Effects of a plant-based low-fishmeal diet on digestive physiology in yellowtail *Seriola quinqueradiata*

Koji Murashita[1*], Hiroyuki Matsunari[1], Haruhisa Fukada[2], Nobuhiro Suzuki[3], Hirofumi Furuita[1], Hiromi Oku[1], Ivar Rønnestad[4], Hazuki Yoshinaga[1], Takeshi Yamamoto[1]

[1]Research Center for Aquaculture Systems, National Research Institute of Aquaculture, Japan Fisheries Research and Education Agency, 224-1 Hiruda, Tamaki, Mie 519-0423, Japan

[2]Laboratory of Fish Nutrition, Faculty of Agriculture, Kochi University, 200 Monobe, Nankoku, Kochi 783-8502, Japan

[3]Department of Fisheries, School of Marine Science and Technology, Tokai University, Shimizu, Shizuoka 424-8610, Japan

[4]Department of Biological Sciences, University of Bergen, PO Box 7803, NO-5020 Bergen, Norway

[*]Corresponding author

Tel: +81-596-58-6411; Fax: +81-596-58-6413; E-mail: kojim@affrc.go.jp (K. Murashita)

Abstract

To characterize the effects of a plant-based low-fishmeal (LFM) diet on the digestive physiology of yellowtail, *Seriola quinqueradiata*, we prepared two isonitrogenous and isolipidic diets; a FM-based diet (diet Control, FM 50%) and a plant protein (soybean meal and corn gluten meal)-based low fishmeal diet (diet LFM, FM 15%), and examined the acute and chronic effects of the diets on the digestive physiology of the fish. In the acute effect trial (fed only a single meal), the fish fed the LFM diet displayed faster gastric emptying, lower pH of the gastrointestinal content and suppressed pancreatic digestive enzymes (trypsin, chymotrypsin and amylase) secretions. In the chronic effect trial (feeding for six weeks), in addition to the effects observed in the acute trial, the fish fed the LFM diet also displayed suppressed stomach pepsin secretion and pancreatic digestive enzymes production (gene expression). Furthermore, gene expression levels of digestion-regulating hormones, *gastrin*, *peptide yy* and *cholecystokinin* were also disrupted by the long-term administration of the LFM diet. Taken together, these results indicate that a plant protein-based low fish meal diet appears to not fully activate or stimulate the digestive system of yellowtail in either the short or long term and that its inhibitory/disruptive effects become more pronounced on a long-term basis. The effects we have identified on yellowtail digestive physiology could serve as important indicators to improve plant-based low-fishmeal diets.

Keyword: fishmeal; soybean meal; corn gluten meal; stomach; pepsin; pancreatic digestive enzymes; gut transit rates

43

44 **Highlights**

45 · Yellowtail were fed fishmeal-based (diet Control) and plant-based low FM diets (diet

46 LFM).

47 · The LFM diet-fed fish displayed more rapid gastric emptying, lower pH of the

48 gastrointestinal contents and suppressed pancreatic digestive enzymes secretion as an

49 acute effect.

50 · In addition to the effects observed in the acute response, the chronic effects (long-term

51 feeding) of the LFM diet included suppressed stomach pepsin secretion and pancreatic

52 digestive enzymes production.

53 · Gene expression levels of digestion-regulating hormones, *gastrin*, *peptide yy* and

54 *cholecystokinin* were also disrupted by long-term administration of the LFM diet.

55

1. Introduction

With the recent global growth of aquaculture, the limited supply of fishmeal (FM) for use in fish feeds has caused feed manufactures to reduce the proportion of FM and introduce alternative ingredients, mainly from plant protein sources (Gatlin et al., 2007; Tacon and Metian, 2008). However, the inclusion of high levels of plant ingredients such as defatted soybean meal (SBM)in fish feeds often results in growth retardation and impaired physiological condition, exemplified by morphological changes in the distal intestine and liver, a reduction in bile production, and changes in conjugated bile-salt composition in some teleost species (Iwashita et al., 2008; Nguyen et al., 2011; Romarheim et al., 2006; Urán et al., 2008; van den Ingh et al., 1991; Yamamoto et al., 2007). In addition to these abnormalities, other detrimental effects related to digestive physiology have been observed. The exocrine pancreas of the red seabream *Pagrus major* fed a SBM-based diet has been shown to be atrophied (Matsunari et al., 2015). Feeding SBM-based diets over a long period of time (chronic exposure) reduces the production and secretion of pancreatic digestive enzymes in Atlantic salmon *Salmo salar* (Lilleeng et al., 2007), yellowtail *Seriola quinueradiata* (Nguyen et al., 2011) and Japanese seabass *Lateolabrax japonicus* (Zhang et al., 2018). Even following short-term exposure (a single meal) the SBM protein does not appear to fully stimulate the digestive process in red seabream (Murashita et al., 2018).

The production and secretion of pancreatic digestive enzymes change dynamically after a meal (Murashita et al., 2007), and in fish fed a SBM diet there is faster gastric transit, faster intestinal emptying and lower growth rates than in fish fed a FM-

78 based diet (Murashita et al., 2018). These results suggest that a balance of the gastric and
79 intestinal (pancreatic) digestion is one of the important factors affecting the maximisation
80 or optimisation of feed utilisation. However, many studies of digestion have focused
81 primarily on the intestinal (pancreatic) part , not taking gastric digestion into account
82 (Lilleeng et al., 2007; Nguyen et al., 2011; Zhang et al., 2018). Furthermore, although
83 gastric and pancreatic enzyme activities are strongly affected by the pH (Murashita et al.,
84 2012; 2014), we still do not know if, and to what extent, feed composition affects
85 gastrointestinal pH.

86 Fish belonging to the genus *Seriola* (*Carangidae*) are among the most
87 important targets for aquaculture all over the world (Sicuro and Luzzana, 2016).
88 Yellowtail, *S. quinqueradiata* is a key species cultured in the Asia-Pacific region,
89 particularly in Japan, and is thus of significant economic importance (Nakada, 2008). In
90 order to characterize the effects of plant-based low fishmeal diets on digestive physiology
91 in yellowtail, we therefore examined the postprandial responses of yellowtail to a SBM
92 and corn gluten meal (CGM)-based low-fishmeal diet in terms of digestive physiology,
93 including intestinal transit rate, gallbladder weight, pH of gastrointestinal content,
94 production/secretion of gastrointestinal digestive enzymes and digestive hormonal genes
95 in both short- and long-term trials.

96

97 **2. Materials and methods**

98 2.1. Diets

99 The formulation and proximate composition of the experimental diets are

100 shown in Table 1. Isonitrogenous (crude protein, 49%) and isolipidic (crude fat, 15%)
101 diets based on FM (diet Control, fishmeal 50%), and on SBM and CGM (diet LFM,
102 fishmeal 15%) were prepared. In the diet LFM, crystalline lysine and methionine were
103 supplemented to complement the indispensible amino acids and bring them to within
104 required limits. In order to avoid taurine deficiency abnormalities (Takagi et al., 2008),
105 crystalline taurine was also supplemented and the level was adjusted between the test
106 diets.
107
108 2.2. Animals and samples
109 2.2.1. Fish
110 Yellowtail used in this study were handled and treated in accordance with the
111 Guidelines for Animal Experimentation at the National Research Institute of Aquaculture
112 (NRIA), Japan. Fertilised yellowtail eggs were produced by artificially induced spawning
113 at the Komame Laboratory of the NRIA (Otsuki, Kochi, Japan). The eggs were transferred
114 to the Nansei Main Station of the NRIA (Minami-Ise, Mie, Japan), and the hatched fish
115 were reared until the experiments were carried out. The fish were maintained in indoor
116 1000 L tanks supplied with a continuous flow of sand-filtered seawater at 21.3 ± 0.9 °C.
117 During acclimation, the fish were hand-fed a commercial pellet feed (Marubeni Nisshin
118 Feed Co. Ltd., Tokyo, Japan) twice a day (08:30 and 16:00).
119
120 2.2.2. Acute effect trial (fed a single meal)
121 Prior to the experiment, 150 fish (29.6 ± 4.5 g) were transferred from the 1,000

L tanks to six 500 L tanks (25 fish/tank) and kept for one week for acclimation to the experimental conditions using the Control diet. After 24 hours of fasting, individual body weight was measured (35.2 ± 5.1 g) and five randomly selected fish from each of the six tanks were transferred to another tank (total 30 fish) for the following sampling. The fish were then kept for 24 hours, still fasting, and then nine fish were sampled from the transferred tank (48 h fasted) representing time 0 fish when the water temperature was 23.0 °C. At this time fish in the original six tanks were hand-fed the test diet, with three tanks for each diet (triplicate). The feeding time was 08:30 and feeding level was adjusted to 4.5 % of body weight (dry matter basis). After feeding, nine fish were sampled from each diet group (three fish/tank) at 2, 6, 12, 24, 36 and 48 hours after feeding. At the time of sampling, the fish were killed by an overdose of 2-phenoxyethanol, and their stomach, pyloric caeca, gallbladder and intestine were collected. A portion of the stomach and pyloric caeca were stored in RNAlater® (Thermo Fisher Scientific, Waltham, MA, USA) at -80 °C until RNA isolations were performed. The remaining stomach and pyloric caeca portions were kept at -20 °C until ready for the enzymatic activity analysis. Content from the stomach, anterior part of the intestine (from the end of stomach to first turn of the intestine) and posterior part of the intestine (from the first turn to the end of the intestine) were removed and its pH was determined using an ISFET probe pH meter H170 (Hach, Loveland, CO, USA). The stomach and intestinal content were freeze-dried, and the dry content weight of the stomach, anterior intestinal and posterior intestine, and the gallbladder weight (with bile) were expressed as percentages of total body weight. The freeze-dried stomach and intestinal content were also used for the analysis of enzymatic

144 activity.

145

146 2.2.3. Chronic effect trial (six weeks feeding)

147 Ahead of the experiment, 210 fish were transferred from the 1000 L tanks to
148 six 500 L tanks (35 fish/tank) and reared for one week under the same conditions as in
149 the acute effects trial. After 48 hours of fasting, the number of fish per tank was reduced
150 to 23 (mean body weight, 2.6 ± 0.5 g) to give a fish density of approximately 60 g $tank^{-1}$.
151 Each test diet was then fed twice daily (08:30 and 16:00) by hand to triplicate tanks to
152 apparent satiation, six days a week for six weeks. After the final body weight of fish after
153 24 hours of fasting had been measured, three fish from each tank (nine fish/diet group)
154 were transferred to a new tank for the following sampling. One day after the body weight
155 measurement, nine fish from each group were sampled from the newly divided tanks as
156 time 0 fish (48 h fasted), and then each test diet was fed to the remaining tanks,(08:30,
157 4.5% of body weight, dry matter basis) when the water temperature was 23.0 °C. Nine
158 fish from each group (three fish per tank) were sampled at 2, 6, 12, 24, 36 and 48 hours
159 after feeding. At the time of sampling, fish were killed by an overdose of 2-
160 phenoxyethanol, and the stomach (tissue and content), pyloric caeca, gallbladder and
161 intestinal contents were taken from each fish in the same way as in the acute trial (see
162 2.2.2).

163

164 2.3. Analysis

165 2.3.1. Chemical analyses of diets

166 Determinations of moisture, crude protein, crude fat and ash of the test diets
167 were performed by drying samples for 10 h at 110 °C, semi-micro Kjeldahl method (N ×
168 6.25), ethyl ether extraction, and 5 h combustion at 600 °C. The crude starch content of
169 each diet was determined by measuring the glucose liberated by boiling in 5% HCl for 2
170 h based on the Somogyi-Nelson method (Nelson, 1944). The amino acid composition of
171 the protein ingredients was determined using an automatic amino acid analyser (L-8500,
172 Hitachi, Tokyo, Japan) after the samples had been hydrolysed in 6 N HCl for 22 h at
173 120 °C.
174
175 2.3.2. Biochemical analyses of blood and bile acids
176 Blood haemoglobin concentration was measured using a clinical investigation
177 kit (Hemoglobin Assay Kit Wako; FUJIFILM Wako Pure Chemical Co., Osaka, Japan).
178 Plasma nutrient constituents were measured with an automatic analyser (Spotchem SP-
179 4410; Arkrey, Kyoto, Japan). Bile acid concentration gall bladder was measured using a
180 commercial kit (Total Bile Acid Test Wako; FUJIFILM Wako Pure Chemical Co). The
181 conjugated bile salt composition was determined by high-performance liquid
182 chromatography (HPLC) composed of a pump (LC-10AT; Shimadzu, Kyoto, Japan) and
183 an ultraviolet-visible (UV-Vis) spectrophotometric detector (SPD-10AV; Shimadzu)
184 according to the method of Goto et al. (1996).
185
186 2.3.3. Enzyme activity assay
187 The stomach tissue, pyloric caeca tissue, stomach content and intestinal content

188 (anterior and posterior parts) were homogenised into nine volumes (v/w) of ice-cold
189 distilled water. The homogenate was then centrifuged at 4 °C at 20,000 g for 15 min. The
190 supernatant was further diluted with cold distilled water and used for enzyme activity
191 assay as a crude enzyme extract. Enzyme activities were expressed as U/g tissue and U/g
192 content.

193 Pepsin (EC 3.4.23.1) activity was assayed according to the method of Anson
194 (1938) with modifications. A 5% hemoglobin solution (pH 3.0 adjusted by HCl) was
195 prepared for use as the substrate. The reaction mixture of 25 µl of enzyme extract, 125 µl
196 of hemoglobin solution and 100 µl of KCl-HCl buffer (pH 2.0) was incubated for 60 min.
197 Following the addition of 250 µl of 10% trichloroacetic acid (TCA), the mixture was
198 centrifuged for 5 min at 10,000 g. The absorbance of the supernatant was measured at
199 280 nm. For the blank reading, TCA was added to the substrate prior to the addition of
200 the enzyme extract. The activity was expressed in U, which was defined as the amount of
201 enzyme that caused an increase of 1 absorbance unit at 280 nm in 1 min.

202 The activity of trypsin (E.C. 3.4.21.4) was assayed using N-benzoyl-L-
203 arginine-*p*-nitroanilide (L-BAPA, Peptide Institute, Inc., Osaka, Japan) as the substrate,
204 which is a modification of the method of Erlanger et al. (1961). Briefly, the reaction
205 mixture was prepared by combining 240 µl of 100 mM Tris buffer (pH 8.5, containing 20
206 mM $CaCl_2$), 100 µl 2.4 mM L-BAPA and 50 µl enzyme extract. Production of *p*-
207 nitroaniline (*p*NA) was measured by monitoring the increase in absorbance at 405 nm per
208 minute for 7 min at 37 °C. One unit (U) of activity was defined as the amount of enzyme
209 that caused an increase of 1 absorbance unit in 1 min.

