality, and taurine)

Liver histology

Figure 3 shows H&E-stained liver sections from Trial 2 fish fed PP1 (Panel A) or PP4 (Panel B). Supplementation with taurine prevents pervasive steatosis. This effect was also seen in fish fed FM1 vs. FM4 (data not shown). Taurine supplementation may have therapeutic potential in the treatment of nonalcoholic fatty liver disease, suggesting that supplementation may be of value even in cases where there is not an underlying deficiency (42).

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

Detection of immune factors capable of binding to wheat gluten

Suspecting that wheat gluten might be a contributing factor to poor growth performance, we assayed in Trial 2 fish plasma an immune response to gliadin, a potentially immunogenic component of wheat gluten. Standard methods for detecting specific antibody responses to wheat gluten in human celiac patients cannot be utilized as they are designed to detect IgA. Cobia and other teleosts have only three classes of immunoglobulins: IgM, IgT, and IgD, and there are currently no reagents to detect these immunoglobulins in cobia (43). It is not known if antibodies designed to detect these adaptive factors have cross-reactivity to these immune components in cobia.

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.

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

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

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

approximately twice as high in the fish fed the diet containing wheat gluten (Figure 7). One possible explanation is that the fish are producing or sequestering more taurine to counter some effect induced by the wheat gluten.

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

Gliadin immunoblotting

To detect plasma factors capable of binding to gliadin, varying concentrations of the protein as well as a fixed amount of eIF4E-1A from the dinoflagellate *A. carterae* were subjected to immunoblotting. eIF4E-1A, a translation protein, was used as a control for loading, transfer efficiency, and specificity of binding. After immunoblotting, membranes were pre-incubated in blocking buffer only or blocking buffer containing plasma. In the immunoblot, bands are visible in the 30-40 kDa range corresponding to various α/β and γ gliadins (Figure 8) (44). These gliadin bands are less visible over the course of 2-fold dilutions. There does not appear to be a component in European sea bass plasma produced in response to dietary wheat gluten that is capable of binding gliadin. This would be evidenced by decreased binding of the primary antibody, and subsequently the secondary signal antibody. This is in contrast to data presented in Chapter 2 showing that juvenile cobia produce a plasma factor capable of binding gliadin when they are fed a diet containing 3.2-3.6% wheat gluten.

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

Ig and IgM

We used immunoblotting to assay for IgT and IgM to see if dietary inclusion of wheat gluten alters their levels in plasma. The results shown in Figure 9 suggest that levels do not change in response to dietary wheat gluten. Any immune response mounted against gliadin or some other component of wheat gluten is not likely due to an adaptive response, in this case. The size of ~73 kDa for the heavy chains of the antibodies is similar to the size of ~78 kDa detected by Picchietti et al. (45).

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

Intestinal microbiome analysis

To characterize the microbiome of the water, feed, and intestinal sections of the European sea bass, 16S rRNA gene analysis was performed using the MiSeq platform. Figure 10 shows a bar graph of order-level taxonomic abundance. Proteobacteria dominate in the tank water. The "cyanobacteria" in feed are most likely chloroplasts from plant ingredients. This is also true for "cyanobacteria" in the pyloric caeca, which likely corresponds to undigested feed despite the fact that food was withheld from these fish for 24 hours before tissue sampling. For 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

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

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

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

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

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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852

853 1. Olsen RL, Hasan MR. A limited supply of fishmeal: Impact on future increases in global
854 aquaculture production. Trends in Food Science and Technology. 2012.
855 2. Rust MB, Barrows FT, Hardy RW, Lazur A, Naughten K, Silverstein J. The Future of
856 Aquafeeds. NOAA Tech Memo NMFS F/SPO-124. 2011.
857 3. Hardy RW. New developments in aquatic feed ingredients , and potential of enzyme
858 supplements. Av en Nutr Acuicola V Memorias del V Simp Int Nutr Acuicola. 2000.
859 4. Apper-Bossard E, Feneuil A, Wagner A, Respondek F. Use of vital wheat gluten in
860 aquaculture feeds. Aquatic Biosystems. 2013.
861 5. Wieser H. Chemistry of gluten proteins. Food Microbiol. 2007.
862 6. Day L, Augustin MA, Batey IL, Wrigley CW. Wheat-gluten uses and industry needs. Trends
863 in Food Science and Technology. 2006.
864 7. Gujral N, Freeman HJ, Thomson ABR. Celiac disease: Prevalence, diagnosis, pathogenesis
865 and treatment. World J Gastroenterol. 2012.
866 8. Sollid LM. Coeliac disease: Dissecting a complex inflammatory disorder. Nature Reviews
867 Immunology. 2002.
868 9. Di Sabatino A, Corazza GR. Coeliac disease. The Lancet. 2009.
869 10. Barrows FT, Gaylord TG, Sealey WM, Porter L, Smith CE. The effect of vitamin premix in
870 extruded plant-based and fish meal based diets on growth efficiency and health of
871 rainbow trout, *Oncorhynchus mykiss*. Aquaculture. 2008.
872 11. Gibson Gaylord T, Barrows FT, Teague AM, Johansen KA, Overturf KE, Shepherd B.
873 Supplementation of taurine and methionine to all-plant protein diets for rainbow trout
874 (*Oncorhynchus mykiss*). Aquaculture. 2007.
875 12. Zhou QC, Wu ZH, Chi SY, Yang QH. Dietary lysine requirement of juvenile cobia
876 (*Rachycentron canadum*). Aquaculture. 2007.
877 13. Salze GP, Davis DA. Taurine: A critical nutrient for future fish feeds. Aquaculture. 2015.
878 14. Watson AM, Kissil GWM, Barrows FT, Place A. Developing a plant-based diet for cobia
879 (*Rachycentron canadum*). Int Aquafeed. 2012;15:34–8.
880 15. Bagni M. Cultured Aquatic Species Information Programme. *Dicentrarchus labrax*.
881 [Internet]. FAO Fisheries and Aquaculture Department. 2005. Available from:
882 http://www.fao.org/fishery/culturedspecies/Dicentrarchus_labrax/en
883 16. Kaushik SJ, Covès D, Dutto G, Blanc D. Almost total replacement of fish meal by plant
884 protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus*
885 *labrax*. Aquaculture. 2004.
886 17. Geay F, Ferraresso S, Zambonino-Infante JL, Bargelloni L, Quentel C, Vandeputte M, et al.
887 Effects of the total replacement of fish-based diet with plant-based diet on the hepatic
888 transcriptome of two European sea bass (*Dicentrarchus labrax*) half-sibfamilies showing
889 different growth rates with the plant-based diet. BMC Genomics. 2011.
890 18. Danilova N, Bussmann J, Jekosch K, Steiner LA. The immunoglobulin heavy-chain locus in

zebrafish: Identification and expression of a previously unknown isotype, immunoglobulin Z. Nat Immunol. 2005.