The activity of chymotrypsin (E.C. 3.4.21.1) was assayed using N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAPFNA, Sigma-Aldrich, St. Louis, MO) as a substrate, which is a modification of the method of Erlanger et al. (1961). The reaction mixture consisted of 240 µl of 100 mM Tris buffer (pH 8.5, containing 20 mM $CaCl_2$), 100 µl 2.4 mM SAPFNA and 50 µl enzyme extract. Production of *p*NA was measured by monitoring the increase in absorbance at 405 nm per minute for 7 min at 37 °C. One U of activity was defined as the amount of enzyme that caused an increase of 1 absorbance unit in 1 min.

The activity of lipase (E.C. 3.1.1) was assayed using *p*-nitrophenyl myristate (PNPM, Sigma-Aldrich) as a substrate, which is a modification of the method of Albro et al. (1985). The reaction mixture consisted of 240 µl of 100 mM Tris-HCl buffer (pH 8.5, containing 10 mM deoxycholic acid), 100 µl of 3.5 mM PNPM (containing 0.5% Triton X-100, Nakalai Tesque, Kyoto, Japan) and 50 µl of enzyme extract. Production of *p*-nitrophenol (*p*NP) was measured by monitoring the increase in absorbance at 405 nm per minute for 7 min at 37 °C. One U of activity was defined as the amount of enzyme that caused an increase of 1 absorbance unit in 1 min.

Amylase (E.C. 3.2.1.1) activity was assayed according to Natalia (2004), with a slight modification. Briefly, a 1% starch solution was prepared for use as a substrate, 25 µl of the substrate solution and 25 µl 20 mM sodium phosphate buffer (pH 6.9, containing 6.0 mM NaCl) were added to 50 µl enzyme extract, and the mixture was incubated at 37°C for 60 min. Following the addition of 50 µl dinitrosalicylic acid reagent (1% dinitrosalicylic acid and 30% sodium potassium tartrate in 0.4 M NaOH) and incubation

232 in boiling water for 5 min, the absorbance of a six-fold dilution of the reaction mixture
233 was recorded at 540 nm and the amount of maltose released was determined by reference
234 to a standard curve. The activity was expressed in U, which was defined as the amount
235 (μmol) of maltose released in 1 min.
236
237 2.3.4. Real-time quantitative RT-PCR
238 Real-time quantitative RT-PCR (qPCR) analysis was performed to estimate the
239 effect of the experimental diets on the expression of pancreatic digestive enzyme genes.
240 Total RNA was isolated from the collected stomach and pyloric caeca using Sepasol®-
241 RNAISuper G (Nakalai Tesque). The purity of the isolated RNA was verified by the
242 optical density (OD) absorption ratio (OD 260 nm/OD 280 nm) using a e-spect
243 spectrophotometer (Malcom, Tokyo, Japan). Samples with ratios of OD 260 nm/ OD 280
244 nm > 2.0 were used for subsequent cDNA synthesis. The first-strand cDNA was
245 synthesized from the total RNA using a Verso cDNA Synthesis Kit (Thermo Fisher
246 Scientific). Acute or chronic effects of the test diet on the mRNA for *pepsin* and *gastrin*
247 in the stomach, *trypsin, pancreatic lipase, amylase, cholecystokinin* (*cck*) and *peptide yy*
248 (*pyy*) in the pyloric caeca were then analysed using a LightCycler®96 System (Roche,
249 Basel, Switzerland). The mRNA of *trypsin*, *lipase*, *amylase* and *pyy* were analysed by the
250 SYBR green dye intercalation method with FastStart Essential DNA Green Master
251 (Roche), and the expression levels of *gastrin* and *cck* were assessed by the FAM-labeled
252 TaqMan probe method using FastStart Universal Probe Master (Roche). The primer sets
253 and probes used in each assay, except for the *pepsin* and *gastrin*, were designed in the

same manner as given in Murashita et al. (2007, for *trypsin*, *lipase*, and *amylase*), Furutani et al. (2012, for *cck*) and Murashita et al. (2006, for *pyy*), and the sequence information about the primers is provided in Table 2. cDNA sequence for full protein coding region of yellowtail *pepsin* was determined (GenBank accession no.: LC435267), and the primer set for the *pepsin* qPCR was based on the sequence obtained. Detailed information on the cDNA cloning of yellowtail *pepsin* is summarized in Supplementary Figure 1. The primer set and probe for *gastrin* were based on the nucleotide sequence deposited in the GenBank data base (GenBank accession no.: xxxxxx, submitted). The PCR parameters consisted of 95°C for 10 s (initial denaturation), followed by 40 cycles at 95 °C for 5 s (denaturation) and 60 °C for 20 s (annealing and extension). Melting temperature analyses revealed a single melting peak and no amplification was observed in the negative control (non-reverse transcribed RNA sample). The amount of mRNAs was calculated as copies per nanogram of total RNA.

2.3.5. Statistical analysis

The results were analysed by one-way analysis of variance (ANOVA). Differences in gallbladder weight, gastrointestinal content pH, enzyme activity and gene expression in the stomach and pyloric caeca after feeding relative to the values at time 0 within the same diet group were assessed by Dunnett's multiple comparison test, and differences in all analysed parameters between the groups at each sampling time were tested by *t*-test, using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA), and a probability level of less than 0.05 was considered significant.

276

277 **3. Results**

278 3.1. Growth performance

279 The growth performance of fish fed the experimental diets for six weeks is
280 shown in Table 3. Final body weight, weight gain, specific growth rate and feed efficiency
281 ratio were significantly lower in fish fed the LFM diet compared to those of fish fed the
282 Control diet. On the other hand, feed intake was significantly higher in fish fed the LFM
283 diet than in fish fed the Control diet.

284

285 3.2. Haematological parameters

286 The haemoglobin, total protein, total cholesterol and glucose of fish fed the
287 LFM diet were lower than in the Control diet group (Supplementary Table 1). No
288 significant differences between the diet groups were observed in plasma alkaline
289 phosphatase, triacylglycerol, inorganic phosphorus and calcium.

290

291 3.3. Gallbladder and bile acid status

292 Relative gallbladder weight (with bile) of fish fed the Control and LFM diets
293 in the acute effects trial fell to around 29% of the pre-feeding values 2 h after feeding and
294 remained at low levels until 12 h after feeding (Fig. 1). In the chronic effects trial, the
295 gallbladder weight of fish fed the Control and LFM diets at 2 h after feeding fell to 26%
296 and 24% of the pre-feeding level and remained at low levels until 12 h after feeding. The
297 relative gallbladder weight of fish fed thweLFM diet was higher than that of fish fed the

298 FM diet except for 2 and 12 h after feeding in the chronic effects trial.

299 The total bile acid concentration of fish fed the LFM diet was lower than that
300 of fish fed the Control diet in the acute effects trial (Table 4). No significant difference
301 between the diet groups was found in total bile acid content. The C-tau/CDC-tau ratio of
302 fish fed the LFM diet was higher than that of fish fed the Control diet.

303

304 3.4. Gastrointestinal content weight

305 In the acute effects trial, the dry weight of stomach contents fell after feeding
306 and approached zero within 24 h after feeding in both the Control and LFM diet groups,
307 while the stomach content of the fish fed the LFM diet was significantly lower than those
308 fed the Control diet at 12 h after feeding (0.54-fold) (Fig. 2). The dry weight of the anterior
309 and posterior intestinal contents in the acute trial gradually fell from 2 h to 48 h after
310 feeding in both the FM and SBM diet groups. In the chronic effects trial, the stomach
311 contents of fish fed the LFM diet rapidly decreased after feeding, and were significantly
312 lower than those of fish fed the Control diet at 6 h (0.59-fold) and 12 h (0.54-fold) after
313 feeding. On the other hand, although no significant differences were observed, the content
314 of the anterior intestine of fish fed the LFM diet was relatively higher than that of fish fed
315 the Control diet at 2 h after feeding. The posterior intestinal contents of fish fed the LFM
316 diet remained at higher levels than those of fish fed the Control diet at 6 h after feeding
317 (2.1-fold).

318

319 3.5. Gastrointestinal content pH

320 In the acute effects trial, the stomach content pH decreased after feeding from
321 2 h (Control: pH 5.0; LFM: pH 4.5) to 12 h (Control: pH 3.2; LFM: pH 3.0), and the pH
322 of fish fed the LFM diet was lower than that of fish fed the Control diet at 2 and 6 h after
323 feeding (Fig. 3). In the anterior intestinal contents of the acute effect trial, the pH of the
324 fish fed the Control diet increased from 6 h (pH 6.8) to 12 h (pH 7.7) after feeding and
325 remained at high levels until 24 h (pH 7.7), while those of fish fed the LFM diet were
326 significantly lower than the Control group at 12 h (pH 7.2) and 24 h (pH 7.5) after feeding.
327 The pH of the content of the posterior part of the intestine fish fed the Control diet
328 increased from 2 h (pH 7.5) to 12 h (pH 8.3) after feeding and then fell at 24 h (pH 6.6).
329 In the acute effect trial, the pH of the posterior intestinal content in fish fed the LFM diet
330 was lower than those of fish fed the Control diet, 12 h after feeding (pH 7.9) in the acute
331 effect trial.
332 In the chronic effect trial, the stomach content pH displayed a similar trend to
333 that of the fish in the acute effect trial, and the pH of fish fed the LFM diet was lower than
334 that of fish fed the Control diet at 2 and 6 h after feeding. The content of the anterior and
335 posterior parts of the intestine of the pH fish fed the Control diet increased from 2 to 6 h
336 after feeding and then decreased at 24 h, while that of fish fed the LFM diet decreased at
337 12 h after feeding and remained at low level until 24 h.
338
339 3.6. Digestive enzyme activity in the stomach and pyloric caeca
340 The effects of the test diets on pepsin activity in the stomach tissue are shown
341 in Fig. 4. In the acute effects trial, pepsin activity was temporally reduced after feeding,

without significant differences between the diet groups being observed. In the chronic effects trial, levels of pepsin activity fell after feeding in fish fed the Control diet, while no significant changes were observed after feeding in fish fed the LFM diet.

The effects of the test diets on the pancreatic digestive enzymes in the pyloric caeca tissue are shown in Fig. 5. Trypsin activity in fish fed the Control diet was reduced after feeding in both the acute and chronic effects trials, whereas that of fish fed the LFM diet did not changes significantly after feeding. Chymotrypsin activity infish fed the Control diet was also reduced after feeding in the acute/chronic effect trials, and chymotrypsin levels were significantly lower than those of fish fed the LFM diet at 2 and 6 h after feeding in the acute effect trial. Lipase activity showed a similar trend in the both diet groups and trials. On the other hand, although amylase activities of fish fed the LFM diet did not change, those of fish fed the Control diet showed higher levels compared to the LFM diet group.

3.7. Expression of digestion-related genes in the stomach and pyloric caeca

The effects of the diets on the gene expression of digestive enzymes and digestion-regulating factors in the stomach and pyloric caeca are shown in Figs. 6 and 7. In the acute effect trial, all of the digestion-related genes displayed similar patterns of expression in fish fed the Control and LFM diets. However, in the chronic effects trial, the stomach *pepsin* expression of fish fed the Control diet was higher than that of fish fed the LFM diet at 0 time (1.3-fold) and decreased after feeding (Fig. 6). The pyloric caeca pancreatic digestive enzyme genes, *trypsin*, *lipase* and *amylase* of fish fed the Control

364 diet in the chronic effect trial were expressed more highly than those of fish fed the LFM
365 diet. On the other hand, in fish fed the LFM diet, there were higher levels of expression
366 of stomach *gastrin* compared to those of fish fed the Control diet in the chronic effect
367 trial (Fig. 7). *cck* expression of fish fed the LFM diet was lower than the Control group at
368 2 and 36 h after feeding, while *pyy* mRNA levels of fish fed the LFM diet were higher
369 than those of the Control group at 12 h after feeding.
370
371 3.8. Digestive enzyme activity in the gastrointestinal content
372 Pepsin activity of stomach content in fish fed the LFM diet was higher than in
373 fish fed the Control diet in the acute effect trial, whereas in the chronic effect trial, lower
374 pepsin activity was found 2 and 12 h after feeding in fish fed the LFM diet than in fish
375 fed the Control diet (Fig. 8). In the acute and chronic effect trials, there were lower levels
376 of pancreatic activities of trypsin, chymotrypsin and amylase in fish fed the LFM diet
377 than in fish fed the Control diet of both the anterior and posterior intestinal contents (Figs.
378 9 and 10). No significant differences in lipase activities were observed in the
379 anterior/posterior intestinal contents between the diet groups in either the acute or chronic
380 effect trials.
381
382 **4. Discussion**
383 In this study, we characterized the effect of a plant (SBM and CGM)-based low
384 FM (15%) diet on yellowtail digestive physiology. In the acute effects trial, in which the
385 fish were fed the test diets only once as a single meal, the gallbladder weights of both the

386 Control and LFM diets groups were reduced after feeding and then recovered within 48
387 h. This is in line with our previous report on rainbow trout, yellowtail and red seabream
388 fed FM-based or SBM-based diets (Murashita et al., 2007; 2013; 2018). Red seabream
389 and rainbow trout that were fed SBM-based diets for six weeks have also been found to
390 have smaller gallbladders than fish fed FM-based diets (Murashita et al., 2013; 2018;
391 Yamamoto et al., 2007). In this study, however, the gallbladders of fish fed the SBM diet
392 for six weeks were larger than those of fish fed the FM diet. Kortner et al. (2013) reported
393 that the bile acid synthesis gene *cyp7a1* is up-regulated in Atlantic salmon fed a 20%
394 SBM diet. Partial replacement of FM by plant proteins such as SBM might stimulate bile
395 production in fish. Gallbladder weight and bile acid quantity are generally positively
396 correlated (Murashita et al., 2013; Yamamoto et al., 2007). However, it should be noted
397 that no significant differences were found in the total bile acid content between the diet
398 groups in the present study, since the bile acid concentration of fish fed the Control diet
399 was higher of fish fed the LFM diet.
400 In red seabream, the gastric transit speed of fish fed a SBM-based diet is faster
401 than that of fish fed an FM-based diet (Murashita et al., 2018). Similarly, we found that
402 the gastric transit speed of yellowtail fed the LFM diet was faster than that of fish fed the
403 Control diet. The stomach digestive enzyme, pepsin, is mainly synthesised and secreted
404 by the gastric chief cells (Gritti et al., 2000). Therefore, changes in pepsin activity in the
405 stomach tissue would reflect the extent of accumulation and secretion of the enzyme.
406 Although the pepsin activity of both diet groups displayed postprandial reduction
407 (secretion) and increase (accumulation) patterns in the acute effect trial, no significant

changes were observed in fish fed the LFM diet in the chronic effects trial. Also, in the chronic effect trial, pepsin activity of the stomach content in fish fed the LFM diet was lower than in fish fed the Control diet. These findings indicate that dietary plant protein (SBM and/or CGM) down-regulates pepsin secretion from the stomach tissue on a long-term basis. On the other hand, in the acute effects trial, higher pepsin activity was found in the stomach content of the LFM group than in the Control group. In this study, pepsin activity in the stomach contents is presented as U/g content. If the results are calculated as U/g BW, they would show different trends from those that used the other unit in the acute effect trial; there were no differences in pepsin activity between the diet groupsies (Supplementary Figure 2). The unit of U/g BW is assumed to reflect the total levels of secretion of the enzyme in the fish, and the total levels of pepsin secretion correlated strongly with the amounts of stomach content in our study (correlation coefficient: 0.73). Since the LFM diet group displayed a faster gastric transit as well as pepsin secretion (in terms of total levels) similar to that of the Control group, the LFM diet might be evacuated without sufficient digestion in the stomach segment. Besides the faster gastric emptying and the lower pepsin secretion, stomach content in fish fed the LFM diet had a lower pH than fish fed the Control diet, which suggests plant protein ingredients (SBM and/or CGM) may stimulate gastric acid secretion. In fact, in the chronic effect trial, higher *gastrin* expression levels were observed in the LFM group than in the Control group; gastrin is a well-known peptide hormone that stimulates gastric acid secretion (Rehfeld, 2014). This is the first report that show a relationship between dietary ingredients and gastrin. Since few reports regarding endogenous gastrin in fish have been published to