19. Hansen JD, Landis ED, Phillips RB. Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. Proc Natl Acad Sci. 2005.
20. Xu Z, Parra D, Gomez D, Salinas I, Zhang Y-A, von Gersdorff Jorgensen L, et al. Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. Proc Natl Acad Sci. 2013.
21. Egerton S, Culloty S, Whooley J, Stanton C, Ross RP. The gut microbiota of marine fish. Frontiers in Microbiology. 2018.
22. Ringø E, Sperstad S, Myklebust R, Refstie S, Krogdahl Å. Characterisation of the microbiota associated with intestine of Atlantic cod (*Gadus morhua*). Aquaculture. 2006;
23. Green TJ, Smullen R, Barnes AC. Dietary soybean protein concentrate-induced intestinal disorder in marine farmed Atlantic salmon, *Salmo salar* is associated with alterations in gut microbiota. Vet Microbiol. 2013.
24. Guerreiro I, Serra CR, Oliva-Teles A, Enes P. Short communication: gut microbiota of European sea bass (*Dicentrarchus labrax*) is modulated by short-chain fructooligosaccharides and xylooligosaccharides. Aquac Int. 2018.
25. Huebner FR, Wall JS, Regional N. Fractionation and Quantitative Differences of Glutenin from Wheat Varieties Varying in Baking Quality. Cereal Chem. 1974.
26. Coleman BR, Iqbal S, Godfrey PP, Billington D, Box PO, Birmingham B. Membranes and Bile Formation: Composition of Several Mammalian Biles and their Membrane-Damaging Properties. J Biochem. 1979.
27. Chaimbault P, Alberic P, Elfakir C, Lafosse M. Development of an LC-MS-MS method for the quantification of taurine derivatives in marine invertebrates. Anal Biochem. 2004.
28. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 2013.
29. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nature Methods. 2010.
30. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res. 2013.
31. Caporaso JG, Bittinger K, Bushman FD, Desantis TZ, Andersen GL, Knight R. PyNAST: A flexible tool for aligning sequences to a template alignment. Bioinformatics. 2010.
32. R Development Core Team R. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. 2011.
33. Watson AM, Barrows FT, Place AR. Effects of Graded Taurine Levels on Juvenile Cobia. N Am J Aquac. 2014;
34. Rehulka J, Minarik B, Adamec V, Rehulkova E. Investigations of physiological and pathological levels of total plasma protein in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquac Res. 2005.
35. Maita M. Dietary Supplements for the Health and Quality of Cultured Fish. Fish Heal Assessment, Intl. 2007;10–34.

36. Peres H, Santos S, Oliva-Teles A. Blood chemistry profile as indicator of nutritional status in European seabass (*Dicentrarchus labrax*). Fish Physiol Biochem. 2014.
37. Gowda S, Desai PB, Hull V V, Math AAK, Vernekar SN, Kulkarni SS. A review on laboratory liver function tests. Pan Afr Med J. 2009.
38. Rosalki SB. Low serum creatine kinase activity. Clinical chemistry. 1998.
39. P Sharma O. Clinical Biochemistry of Hepatotoxicity. J Clin Toxicol. 2014.
40. Wyllie R, Hyams JS, Kay M. Pediatric Gastrointestinal and Liver Disease. Pediatric Gastrointestinal and Liver Disease. 2006.
41. Eames SC, Philipson LH, Prince VE, Kinkel MD. Blood Sugar Measurement in Zebrafish Reveals Dynamics of Glucose Homeostasis. Zebrafish. 2010.
42. Gentile CL, Nivala AM, Gonzales JC, Pfaffenbach KT, Wang D, Wei Y et al. Experimental evidence for therapeutic potential of taurine in the treatment of nonalcoholic fatty liver disease. Am J Physiol Regul Integr Comp Physiol. 2011;301(6):1710–22.
43. Fillatreau S, Six A, Magadan S, Castro R, Sunyer JO, Boudinot P. The astonishing diversity of Ig classes and B cell repertoires in teleost fish. Frontiers in Immunology. 2013.
44. Urade R, Sato N, Sugiyama M. Gliadins from wheat grain: an overview, from primary structure to nanostructures of aggregates. Biophysical Reviews. 2018.
45. Picchietti S, Nuñez-Ortiz N, Stocchi V, Randelli E, Buonocore F, Guerra L, et al. Evolution of lymphocytes. Immunoglobulin T of the teleost sea bass (*Dicentrarchus labrax*): Quantitation of gene expressing and immunoreactive cells. Fish Shellfish Immunol. 2017.
46. Glencross BD, Booth M, Allan GL. A feed is only as good as its ingredients - A review of ingredient evaluation strategies for aquaculture feeds. Aquaculture Nutrition. 2007.
47. Gómez-Requeni P, Mingarro M, Calduch-Giner JA, Médale F, Martin SAM, Houlihan DF, et al. Protein growth performance, amino acid utilisation and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). Aquaculture. 2004.
48. Unsworth DJ, Würzner R, Brown DL, Lachmann PJ. Extracts of wheat gluten activate complement via the alternative pathway. Clin Exp Immunol. 1993.
49. Moossavi S. Gliadin is an uncatalogued Toll-like receptor ligand. J Med Hypotheses Ideas. 2014.
50. Briani C, Samaroo D, Alaedini A. Celiac disease: From gluten to autoimmunity. Autoimmunity Reviews. 2008.
51. Kuiper HA KG. The scientific basis for risk assessment and regulation of genetically modified foods. Trends Food Sci Technol. 2003;14(5):277–93.
52. Penttila IA, Devery JM, Gibson CE, LaBrooy JT, Skerritt JH. Cellular and humoral responses in coeliac disease. 1. Wheat protein fractions. Clin Chim Acta. 1991.
53. Rivabene R, Mancini E, De Vincenzi M. In vitro cytotoxic effect of wheat gliadin-derived peptides on the Caco-2 intestinal cell line is associated with intracellular oxidative imbalance: Implications for coeliac disease. Biochim Biophys Acta - Mol Basis Dis. 1999.
54. Heyman M, Menard S. Pathways of gliadin transport in celiac disease. Ann N Y Acad Sci. 2009.
55. Matysiak-Budnik T, Moura IC, Arcos-Fajardo M, Lebreton C, Ménard S, Candalh C, et al. Secretory IgA mediates retrotranscytosis of intact gliadin peptides via the transferrin receptor in celiac disease. J Exp Med. 2008.

56. Gianfrani C, Siciliano RA, Facchiano AM, Camarca A, Mazzeo MF, Costantini S, et al. Transamidation of Wheat Flour Inhibits the Response to Gliadin of Intestinal T Cells in Celiac Disease. Gastroenterology. 2007.
57. Smith DL, Barry RJ, Powell ML, Nagy TR, D'Abramo LR, Watts SA. Dietary Protein Source Influence on Body Size and Composition in Growing Zebrafish. Zebrafish. 2013.
58. Day RD, Tibbetts IR, Secor SM. Physiological responses to short-term fasting among herbivorous, omnivorous, and carnivorous fishes. J Comp Physiol B Biochem Syst Environ Physiol. 2014.
59. Fleckenstein B, Molberg Ø, Qiao SW, Schmid DG, Von Mülbe F Der, Elgstøen K, et al. Gliadin T cell epitope selection by tissue transglutaminase in celiac disease. Role of enzyme specificity and pH influence on the transamidation versus deamidation reactions. J Biol Chem. 2002.
60. Çetiner M, Şener G, Şehirli AÖ, Ekşioğlu-Demiralp E, Ercan F, Şirvanci S, et al. Taurine protects against methotrexate-induced toxicity and inhibits leukocyte death. Toxicol Appl Pharmacol. 2005.
61. Romarheim OH, Overland M, Mydland LT, Skrede A, Landsverk T. Bacteria Grown on Natural Gas Prevent Soybean Meal-Induced Enteritis in Atlantic Salmon. J Nutr. 2011.
62. Urán PA, Gonçalves AA, Taverne-Thiele JJ, Schrama JW, Verreth JAJ, Rombout JHWM. Soybean meal induces intestinal inflammation in common carp (*Cyprinus carpio L*.). Fish Shellfish Immunol. 2008.
63. Son M, Kim HK, Kim WB, Yang J, Kim BK. Protective effect of taurine on indomethacin-induced gastric mucosal injury. Arch Pharm Res. 1996.
64. Ripps H, Shen W. Review: Taurine: A "very essential" amino zcid. Mol Vis. 2012;
65. Myléus A, Ivarsson A, Webb C, Danielsson L, Hernell O, Högberg L, et al. Celiac disease revealed in 3% of Swedish 12-year-olds born during an epidemic. J Pediatr Gastroenterol Nutr. 2009.
66. Cook AM, Denger K. Metabolism of taurine in microorganisms: A primer in molecular biodiversity? In: Advances in Experimental Medicine and Biology. 2006.
67. Backus RC, Rogers QR, Morris JG. Microbial degradation of taurine in fecal cultures from cats given commercial and purified diets. J Nutr. 1994.
68. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014.
69. Sjöberg V, Sandström O, Hedberg M, Hammarström S, Hernell O, Hammarström ML. Intestinal T-cell Responses in Celiac Disease - Impact of Celiac Disease Associated Bacteria. PLoS One. 2013.
70. Roque A, Yildiz HY, Carazo I, Duncan N. Physiological stress responses of sea bass (*Dicentrarchus labrax*) to hydrogen peroxide (H2O2) exposure. Aquaculture. 2010.
71. Al-Khashali MS, Al-Shawi SAS. Effect of salt stress on ALT and AST enzymes activity and cortisol level in adults of *Carassius auratus*. Pakistan J Nutr. 2013.
72. Messina M, Piccolo G, Tulli F, Messina CM, Cardinaletti G, Tibaldi E. Lipid composition and metabolism of European sea bass (*Dicentrarchus labrax L*.) fed diets containing wheat gluten and legume meals as substitutes for fish meal. Aquaculture. 2013.
73. Castro, C, Corraze G, Perez-Jimenez A, Larroquet L, Cluzeaud M, Panserat S O-TA. Dietary carbohydrate and lipid source affect cholesterol metabolism of Eu.opean sea bass

(*Dicentrarchus labrax*) juveniles. Br J Nutr. 2015;114(8):1143–56.

74. Schmidt V, Amaral-Zettler L, Davidson J, Summerfelt S, Good C. Influence of fishmeal-free diets on microbial communities in Atlantic Salmon (*Salmo Salar*) recirculation aquaculture systems. Appl Environ Microbiol. 2016.

75. Freeman LM. A broken heart: Risk of heart disease in boutique or grain-free diets and exotic ingredients [Internet]. 2018. Available from: http://vetnutrition.tufts.edu/2018/06/a-broken-heart-risk-of-heart-disease-in-boutique-or-grain-free-diets-and-exotic-ingredients/

76. Bonder MJ, Tigchelaar EF, Cai X, Trynka G, Cenit MC, Hrdlickova B, et al. The influence of a short-term gluten-free diet on the human gut microbiome. Genome Med. 2016.

77. Nylund L, Kaukinen K, Lindfors K. The microbiota as a component of the celiac disease and non-celiac gluten sensitivity. Clin Nutr Exp. 2016.

78. Koo H, Hakim JA, Powell ML, Kumar R, Eipers PG, Morrow CD, et al. Metagenomics approach to the study of the gut microbiome structure and function in zebrafish *Danio rerio* fed with gluten formulated diet. J Microbiol Methods. 2017.

79. Savarese K, Davis Tousignant K, Uno J. The effect of wheat products on the colonization of the microbiota in the intestines of zebrafish (903.2). FASEB. 2014;28(1).

80. Gajardo K, Jaramillo-Torres A, Kortner TM, Merrifield DL, Tinsley J, Bakke AM, et al. Alternative protein sources in the diet modulate microbiota and functionality in the distal intestine of Atlantic salmon (*Salmo salar*). Appl Environ Microbiol. 2017.