430 date (Kurokawa et al., 2003), further studies are required to understand the role played by
431 gastrinin fish digestive physiology, in order to improve the utilisation of plant-based diets.
432 In mammals, acidic chyme from the stomach is neutralised by the bicarbonate
433 of the pancreatic juice, while the bicarbonate of marine teleosts is mainly secreted from
434 the intestinal epithelium itself (Grosell and Jensen, 1999; Wilson et al., 1996; 2002). In
435 our study, the pH of the intestinal content of fish fed the LFM diet was lower than tthat
436 of fish fed the Control diet 12 h after feeding, when the intestine was still full of chyme,
437 which suggests that plant protein may might suppress the bicarbonate secretion. Since
438 most of the pancreatic digestive enzymes in fish display maximum activity under basic
439 conditions (Murashita et al., 2014; 2012), our fish fed the LFM diet probably had lower
440 intestinal enzymatic activity. It is known that fish pancreatic digestive enzymes are
441 secreted into intestine from the embedded acinar cells of pancreatic tissue surrounding
442 the pyloric caeca (Einarsson and Davies, 1996). Thus, like the stomach tissue pepsin
443 mentioned above, rises and falls in the enzyme activity of the pyloric caeca tissue may
444 reflect the accumulation and secretion of pancreatic enzymes. In both the acute and
445 chronic effects trials, the activity of proteases, trypsin and chymotrypsin displayed
446 postprandial secretion and accumulation patterns, which were clearer in the Control group
447 than the LFM group. A similar finding has been reported in red seabream fed FM-based
448 and SBM-based diets (Murashita et al., 2018). On the other hand, the amylase activity of
449 pyloric caeca tissue increased after feeding in fish fed the Control diet, whereas such
450 postprandial changes were not found in those on the LFM diet. This is in line with our
451 previous report of yellowtail fed an FM-based commercial diet (Murashita et al., 2007);

452 in yellowtail, most of the amylase required for digestion seems to be produced after
453 feeding. Moreover, except for lipase, the activity of the pancreatic digestive enzymes in
454 the intestinal contents of fish fed the LFM diet was markedly higher than that of fish fed
455 the Control diet. Similar trends were also confirmed in the total secretion levels (U/g BW,
456 Supplementary Figures 3 and 4). The total secretion levels of trypsin and amylase
457 correlated highly with the amount of intestinal content (dry weight, anterior part,
458 correlation coefficient for amylase, 0.90; trypsin, 0.72) while lower correlations were
459 observed in chymotrypsin (0.45) and lipase (0.42); in other words, secretion of trypsin
460 and amylase seems to be strongly affected by the amount of intestinal content. In red
461 seabream, activities of lipase and amylase activity levels correlate highly with the amount
462 of intestinal content (wet weight, anterior part), whereas trypsin and chymotrypsin
463 secretion levels are affected by the kind of protein ingredients rather than the amount of
464 intestinal content (Murashita et al., 2018), suggesting that the response of digestive
465 enzymes secretion to diet ingredients is species-specific even within carnivorous species.
466 Recently, 19 different types of *trypsin* genes have been identified in the
467 yellowtail genome, and RNA-Seq analysis reveals that one of these, called g15220 in the
468 article, is the principal *trypsin* in the intestinal segment; and this paralogue accounts for
469 47% of the total *trypsin* expression in the intestine (Yasuike et al., 2018). In this study,
470 although we subjected only a single gene to trypsin qPCR analysis, the primer set
471 specifically detects g15220. In the acute effects trial, similar trends in the expression
472 levels of all the digestive enzymes genes in the pyloric caeca were observed between the
473 Control and LFM groups, whereas all of the gene expression levels in the LFM group

474 were lower than those of the Control group in the chronic effects trial, indicating that the
475 production of pancreatic digestive enzymes is suppressed by long-term LFM
476 administration. Nguyen et al. (2011; 2017) reported that an alcohol extract of SBM can
477 inhibit trypsin and lipase secretion from yellowtail pancreatic tissues. Furthermore, the
478 exocrine pancreatic tissue of red seabream fed a SBM-based diet has been shown to be
479 atrophied (Matsunari et al., 2015). The SBM-derived anti-nutritional factor(s) might have
480 resulted in such a pathological condition in yellowtail, which might ?? lead to a reduction
481 or loss of the pancreatic function.

482 The secretion of pancreatic digestive enzymes is controlled by both neuronal
483 and hormonal factors, and Cck and Pyy are the two best-known hormonal factors; Cck
484 stimulates the exocrine pancreas while Pyy inhibits pancreatic secretion (Konturek et al.,
485 2003). In yellowtail, as a response to protein and fat, gene expression levels of *cck* and
486 *pyy* (*-b* type, formerly called *peptide y*, *py*) increase and decrease after feeding,
487 respectively (Murashita et al., 2007; 2006; 2008). Furthermore, decreased *cck* gene
488 expression levels have been found in yellowtail administered SBM (Furutani et al., 2012;
489 Nguyen et al., 2017). In the chronic effects trial of the present study, although clear
490 postprandial responses of these genes were not confirmed, lower *cck* and higher *pyy*
491 expression levels were found in fish fed the LFM diet than in fish fed the Control diet.
492 Plant protein ingredients may have suppressed pancreatic digestive enzyme secretion via
493 Cck/Pyy regulation on a long-term basis in this study.

494 In conclusion, fish fed the LFM diet as a single meal displayed faster gastric
495 emptying, lower gastrointestinal content pH and suppressed pancreatic digestive enzymes

496 secretion, indicating that the yellowtail digestive system is not fully stimulated by plant
497 protein, even on a short-term basis. In the chronic effects trial, in addition to the
498 detrimental effects seen in the acute effects trial, fish fed the LFM diet also had
499 suppressed stomach pepsin secretion and low pancreatic digestive enzyme production
500 (gene expression). These effects on digestive physiology could be good indicators for
501 how to improve plant-based low-fishmeal diet utilisation in yellowtail.
502
503 **Acknowledgements**
504 We are grateful to the staff of Komame Laboratory, NRIA for providing
505 yellowtail eggs. This study was supported by the Japan Fisheries Research and Education
506 Agency, and in part by JSPS KAKENHI Grant Number JP24380117 (H.F. and K.M.).
507

508 **Figure legends**

509

510 Fig. 1. Relative gallbladder weight in yellowtail fed a fishmeal-based diet (Control) and
511 a plant-based low-fishmeal diet (LFM). Values are mean ± SE ($n = 9$). †p < 0.05 compared
512 with 0 time 0 fish fed the Control diet. ‡p < 0.05 compared with time 0 fish fed the LFM
513 diet. Values with asterisks (*) are significantly different at the same point in time between
514 the test groups ($p < 0.05$).

515

516 Fig. 2. Stomach and intestinal content weight in yellowtail fed a fishmeal-based diet
517 (Control) and a plant-based low-fishmeal diet (LFM). Values are mean ± SE ($n = 9$).
518 Values with asterisks (*) are significantly different at the same point in time between the
519 test groups ($p < 0.05$).

520

521 Fig. 3. Stomach and intestinal content pH in yellowtail fed a fishmeal-based diet (Control)
522 and a plant-based low-fishmeal diet (LFM). Values are mean ± SE ($n = 9$). †$p < 0.05$
523 compared with time 0fish fed the Control diet. ‡$p < 0.05$ compared with time 0 fish fed
524 the LFM diet. Values with asterisks (*) are significantly different at the same point in time
525 between the test groups ($p < 0.05$).

526

527 Fig. 4. Pepsin activities in the stomach of yellowtail fed a fishmeal-based diet (Control)
528 and a plant-based low-fishmeal diet (LFM). Values are mean ± SE ($n = 9$). †$p < 0.05$
529 compared with time 0 fish fed the Control diet. ‡$p < 0.05$ compared with time 0 fish fed

530 the LFM diet. Values with asterisks (*) are significantly different at the same point in
531 time between the test groups ($p < 0.05$).
532
533 Fig. 5. Pancreatic digestive enzymes activities in the pyloric caeca of yellowtail fed a
534 fishmeal-based diet (Control) and a plant-based low-fishmeal diet (LFM). Values are
535 mean ± SE ($n = 9$). †$p < 0.05$ compared with time 0 fish fed the Control diet. ‡$p < 0.05$
536 compared with time 0 fish fed the LFM diet. Values with asterisks (*) are significantly
537 different at the same point in time between the test groups ($p < 0.05$).
538
539 Fig. 6. Gene expression of digestive enzymes in the stomach and pyloric caeca tissues of
540 yellowtail fed a fishmeal-based diet (Control) and a plant-based low-fishmeal diet (LFM).
541 Values are mean ± SE ($n = 9$). †$p < 0.05$ compared with time 0 fish fed the Control diet.
542 ‡$p < 0.05$ compared with time 0 fish fed the LFM diet. Values with asterisks (*) are
543 significantly different at the same point in time between the test groups ($p < 0.05$).
544
545 Fig. 7. Gene expression of digestion regulating factors in the stomach and pyloric caeca
546 tissues of yellowtail fed a fishmeal-based diet (Control) and a plant-based low-fishmeal
547 diet (LFM). Values are mean ± SE ($n = 9$). †$p < 0.05$ compared with time 0 fish fed the
548 Control diet. ‡$p < 0.05$ compared with time 0 fish fed the LFM diet. Values with asterisks
549 (*) are significantly different at the same point in time between the test groups ($p < 0.05$).
550

551 Fig. 8. Pepsin activities in the stomach content of yellowtail fed a fishmeal-based diet
552 (Control) and a plant-based low-fishmeal diet (LFM). Values are mean ± SE (n = 9). †p
553 < 0.05 compared with time 0 fish fed the Control diet. ‡p < 0.05 compared with time 0
554 fish fed the LFM diet. Values with asterisks (*) are significantly different at the same
555 point in time between the test groups (p < 0.05).
556
557 Fig. 9. Pancreatic digestive enzymes activities in the anterior intestinal content of
558 yellowtail fed a fishmeal-based diet (Control) and a plant-based lowfishmeal diet (LFM).
559 Values are mean ± SE (n = 9). †p < 0.05 compared with time 0 fish fed the Control diet.
560 ‡p < 0.05 compared with time 0 fish fed the LFM diet. Values with asterisks (*) are
561 significantly different at the same point in time between the test groups (p < 0.05).
562
563 Fig. 10. Pancreatic digestive enzymes activities in the posterior intestinal content of
564 yellowtail fed a fishmeal-based diet (Control) and a plant-based low-fishmeal diet (LFM).
565 Values are mean ± SE (n = 9). †p < 0.05 compared with time 0 fish fed the Control diet.
566 ‡p < 0.05 compared with time 0 fish fed the LFM diet. Values with asterisks (*) are
567 significantly different at the same point in time between the test groups (p < 0.05).
568
569
570

571 **References**

572 Albro, P.W., Hall, R.D., Corbett, J.T., Schroeder, J., 1985. Activation of nonspecific
573 lipase (EC 3.1. 1.-) by bile salts. Biochim. Biophys. Acta 835, 477–490.
574 doi:10.1016/0005-2760(85)90117-1

575 Anson, M.L., 1938. The estimation of pepsin, trypsin, papain, and cathepsin with
576 hemoglobin. The Journal of General Physiology.

577 Einarsson, S., Davies, P.S., 1996. On the localisation and ultrastructure of pepsinogen,
578 trypsinogen and chymotrypsinogen secreting cells in the Atlantic salmon, *Salmo*
579 *salar* L. Comp. Biochem. Physiol. B. 114, 295–301. doi:10.1016/0305-
580 0491(96)00036-3

581 Erlanger, B.F., Kokowsky, N., Cohen, W., 1961. The preparation and properties of two
582 new chromogenic substrates of trypsin. Arch. Biochem Biophys. 95, 271–278.

583 Furutani, T., Masumoto, T., Fukada, H., 2012. Response of cholecystokinin and digestive
584 enzyme mRNA levels to various feed ingredients in yellowtail *Seriola*
585 *quinqueradiata*. Fish Sci. 78, 1075–1082. doi:10.1007/s12562-012-0537-x

586 Gatlin, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W.,
587 Herman, E., Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., Rust, M., Sealey, W.,
588 Skonberg, D., J Souza, E., Stone, D., Wilson, R., Wurtele, E., 2007. Expanding the
589 utilization of sustainable plant products in aquafeeds: a review. Aquac. Research 38,
590 551–579. doi:10.1111/j.1365-2109.2007.01704.x

591 Gritti, I., Banfi, G., Roi, G.S., 2000. Pepsinogens: physiology, pharmacology
592 pathophysiology and exercise. Pharmacol. Res. 41, 265–281.
593 doi:10.1006/phrs.1999.0586

594 Iwashita, Y., Suzuki, N., Yamamoto, T., Shibata, J.-I., Isokawa, K., Soon, A.H., Ikehata,
595 Y., Furuita, H., Sugita, T., Goto, T., 2008. Supplemental effect of cholyltaurine and
596 soybean lecithin to a soybean meal-based fish meal-free diet on hepatic and intestinal
597 morphology of rainbow trout *Oncorhynchus mykiss*. Fish. Sci. 74, 1083–1095.
598 doi:10.1111/j.1444-2906.2008.01628.x

599 Konturek, S.J., Pepera, J., Zabielski, K., Konturek, P.C., Pawlik, T., Szlachcic, A., Hahn,
600 E.G., 2003. Brain-gut axis in pancreatic secretion and appetite control. J. Physiol.
601 Pharmacol. 54, 293–317.