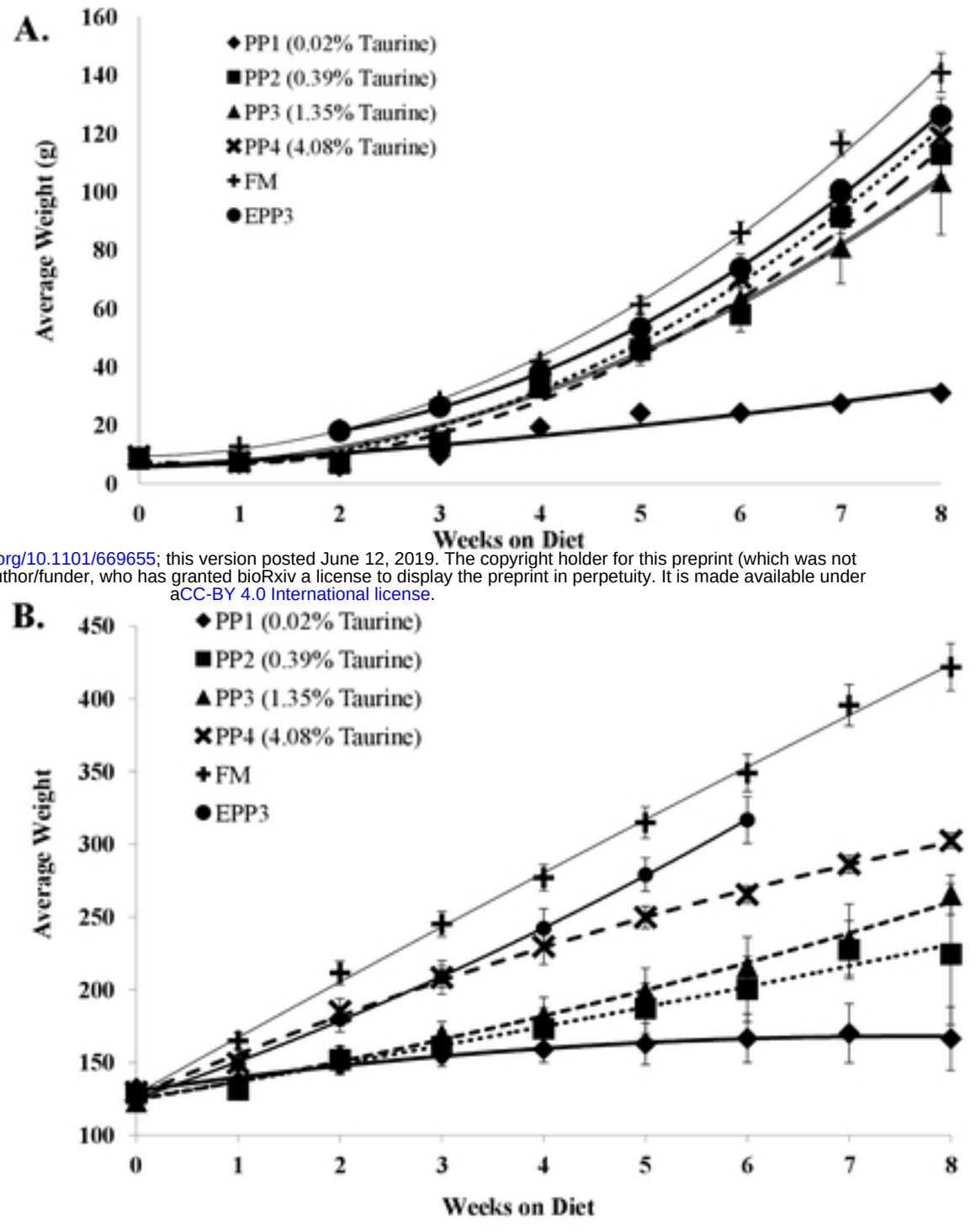


Figure 1

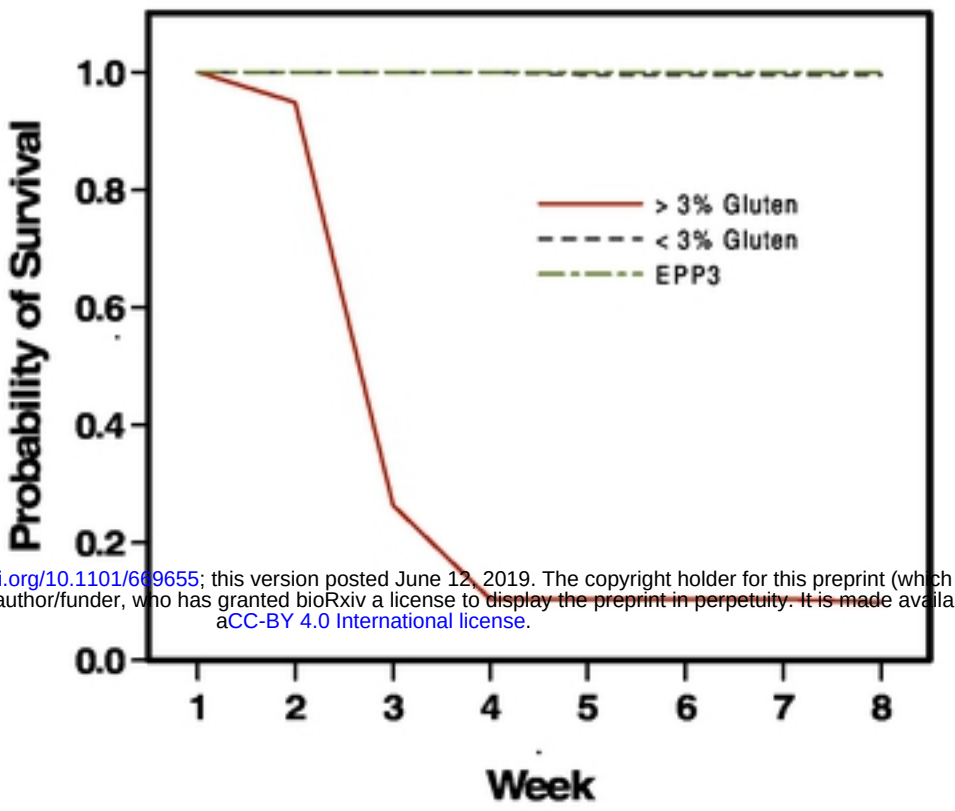


Figure 2

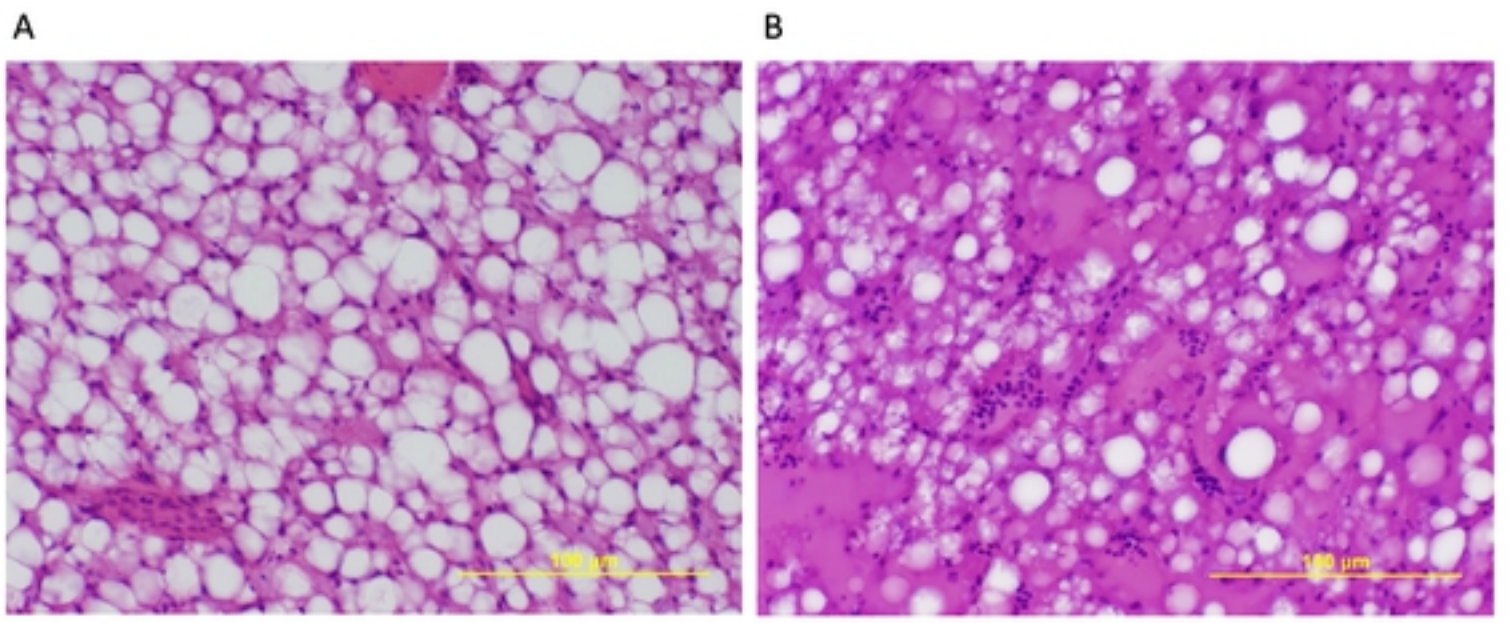