602 Kortner, T.M., Gu, J., Krogdahl, Å., Bakke, A.M., 2013. Transcriptional regulation of

603 cholesterol and bile acid metabolism after dietary soyabean meal treatment in
604 Atlantic salmon (*Salmo salar* L.). Br. J. Nutr. 109, 593–604.
605 doi:10.1017/S0007114512002024
606 Kurokawa, T., Suzuki, T., Hashimoto, H., 2003. Identification of gastrin and multiple
607 cholecystokinin genes in teleost. Peptides 24, 227–235. doi:10.1016/S0196-
608 9781(03)00034-2
609 Lilleeng, E., Frøystad, M.K., Ostby, G.C., Valen, E.C., Krogdahl, Å., 2007. Effects of
610 diets containing soybean meal on trypsin mRNA expression and activity in Atlantic
611 salmon (*Salmo salar* L). Comp. Biochem. Physiol. A 147, 25–36.
612 doi:10.1016/j.cbpa.2006.10.043
613 Matsunari, H., Iwashita, Y., Amano, S., Suzuki, N., Furuita, H., Yamamoto, T., 2015.
614 Effects of alcohol treatment of dietary soybean meal on growth performance and
615 hepatopancreatic histology of juvenile red sea bream *Pagrus major*. Aquacul. Sci. 63,
616 71–78. doi:10.11233/aquaculturesci.63.71
617 Murashita, K., Akimoto, A., Iwashita, Y., Amano, S., Suzuki, N., Matsunari, H., Furuita,
618 H., Sugita, T., Yamamoto, T., 2013. Effects of biotechnologically processed soybean
619 meals in a nonfishmeal diet on growth performance, bile acid status, and
620 morphological condition of the distal intestine and liver of rainbow trout
621 *Oncorhynchus mykiss*. Fish. Sci. 79, 447–457. doi:10.1007/s12562-013-0617-6
622 Murashita, K., Fukada, H., Hosokawa, H., Masumoto, T., 2007. Changes in
623 cholecystokinin and peptide Y gene expression with feeding in yellowtail (*Seriola*
624 *quinqueradiata*): Relation to pancreatic exocrine regulation. Comp. Biochem.
625 Physiol. B 146, 318–325. doi:10.1016/j.cbpb.2006.11.009
626 Murashita, K., Fukada, H., Hosokawa, H., Masumoto, T., 2006. Cholecystokinin and
627 peptide Y in yellowtail (*Seriola quinqueradiata*): Molecular cloning, real-time
628 quantitative RT-PCR, and response to feeding and fasting. Gen. Comp. Endocrinol.
629 145, 287–297. doi:10.1016/j.ygcen.2005.09.008
630 Murashita, K., Fukada, H., Rønnestad, I., Kurokawa, T., Masumoto, T., 2008. Nutrient
631 control of release of pancreatic enzymes in yellowtail (*Seriola quinqueradiata*):
632 Involvement of CCK and PY in the regulatory loop. Comp. Biochem. Physiol. P A
633 150, 438–443. doi:10.1016/j.cbpa.2008.05.003
634 Murashita, K., Furuita, H., Matsunari, H., Yamamoto, T., Awaji, M., Nomura, K., Nagao,
635 J., Tanaka, H., 2012. Partial characterization and ontogenetic development of

636 pancreatic digestive enzymes in Japanese eel *Anguilla japonica* larvae. Fish Physiol.
637 Biochem. 39, 895–905. doi:10.1007/s10695-012-9749-3
638 Murashita, K., Matsunari, H., Furuita, H., Rønnestad, I., Oku, H., Yamamoto, T., 2018.
639 Effects of dietary soybean meal on the digestive physiology of red seabream *Pagrus*
640 *major*. Aquaculture 493, 219–228. doi:10.1016/j.aquaculture.2018.05.005
641 Murashita, K., Matsunari, H., Kumon, K., Tanaka, Y., Shiozawa, S., Furuita, H., Oku, H.,
642 Yamamoto, T., 2014. Characterization and ontogenetic development of digestive
643 enzymes in Pacific bluefin tuna *Thunnus orientalis* larvae. Fish Physiol. Biochem.
644 40, 1741–1755. doi:10.1007/s10695-014-9964-1
645 Natalia, Y., 2004. Characterization of digestive enzymes in a carnivorous ornamental fish,
646 the Asian bony tongue *Scleropages* formosus (*Osteoglossidae*). Aquaculture 233,
647 305–320. doi:10.1016/j.aquaculture.2003.08.012
648 Nguyen, H.P., Khaoian, P., Furutani, T., Nagano, J., 2011. Effects of alcohol extract from
649 soybean meal on pancreatic digestive enzyme and bile acid secretion in yellowtail
650 *Seriola quinqueradiata*. Aquacul. Sci. doi:10.11233/aquaculturesci.59.465
651 Nguyen, H.P., Khaoian, P., Furutani, T., Nagano, J., Fukada, H., Masumoto, T., 2017.
652 Effects of alcohol extract of defatted soybean meal on growth performance and
653 digestive physiology of yellowtail *Seriola quinqueradiata*. Fish. Sci. 83, 99–106.
654 doi:10.1007/s12562-016-1049-x
655 Rehfeld, J.F., 2014. Gastrointestinal hormones and their targets. Adv. Exp. Med. Biol.
656 817, 157–175. doi:10.1007/978-1-4939-0897-4_7
657 Romarheim, O.H., Skrede, A., Gao, Y., Krogdahl, Å., Denstadli, V., Lilleeng, E.,
658 Storebakken, T., 2006. Comparison of white flakes and toasted soybean meal partly
659 replacing fish meal as protein source in extruded feed for rainbow trout
660 (*Oncorhynchus mykiss*). Aquaculture 256, 354–364.
661 doi:10.1016/j.aquaculture.2006.02.006
662 Tacon, A.G.J., Metian, M., 2008. Global overview on the use of fish meal and fish oil in
663 industrially compounded aquafeeds: Trends and future prospects. Aquaculture 285,
664 146–158. doi:10.1016/j.aquaculture.2008.08.015
665 Takagi, S., Murata, H., Goto, T., Endo, M., Yamashita, H., Ukawa, M., 2008. Taurine is
666 an essential nutrient for yellowtail *Seriola quinqueradiata* fed non-fish meal diets
667 based on soy protein concentrate. Aquaculture 280, 198–205.
668 doi:10.1016/j.aquaculture.2008.05.012

669 Urán, P.A., Gonçalves, A.A., Taverne-Thiele, J.J., Schrama, J.W., Verreth, J.A.J.,
670 Rombout, J.H.W.M., 2008. Soybean meal induces intestinal inflammation in
671 common carp (*Cyprinus carpio* L.). Fish Shellfish Immunol. 25, 751–760.
672 doi:10.1016/j.fsi.2008.02.013
673 van den Ingh, T.S.G.A.M., Krogdahl, A., Olli, J.J., Hendriks, H.G.C.J.M., Koninkx,
674 J.G.J.F., 1991. Effects of soybean-containing diets on the proximal and distal
675 intestine in Atlantic salmon (*Salmo salar*): a morphological study. Aquaculture 94,
676 297–305. doi:10.1016/0044-8486(91)90174-6
677 Yamamoto, T., Suzuki, N., Furuita, H., Sugita, T., Tanaka, N., Goto, T., 2007.
678 Supplemental effect of bile salts to soybean meal-based diet on growth and feed
679 utilization of rainbow trout *Oncorhynchus mykiss*. Fish. Sci 73, 123–131.
680 doi:10.1111/j.1444-2906.2007.01310.x
681 Yasuike, M., Iwasaki, Y., Nishiki, I., Nakamura, Y., Matsuura, A., Yoshida, K., Noda, T.,
682 Andoh, T., Fujiwara, A., 2018. The yellowtail (*Seriola quinqueradiata*) genome and
683 transcriptome atlas of the digestive tract. DNA Res. 93, 274.
684 doi:10.1093/dnares/dsy024
685 Zhang, C., Rahimnejad, S., Wang, Y.-R., Lu, K., Song, K., Wang, L., Mai, K., 2018.
686 Substituting fish meal with soybean meal in diets for Japanese seabass (*Lateolabrax*
687 *japonicus*): Effects on growth, digestive enzymes activity, gut histology, and
688 expression of gut inflammatory and transporter genes. Aquaculture 483, 173–182.
689 doi:10.1016/j.aquaculture.2017.10.029
690

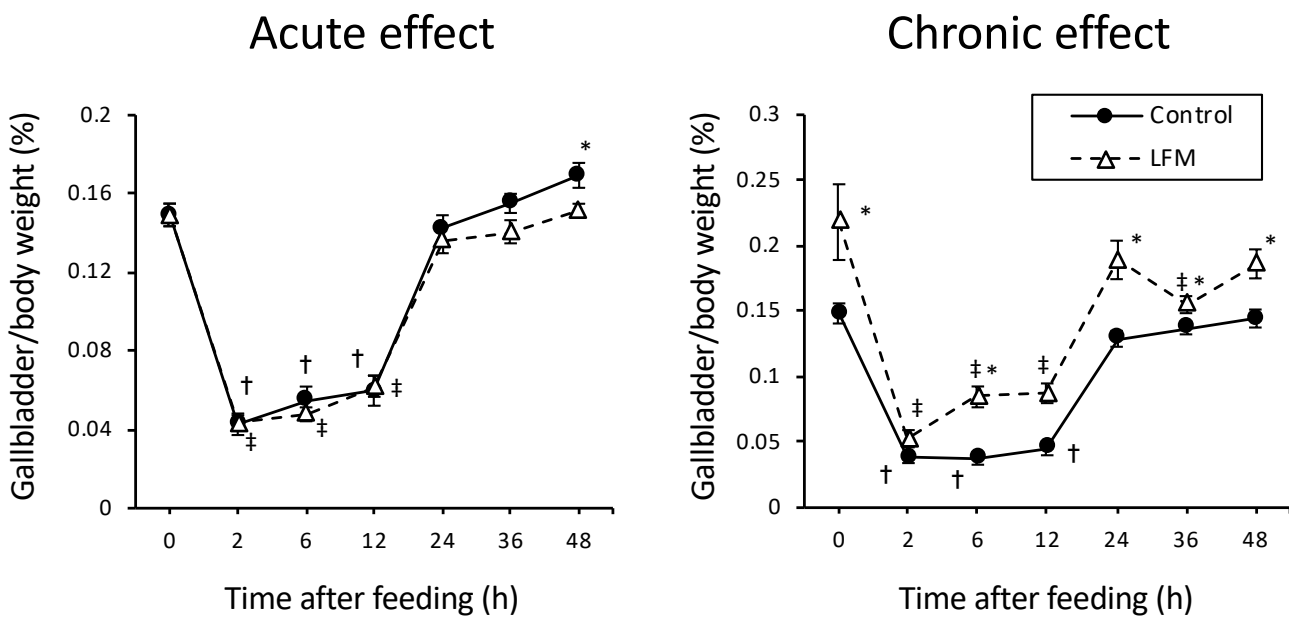

Acute effect
Chronic effect
Gallbladder/body weight (%)
Time after feeding (h)
Control
LFM
0
2
6
12
24
36
48
0.2
0.16
0.12
0.08
0.04
0.3
0.25
0.15
0.1
0.05

Fig. 1

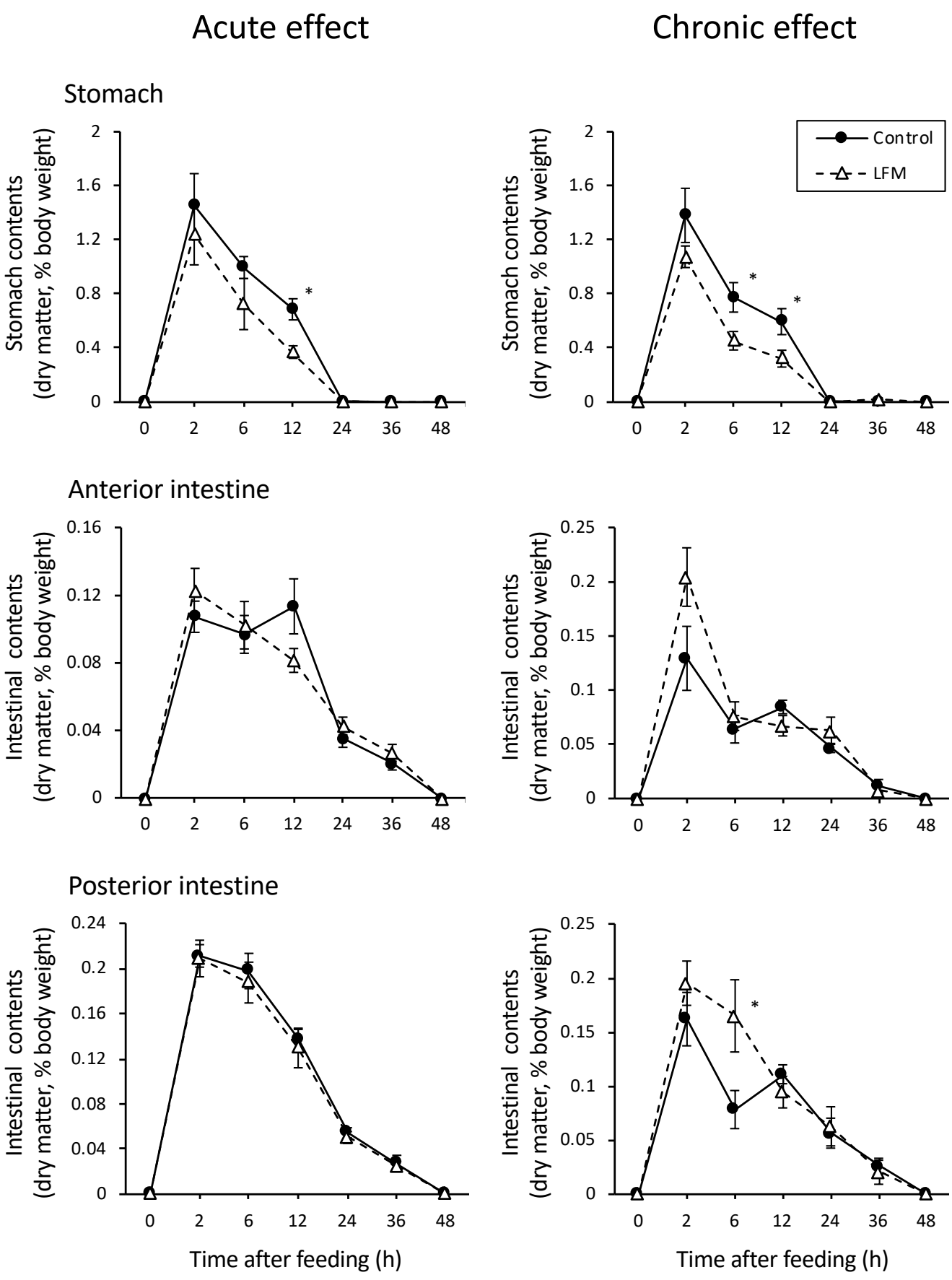

Acute effect
Chronic effect
Stomach
Anterior intestine
Posterior intestine
Stomach contents (dry matter, % body weight)
Intestinal contents (dry matter, % body weight)
Control
LFM
Time after feeding (h)
0
2
6
12
24
36
48
*