Figure 3

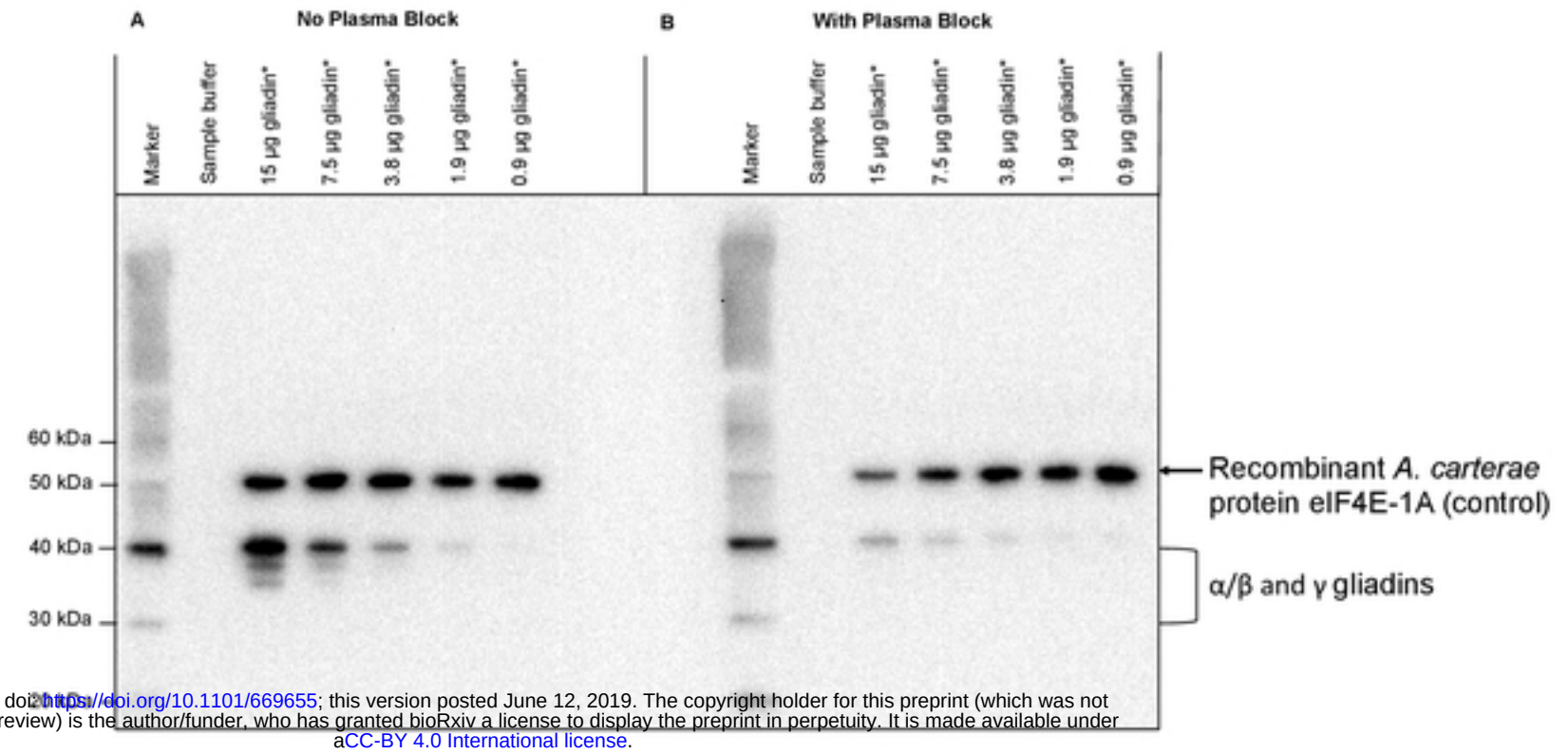


Figure 4

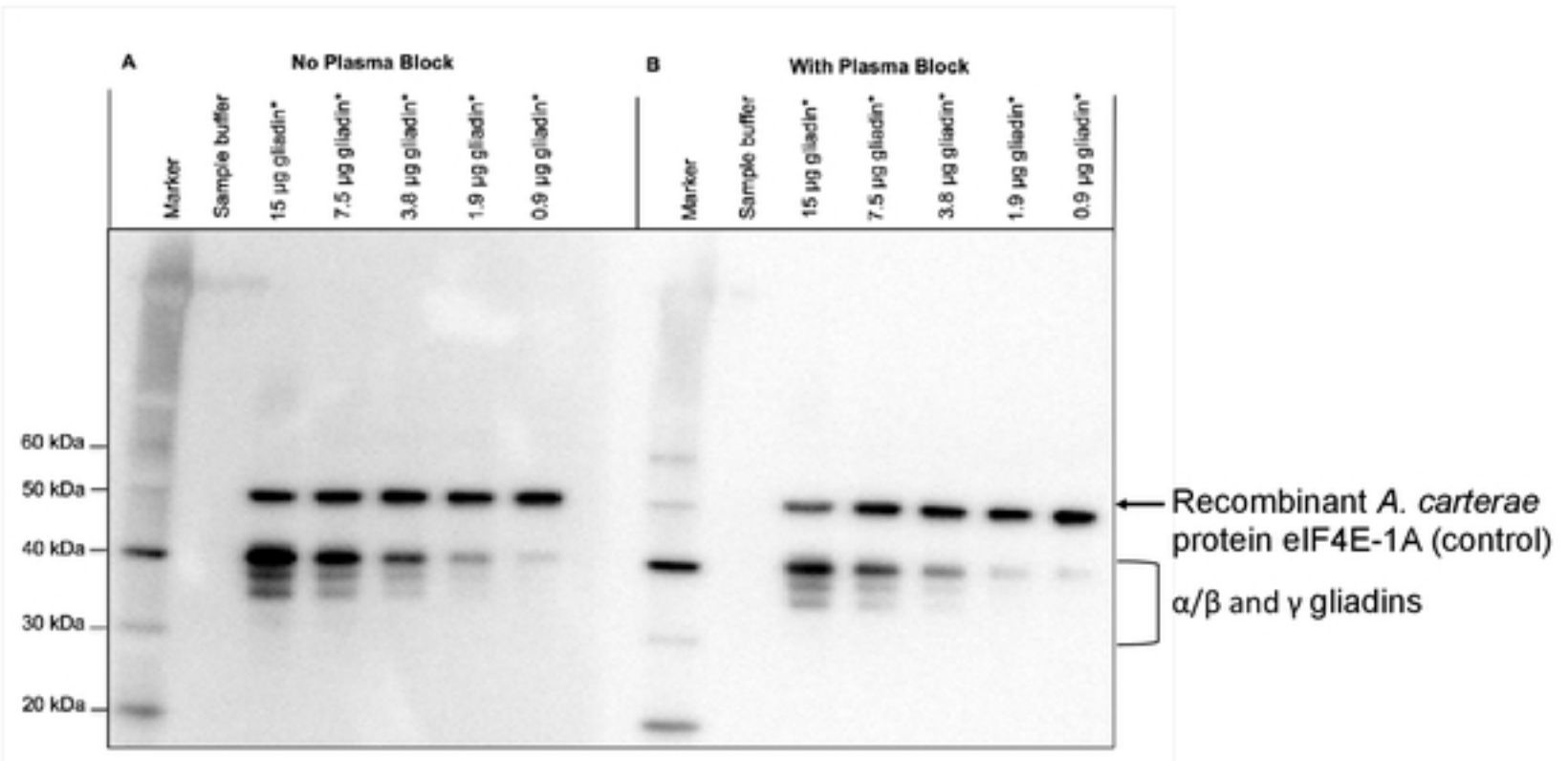
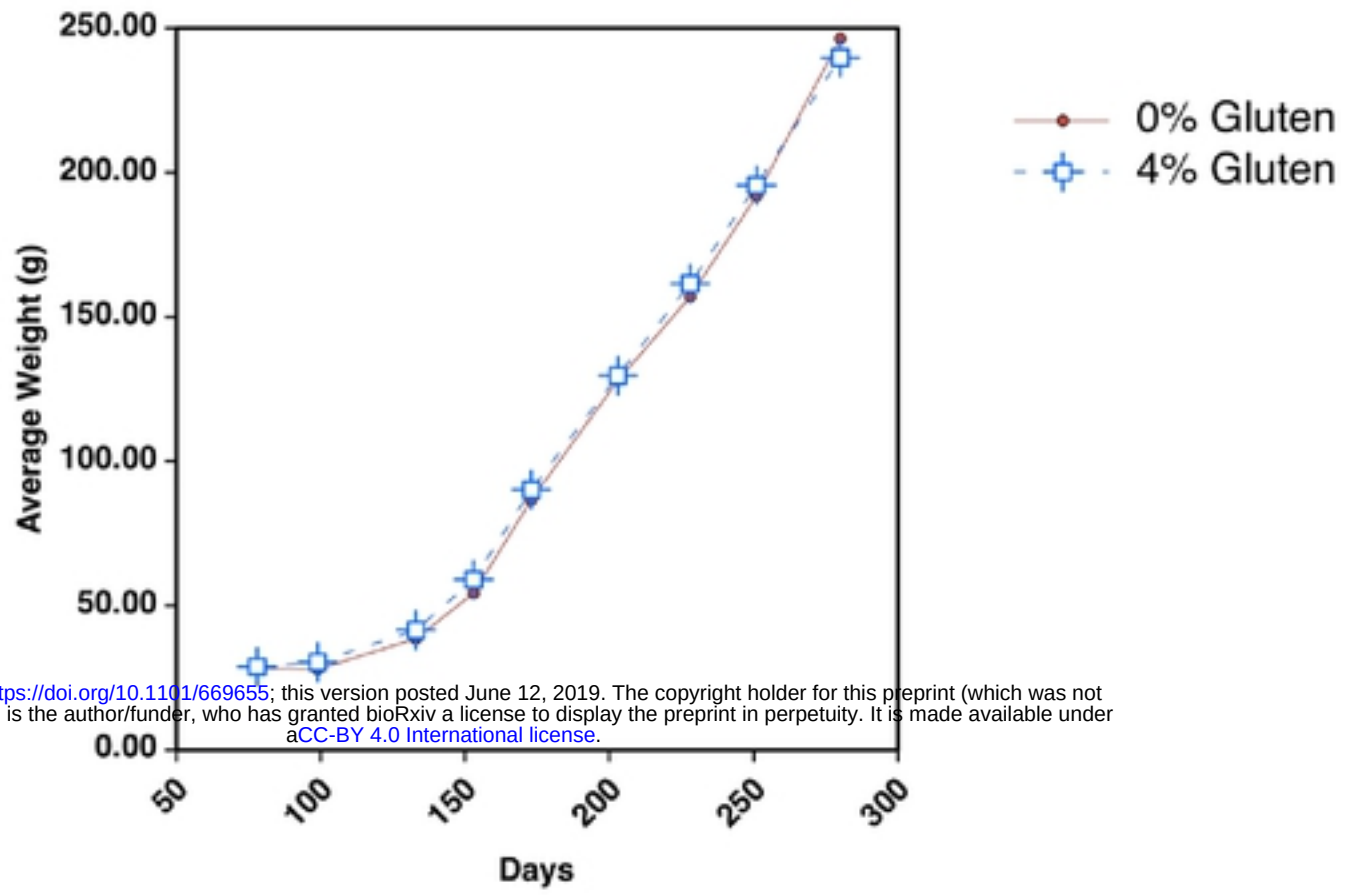


Figure 5



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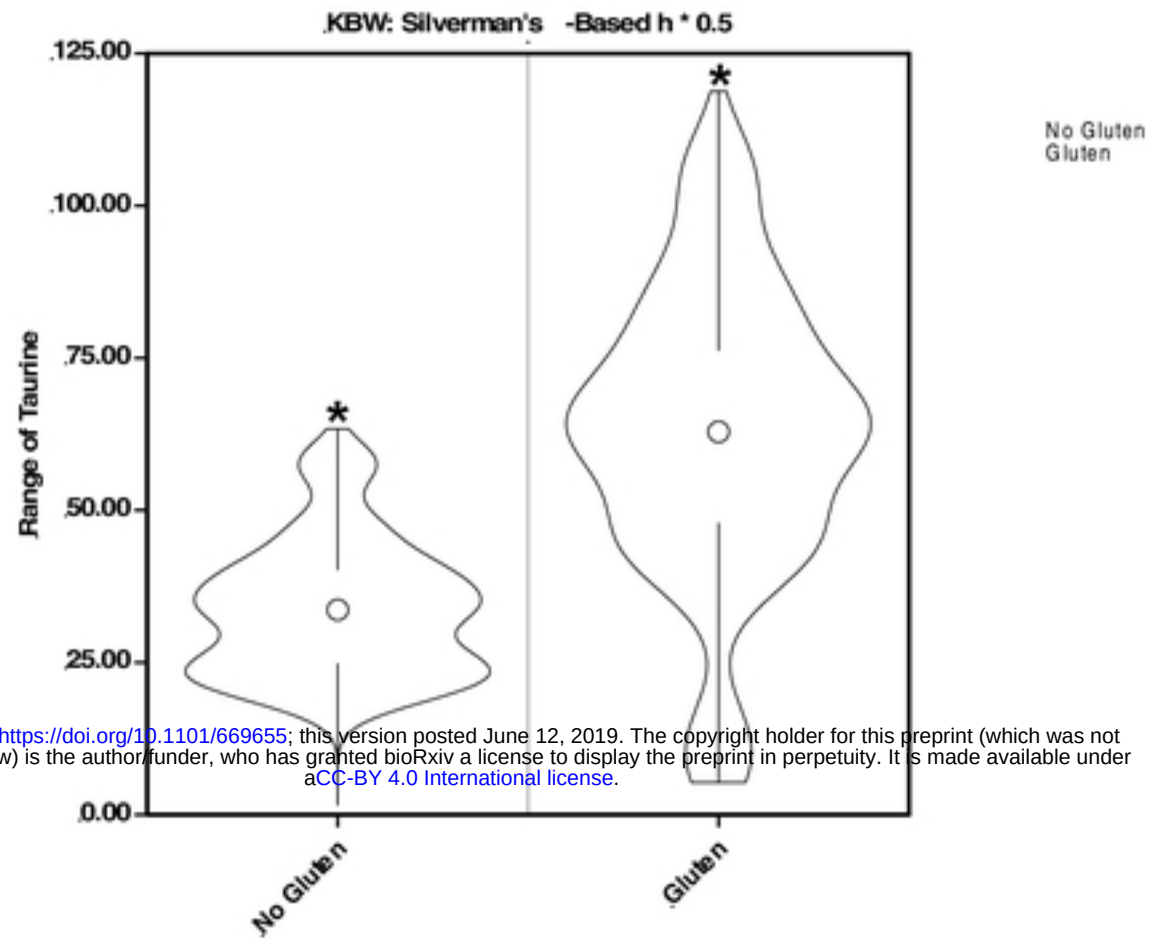
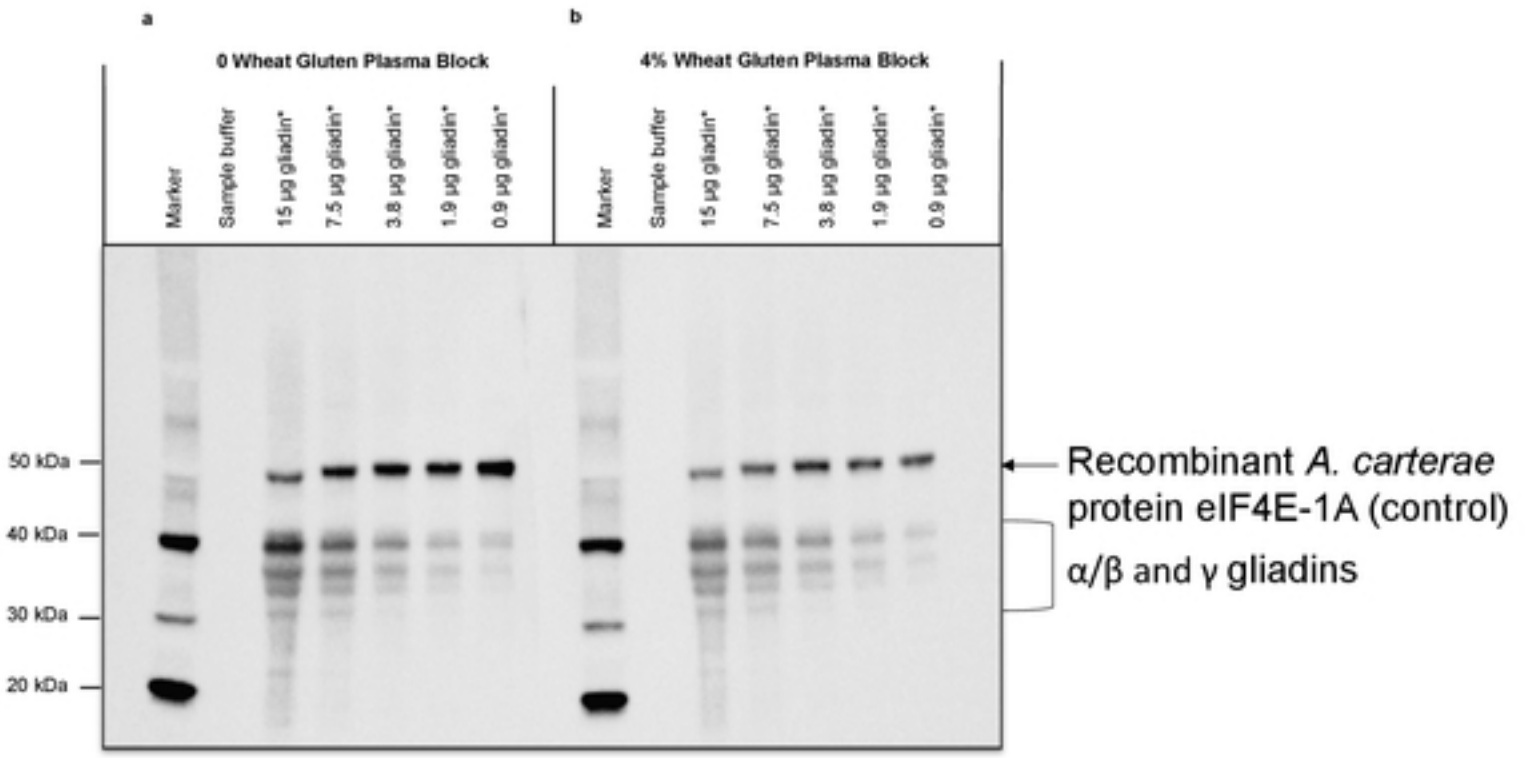