Fig. 2

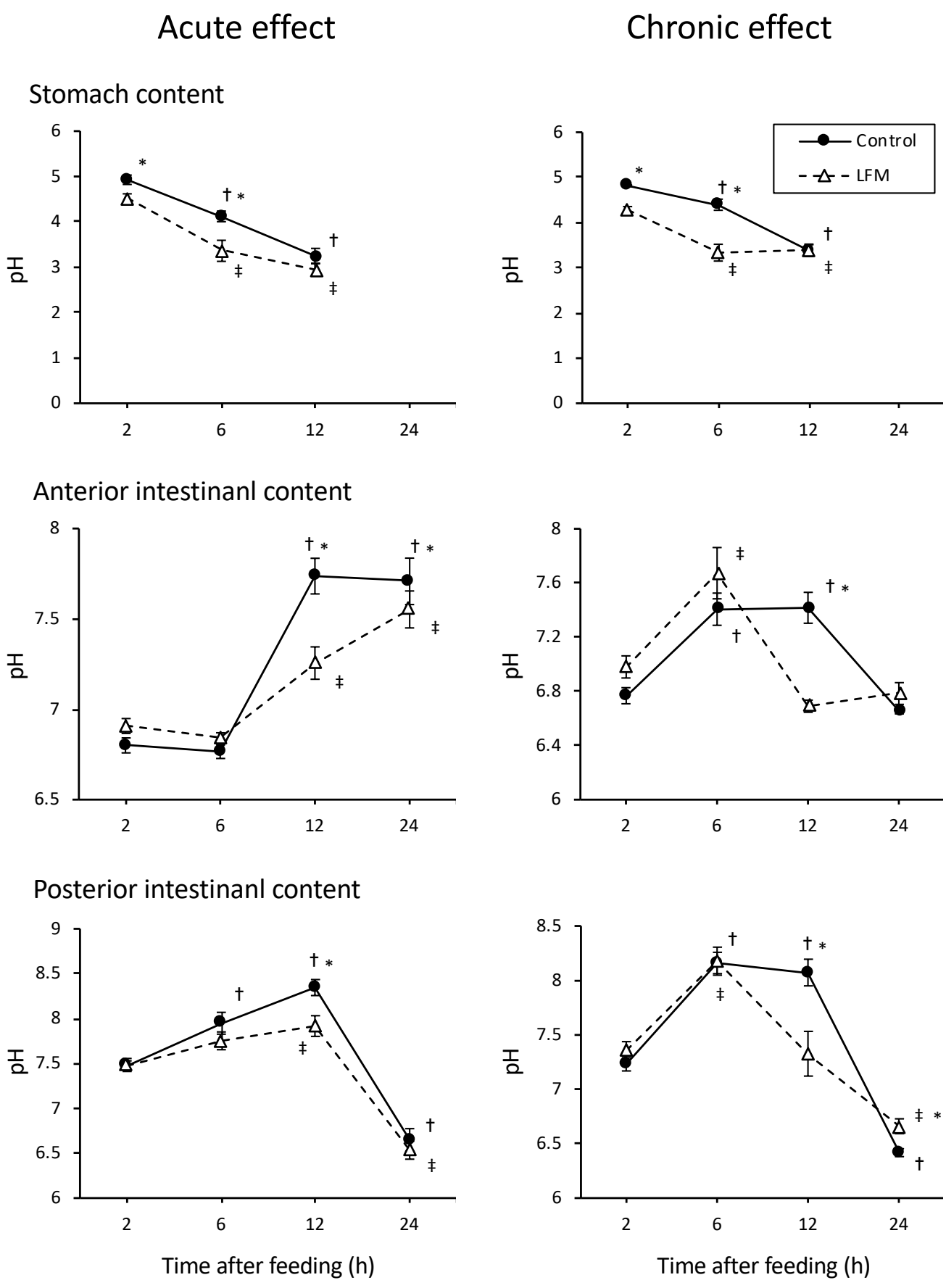

Acute effect
Chronic effect
Stomach content
Anterior intestinanl content
Posterior intestinanl content
pH
Control
LFM
Time after feeding (h)
Time after feeding (h)

Fig. 3

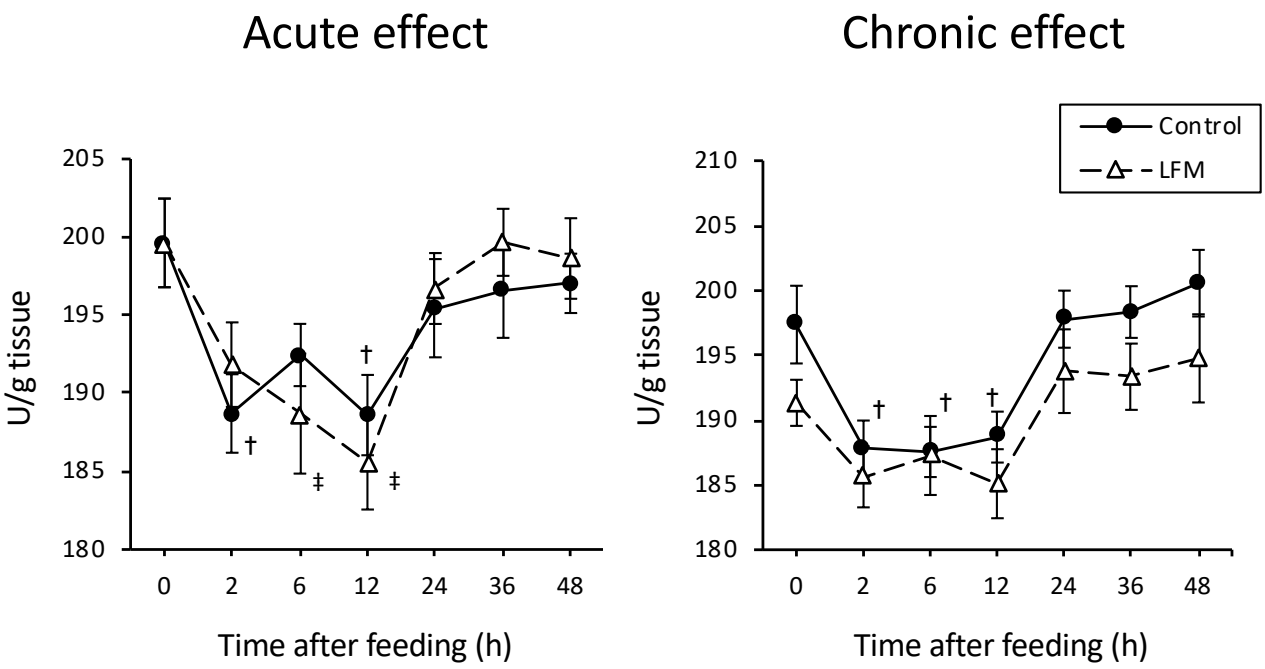

Acute effect
Chronic effect
Control
LFM
U/g tissue
Time after feeding (h)
205
200
195
190
185
180
210
0
2
6
12
24
36
48
†
‡

Fig. 4

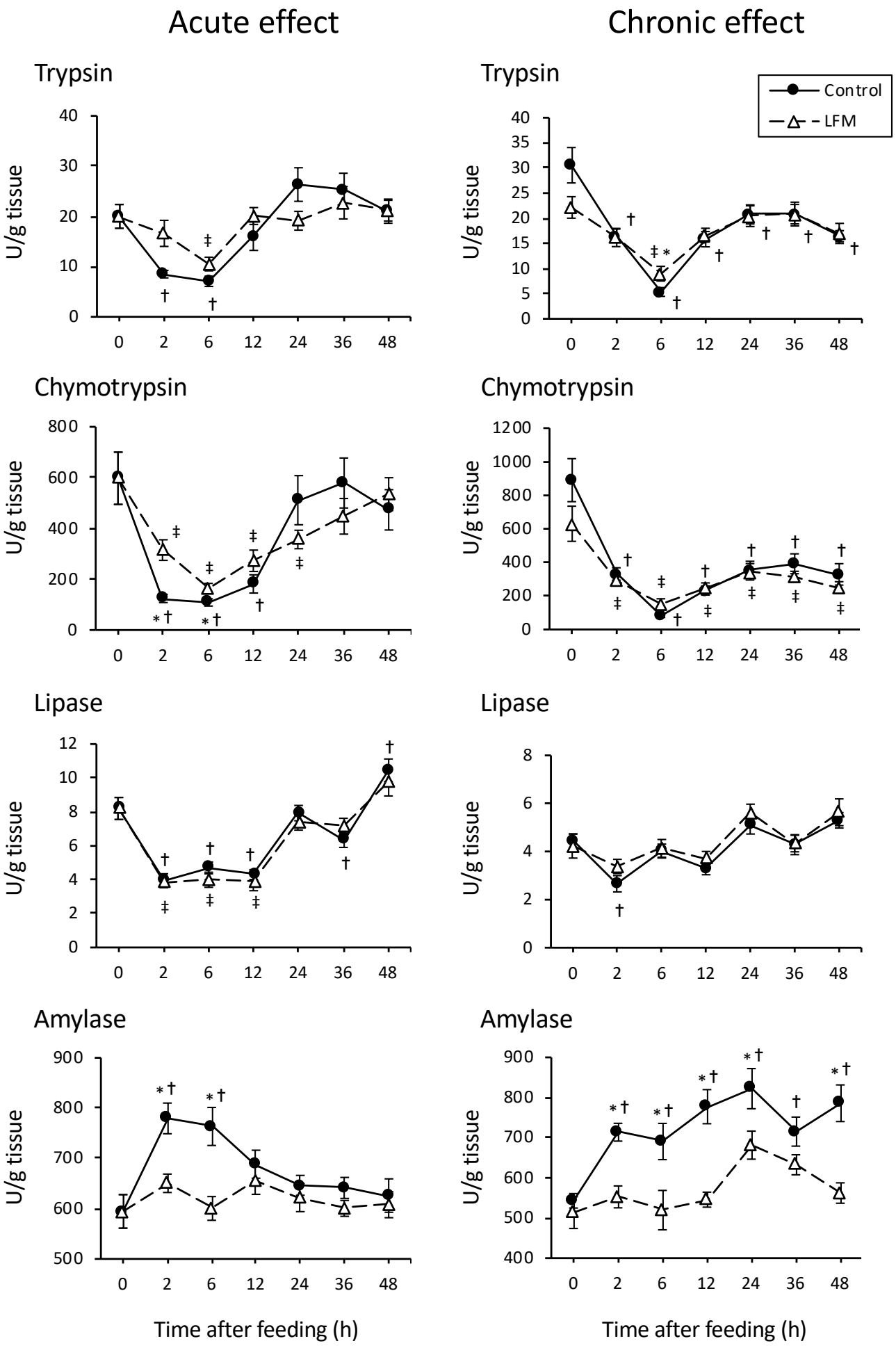

Acute effect
Chronic effect
Trypsin
Chymotrypsin
Lipase
Amylase
Control
LFM
U/g tissue
Time after feeding (h)

Fig. 5

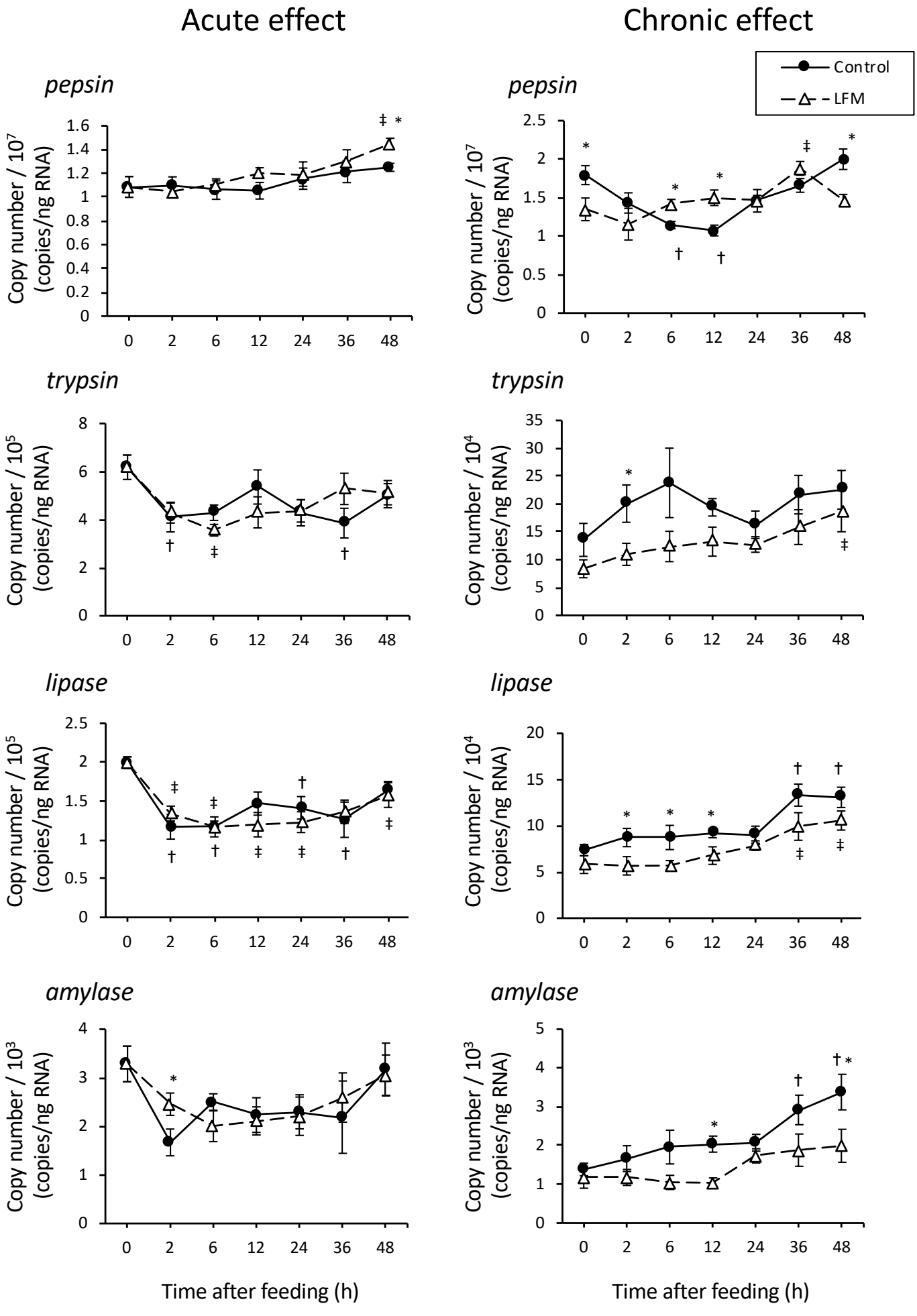

Acute effect
Chronic effect
Control
LFM
pepsin
trypsin
lipase
amylase
Copy number / 10^7 (copies/ng RNA)
Copy number / 10^5 (copies/ng RNA)
Copy number / 10^4 (copies/ng RNA)
Copy number / 10^3 (copies/ng RNA)
0 2 6 12 24 36 48
Time after feeding (h)

Fig. 6

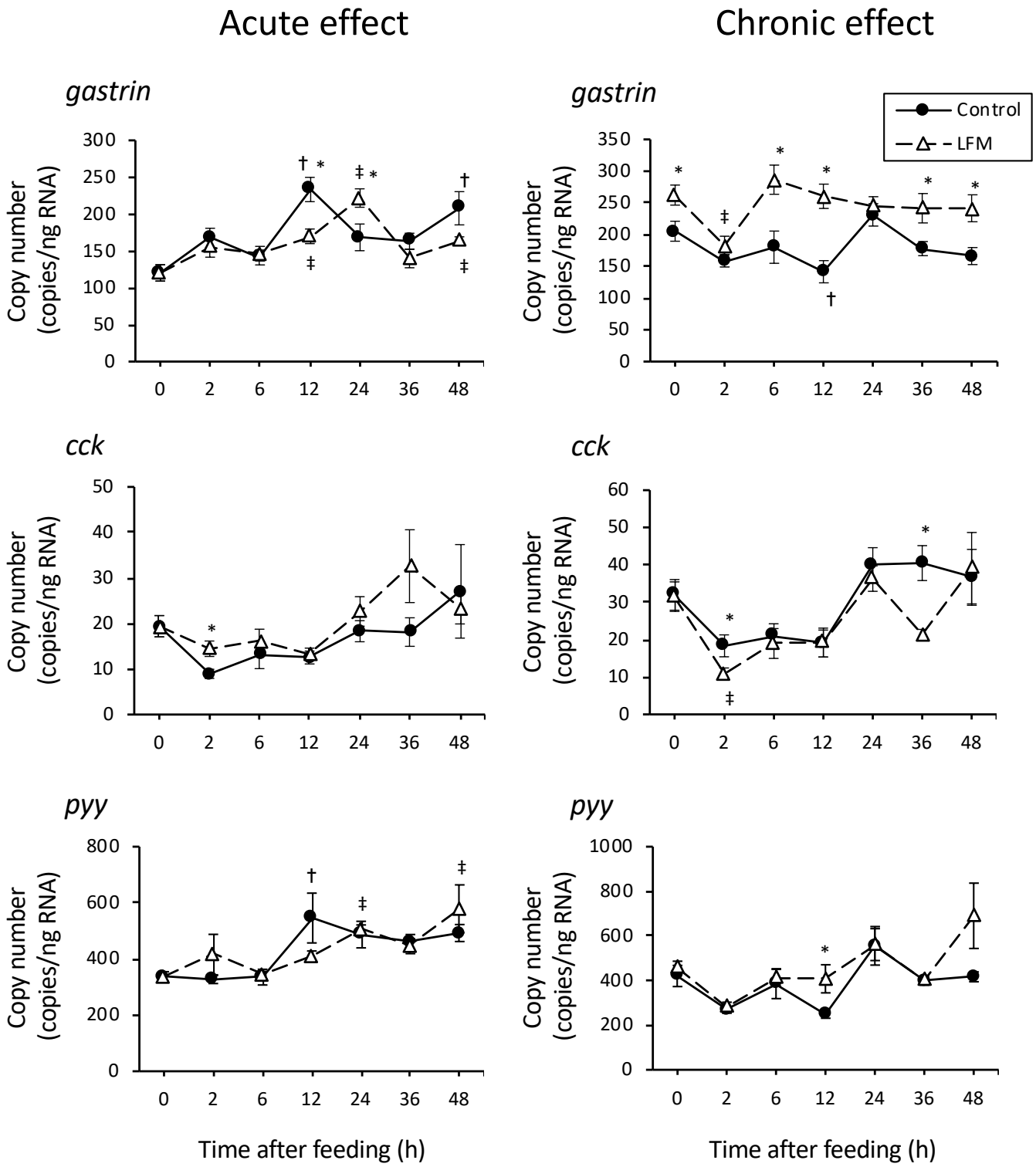

Acute effect
Chronic effect
gastrin
gastrin
Control
LFM
Copy number (copies/ng RNA)
cck
cck
pyy
pyy
Time after feeding (h)
Time after feeding (h)

Fig. 7

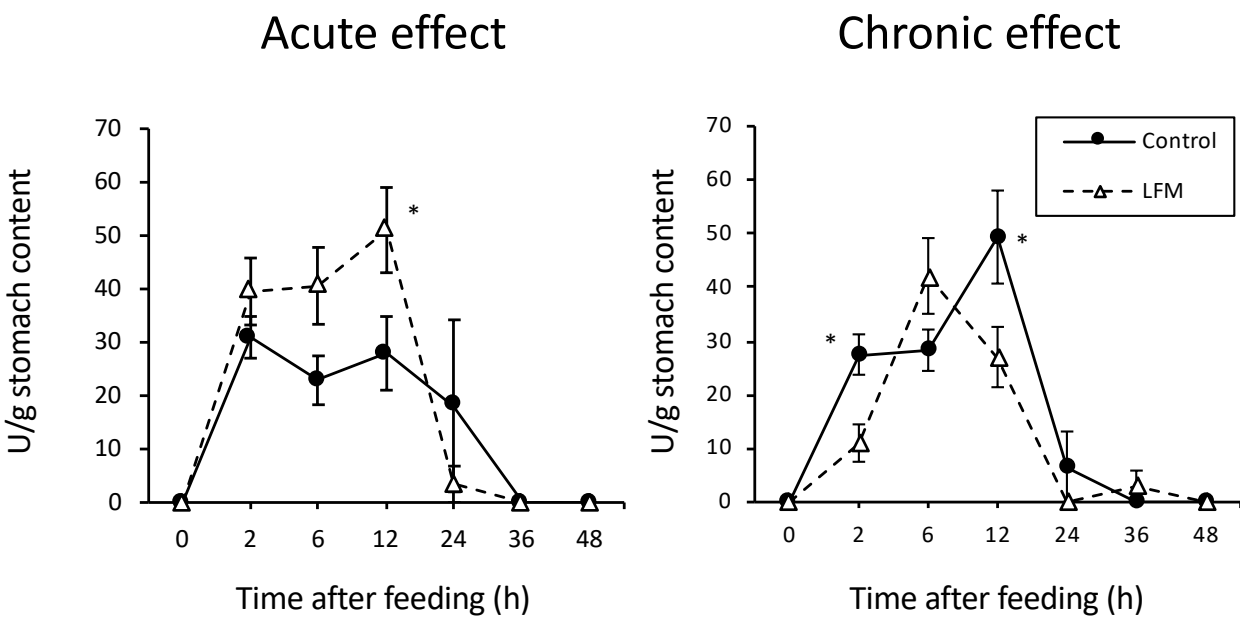

Acute effect
Chronic effect
U/g stomach content
70
60
50
40
30
20
10
0
0
2
6
12
24
36
48
Time after feeding (h)
*
Control
LFM

Fig. 8

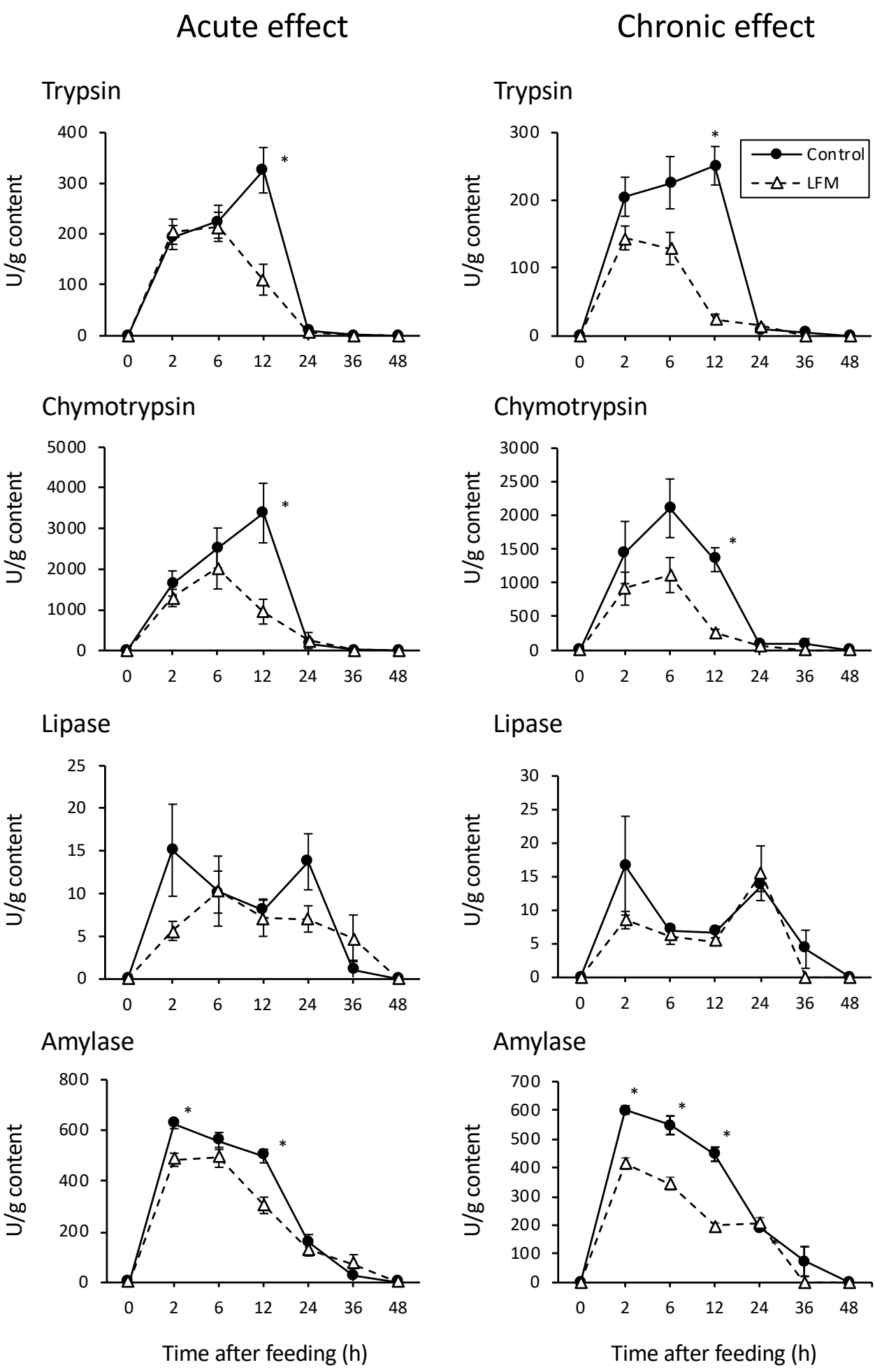

Acute effect
Chronic effect
Trypsin
Trypsin
Chymotrypsin
Chymotrypsin
Lipase
Lipase
Amylase
Amylase
U/g content
Control
LFM
Time after feeding (h)
Time after feeding (h)