*Statistically different between diets

Figure 7



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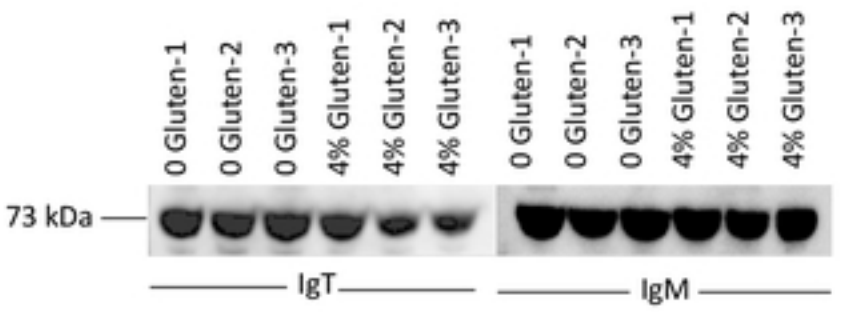

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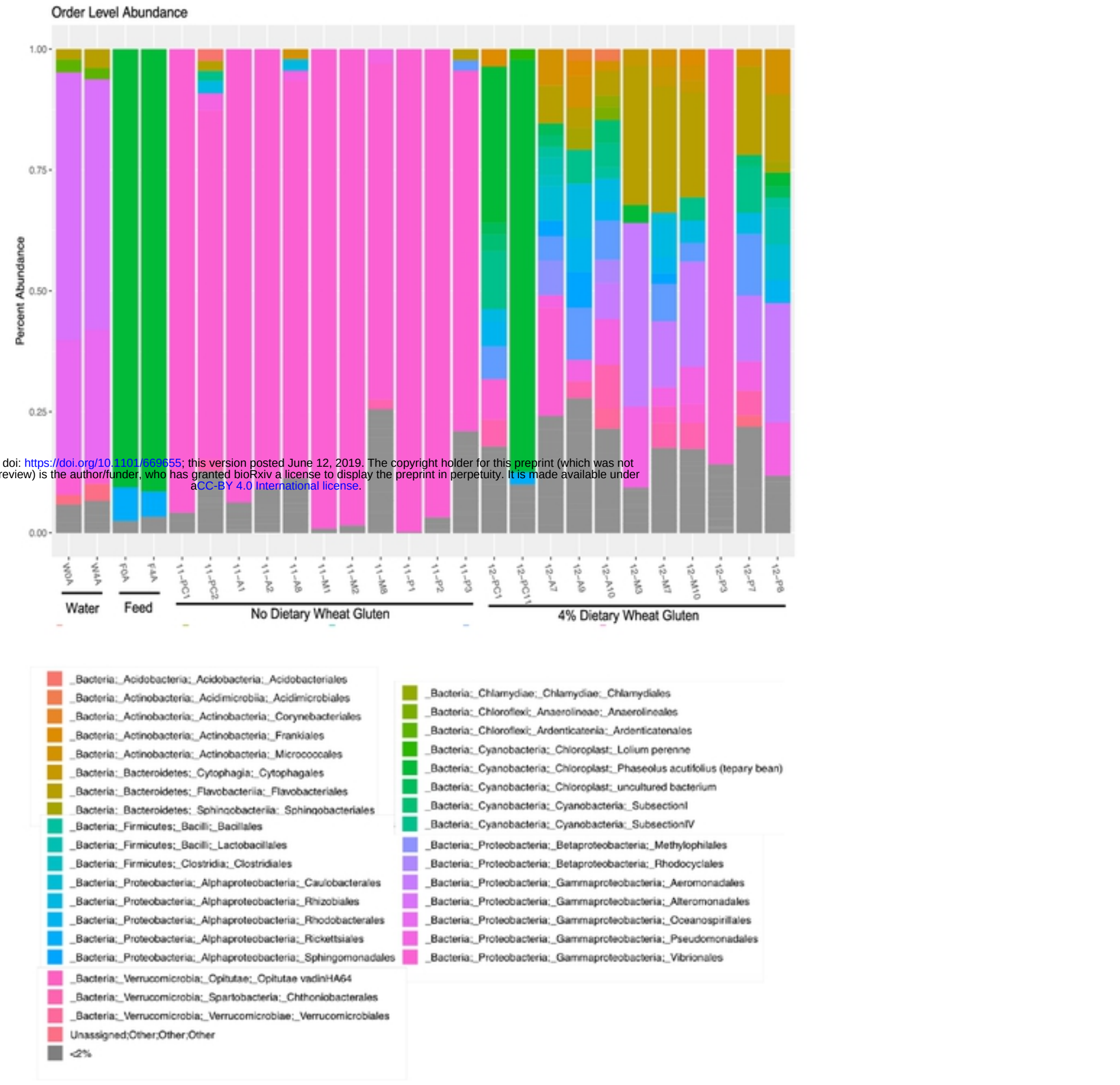