Fig. 9

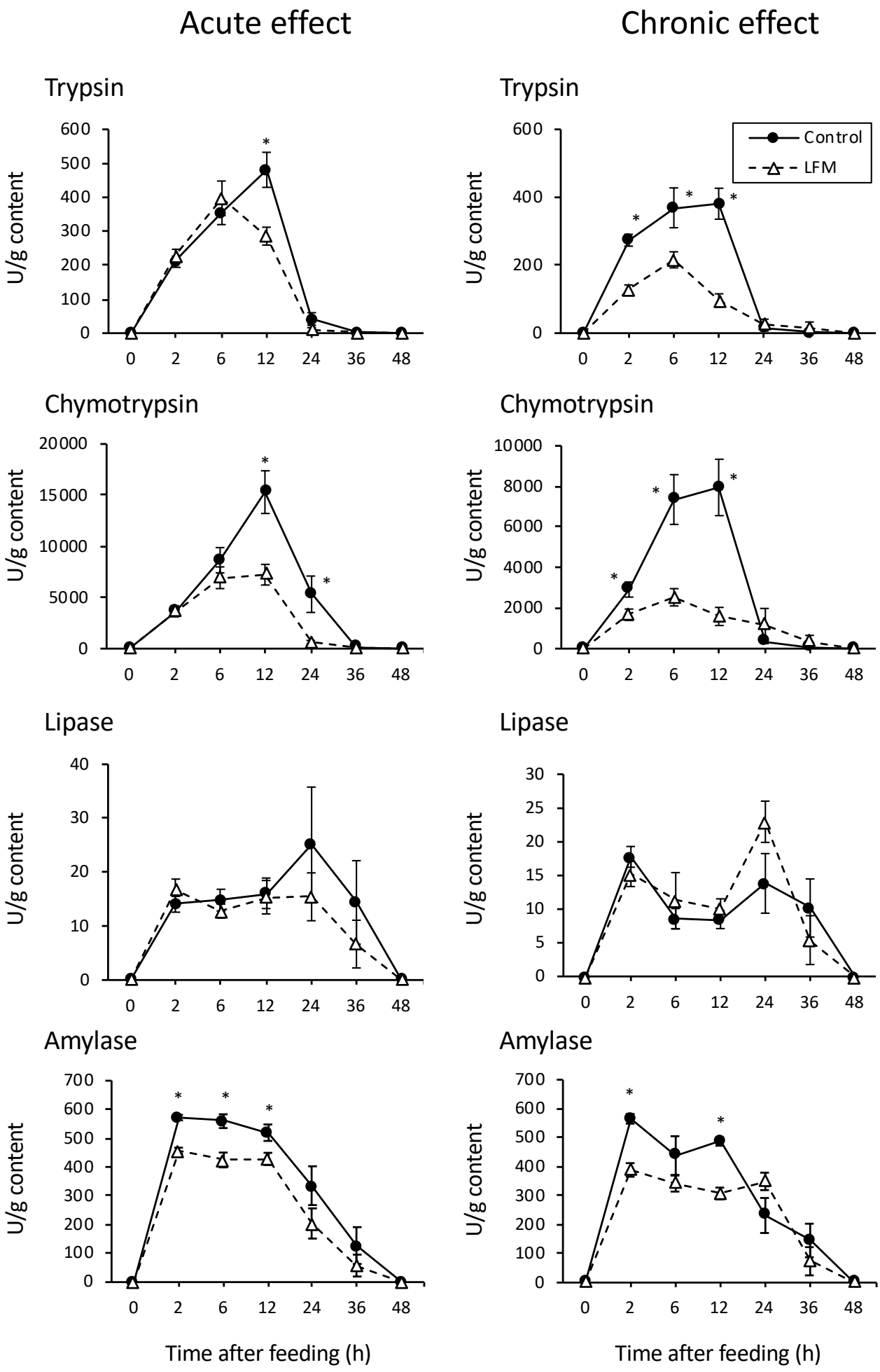
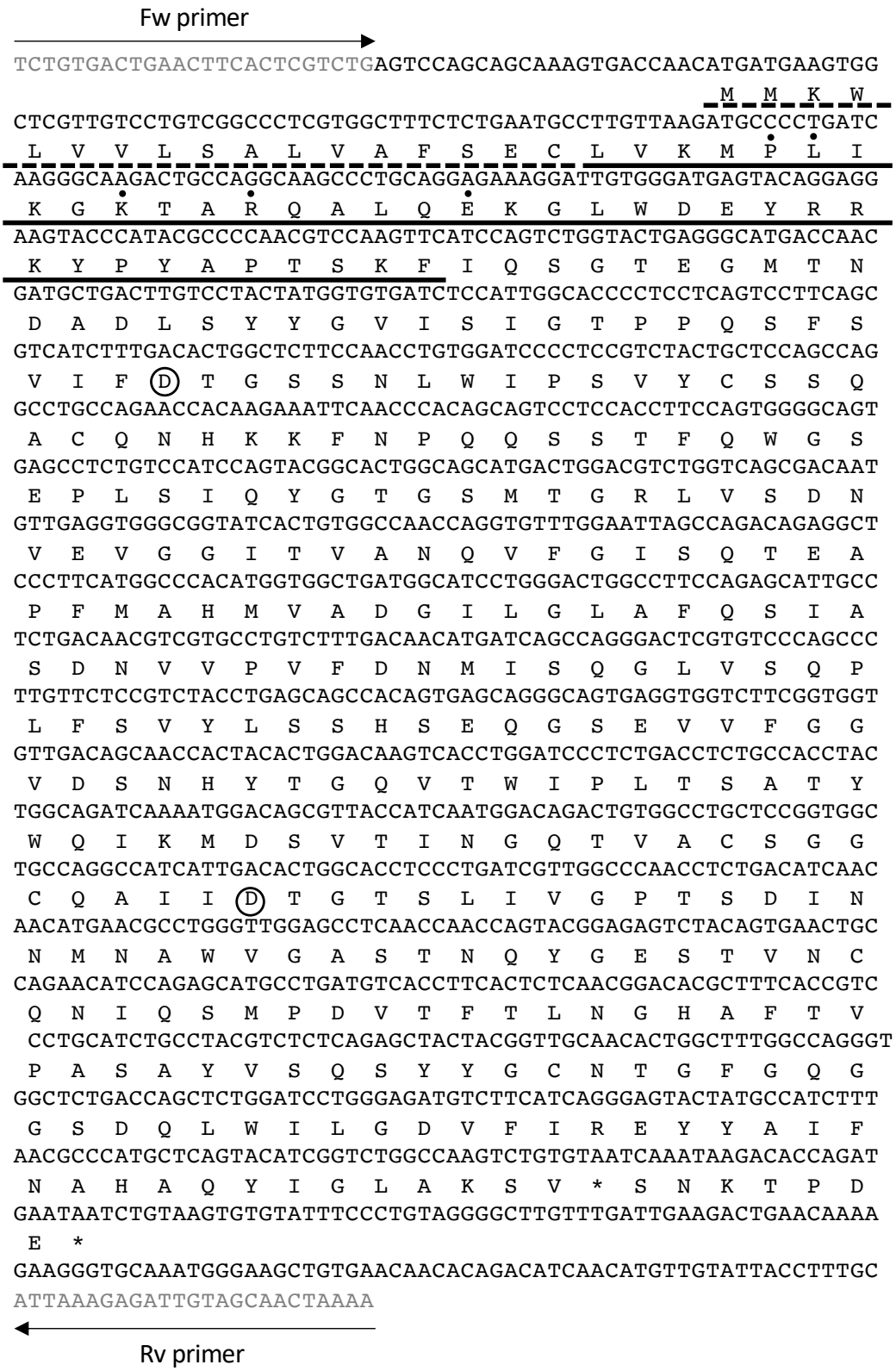
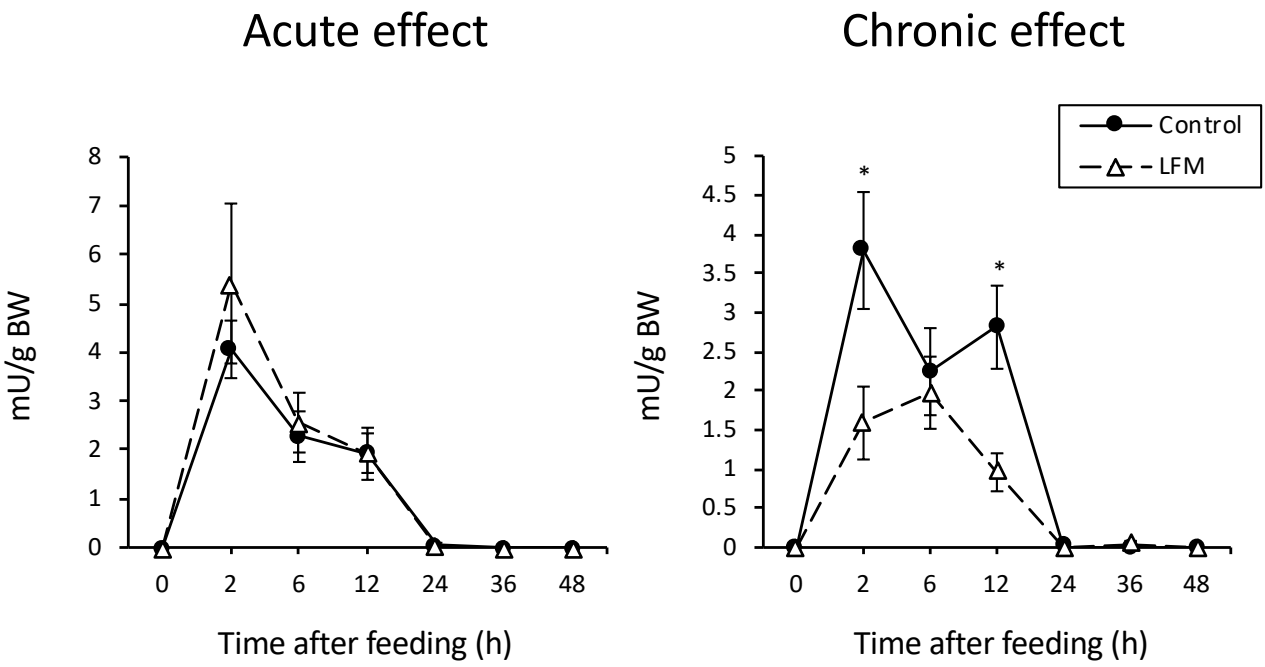

Acute effect
Chronic effect
Trypsin
Trypsin
Control
LFM
Chymotrypsin
Chymotrypsin
Lipase
Lipase
Amylase
Amylase
U/g content
Time after feeding (h)
0
2
6
12
24
36
48

Fig. 10

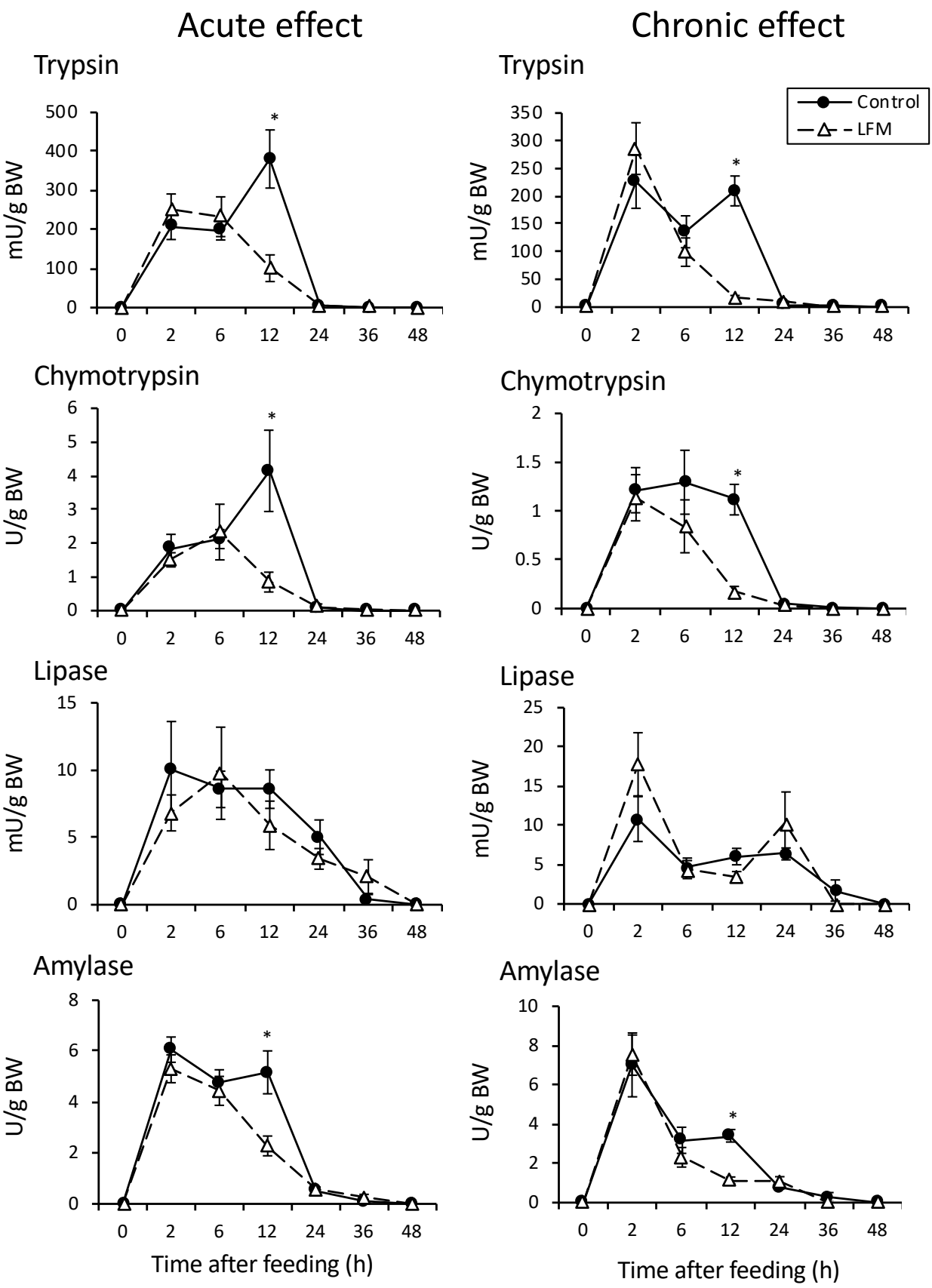


Supplementary figure 1

The cDNA and deduced amino acid sequence of yellowtail *pepsin* assessed in this study (GenBank accession no.: xxxxx). A broken line indicates estimated signal peptide. A solid line indicates activation segment. Five highly conserved residues of the activation segment are indicated with dot. Two aspartic acids in the catalytic site are circled. The primer set for cDNA cloning of the yellowtail *pepsin* was designed based on predicted *pepsin* nucleotide sequence of the another *Seriola* species of California yellowtail, *S. lalandi* (GenBank accession no.: XM_023427456). The first-strand cDNA, synthesized as described in section 2.3.4., was used as a PCR template. The PCR products were purified by agarose gel electrophoresis and a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and were then cloned into the pCR4-TOPO vector (ThermoFisher SCIENTIFIC). The inserts were sequenced by a 3130 Genetic Analyzer using a BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher SCIENTIFIC).

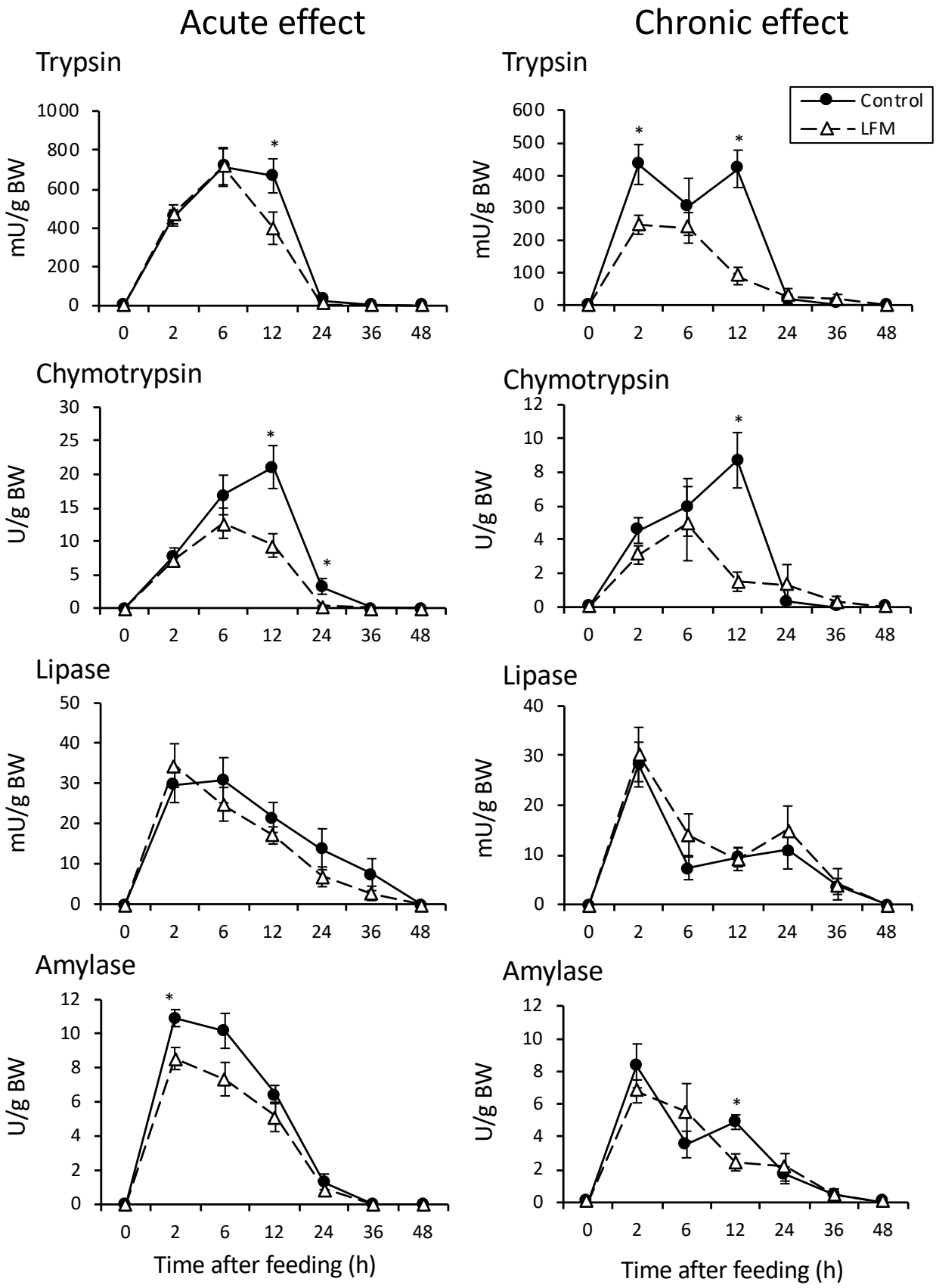


Supplementary figure 2
Pepsin activities in the stomach content of yellowtail fed a fishmeal based diet (Control) and a plant based low fish meal diet (LFM). Values are mean ± SE (n = 9), and expressed as U/g body weight. The unit was calculated as: U/g body weight = U/g intestinal content × Intestinal content (g) / body weight (g). †$p < 0.05$ compared with 0 time fish fed the Control diet. ‡$p < 0.05$ compared with 0 time fish fed the LFM diet. Values with asterisk (*) are significantly different at the same point in time between the test groups ($p < 0.05$).