Figure 10

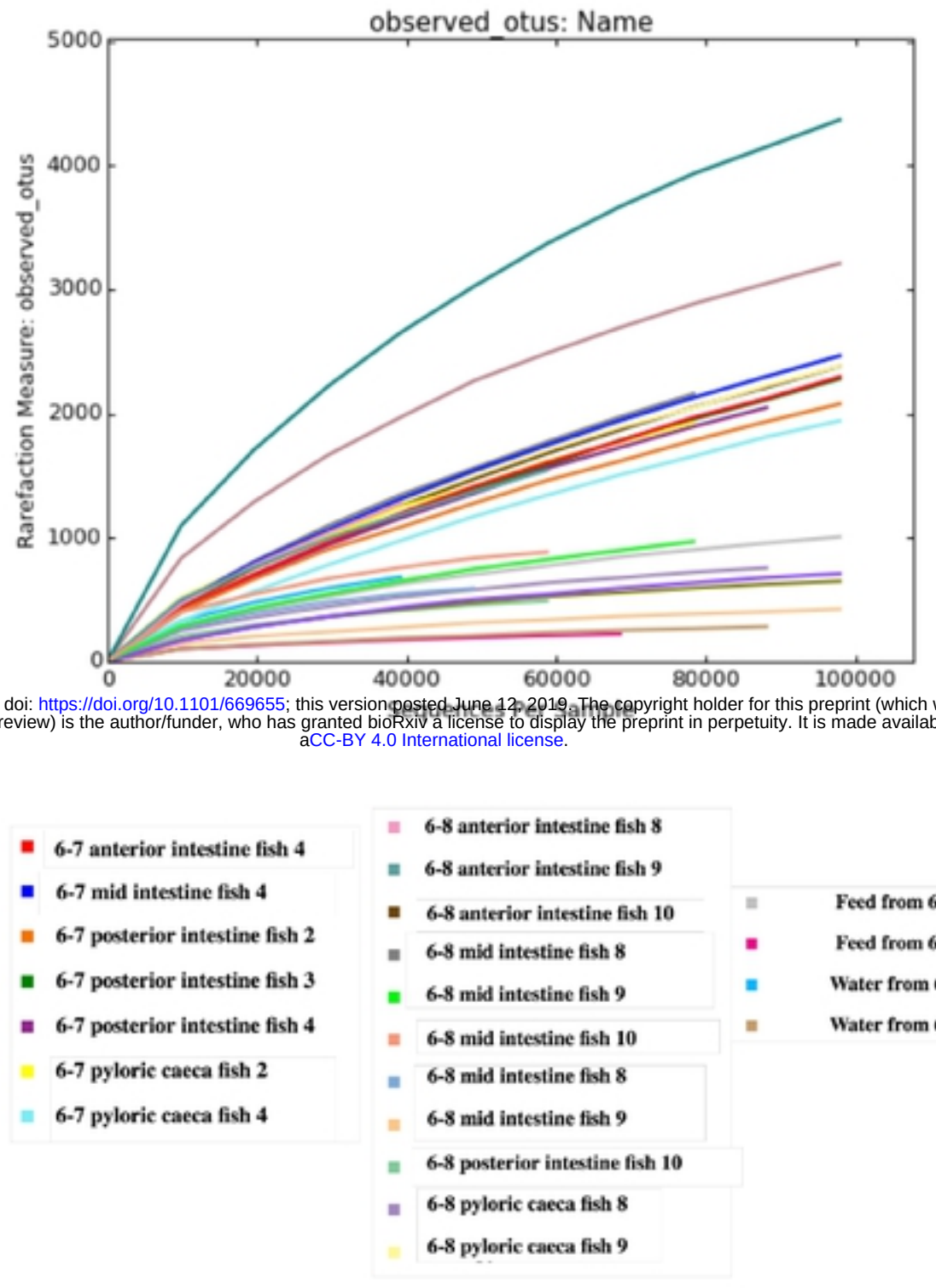

Figure 11

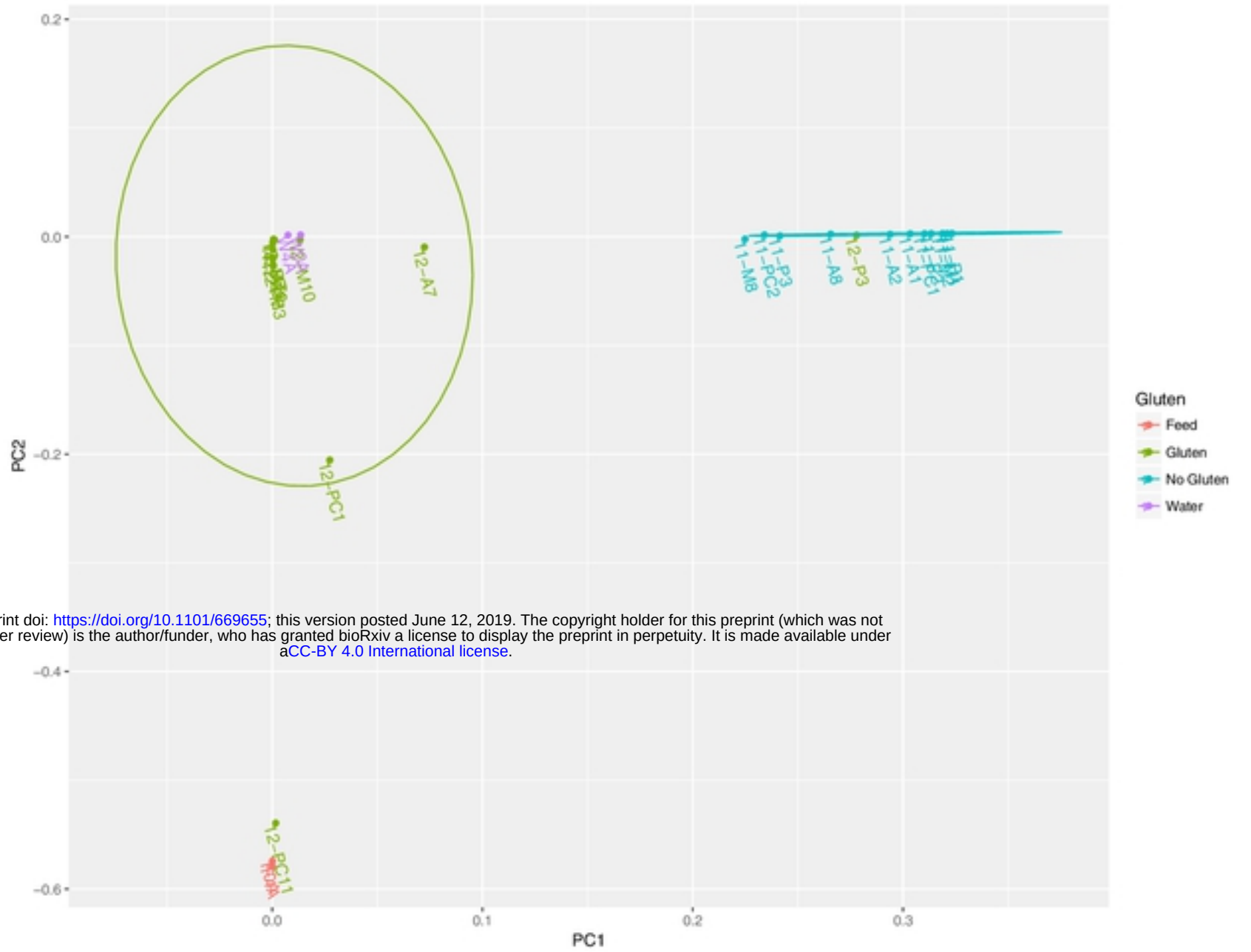

Figure 12