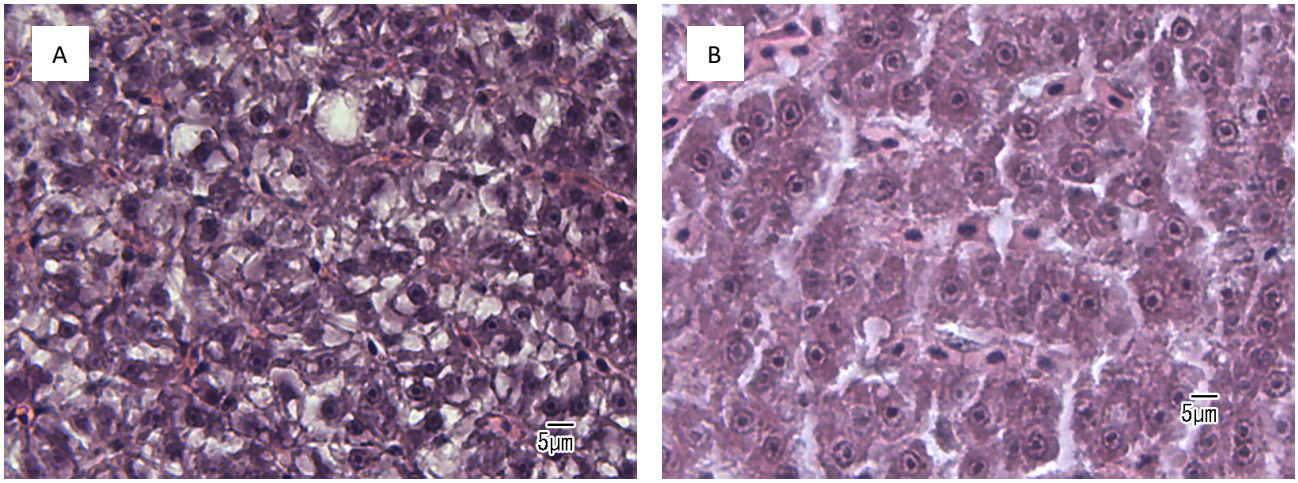
Supplementary figure 3

Pancreatic digestive enzymes activities in the anterior intestinal content of yellowtail fed a fishmeal based diet (Control) and a plant based low fish meal diet (LFM). Values are mean ± SE (n = 9), and expressed as U/g body weight. The unit was calculated as: U/g body weight = U/g intestinal content × Intestinal content (g) / body weight (g). †$p < 0.05$ compared with 0 time fish fed the Control diet. ‡$p < 0.05$ compared with 0 time fish fed the LFM diet. Values with asterisk (*) are significantly different at the same point in time between the test groups ($p < 0.05$).



Supplementary figure 4

Pancreatic digestive enzymes activities in the posterior intestinal content of yellowtail fed a fishmeal based diet (Control) and a plant based low fish meal diet (LFM). Values are mean ± SE (n = 9), and expressed as U/g body weight. The unit was calculated as: U/g body weight = U/g intestinal content × Intestinal content (g) / body weight (g). †$p < 0.05$ compared with 0 time fish fed the Control diet. ‡$p < 0.05$ compared with 0 time fish fed the LFM diet. Values with asterisk (*) are significantly different at the same point in time between the test groups ($p < 0.05$).



C

	Control	LFM	*P*
HSI (%)[a]	1.03±0.22	0.79±0.18	< 0.01
Cytoplasm (μm)[b]	12.8±1.9	12.1±0.8	0.287
Nucleus (μm)[b]	5.95±0.37	5.51±0.79	0.154
Adipocyte (sqμm)[c]	12155±1160	10167±1995*	0.020

[a]Hepatosomatic index = (100 × liver weight) / fish weight. Values are means ± SD (*n* = 63). * Signicicantly different from the control diet (P > 0.05).

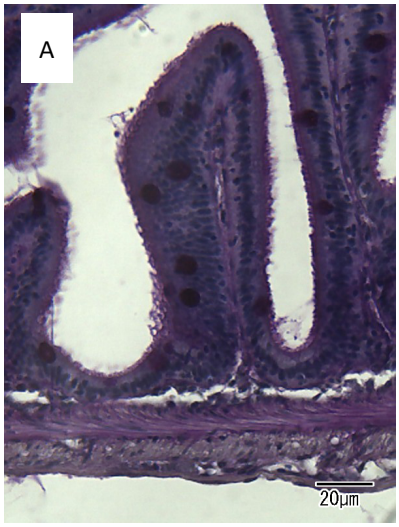
[b]Values are means ± SD (*n* = 18), mean of 30 cells. * Signicicantly different from the control diet (P > 0.05).

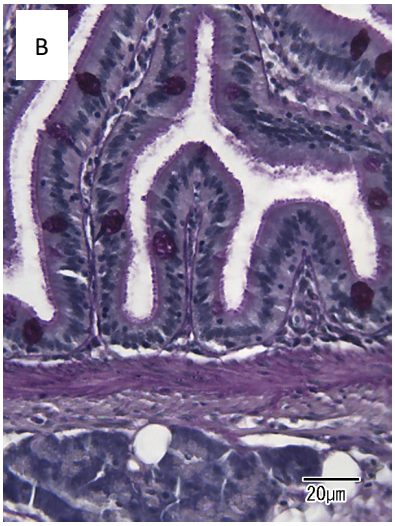
[c]Values are means ± SD (*n* = 18), mean of five observation fields. * Signicicantly different from the control diet (P > 0.05).

Supplementary figure 5

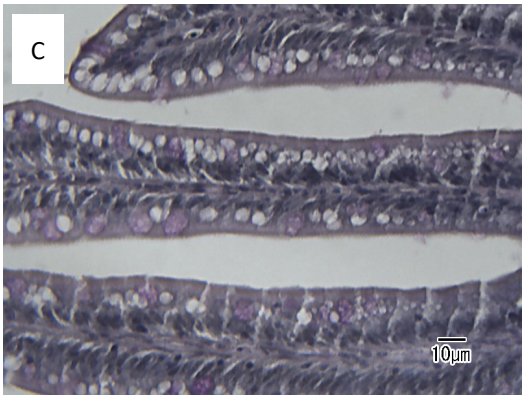
Effects of the test diets on the liver morphology of yellowtail. Typical histological sections of the liver of yellowtail fed (A) a fishmeal based diet (Control) and (B) a plant based low fish meal diet (LFM) for 6 weeks. (C) Hepatosomatic index and cytoplasm, nucleus and adipocyte sizes of the liver in yellowtail fed the experimental diets. Hepatosomatic index of fish fed the LFM diet was smaller than those of fish fed the Control. Although no differences were observed in the cytoplasm and nucleus sizes between the diet groups, total adipocyte area of fish fed the LFM diet was smaller than those of fish fed the Control diet.

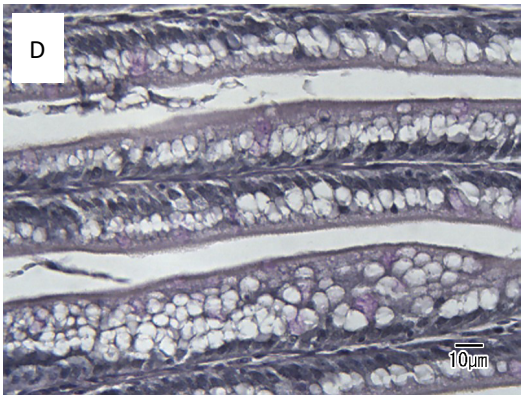
Pyloric caeca

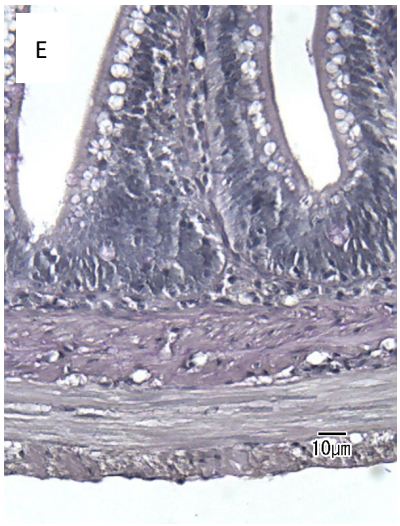


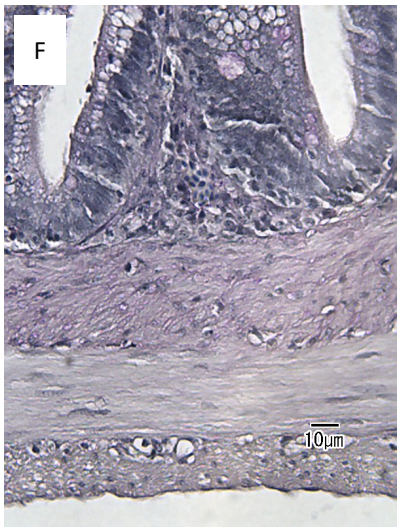


Hind-gut









Supplementary figure 6
Effects of the test diets on the intestinal tract morphology of yellowtail. Typical histological sections of (A) the pyloric caeca of fish fed a fishmeal based diet (Control) and (B) a plant based low fish meal diet (LFM) for 6 weeks. Hstological sections of (C) the mucosal fold in the hind-gut of fish fed the Control diet and (D) the LFM diet. Histological sections of (E) the lamia propria of submucosa in the hind-gut of fish fed Control diet and (F) the LFM diet. The morphological conditions of the mucosal folds and submucosa of the hind-gut in fish fed the LFM diet showed normal features as seen in fish fed the Control diet.

Table 1

Ingredients and proximate composition of the experimental diets.

	Control	LFM
Ingredients (% wet weight)		
Jack mackarel meal	50.00	15.00
Defatted soybean meal	5.30	17.00
Soy protein concentrate	1.50	18.00
Corn gluten meal	7.00	18.00
Wheat flour	17.00	8.00
Pollock oil	4.49	7.75
Soybean oil	3.20	2.44
α-Starch	5.30	5.30
Vitamins[a]	0.50	0.50
Choline chloride	0.25	0.25
$Ca(H_2PO_4)_2H_2O$	1.60	3.20
Minerals[b]	1.20	1.20
Cellulose	0.98	0.03
L-Lysine HCl	-	1.09
L-Methionine	-	0.34
Taurine	1.68	1.90
Analytical contents (% dry matter basis)		
Crude protein	49.3	48.5
Crude fat	14.6	14.6
Crude starch	17.7	17.7
Ash	10.7	7.9
Taurine	1.9	1.9

[a]Vitamin mix (mg/100 g mix): Vitamin B_1, 900; Vitamin B_2, 1500; Vitamin B_6, 600; Vitamin B_{12}, 1.5; niacin, 6×10^3; Ca-pantotenate, 1 500; inositol, 30×10^3; biotine, 90; folic acid, 225; *p* -Aminobenzoic acid, 750; Vitamin K_3, 750; Vitamin A, 600 000 IU; Vitamin D_3, 600 000 IU; cellulose, 57.

[b]Mineral mix (g/100 g mix): Calcium lactate, 3.5; Ferric citrate, 2.5; Magnesium salfate, 15.0; KH_2PO_4, 32.0; NaCl, 1.0; $AlCl_3 \cdot 6H_2O$, 0.015; KlO_3, 0.003; $CuSO_4 \cdot 5 \cdot H2O$, 0.031; $MnSO_4 \cdot 5H_2O$, 0.175; $CoCl_2 \cdot 6H_2O$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.353; celluose, 13.422

Table 2

Primers and probes used for qPCR analyses in the present study.

Gene	Direction / Probe	Sequence (5'-3')
trypsin	Foward[a]	ACCCCGGCATGATTGACAACA
	Riverse[a]	CCACCAGAGTCACCCTGGCAAGA
lipase	Foward[a]	GCCAACTACAAGCAGAATGCCAG
	Riverse[a]	GCTGGGTCCAGTCCTGTGATACG
amylase	Foward[a]	GACTCCAGGCTCCATAAGATGGCTGTT
	Riverse[a]	GACATCACCCTAGCCACTCCATAAGG
pepsin	Forward	CTACAGTGAACTGCCAGAAC
	Riverse	TGCAACCGTAGTAGCTCTGA
gastrin	Forward	CCAGGGCTGGGTGGACTT
	Riverse	AAGCTTGTTGTTTTCACTGTTGTGT
	Probe	CATTCAGCGTCCCGGCGTCC
cck	Foward[b]	TGAGCTGCTGGCAAGACTCA
	Riverse[b]	GCTGTTCGCTGTGGAGTTTCT
	Probe[b]	CTCCTCCAGGAAAGGCTCTGTGCG
pyy	Foward[c]	AACCCTTCCCTGATCCTTTGATTAAGC
	Riverse[c]	CCAGTGCAGGTTGTCTTTTGAAAATCTC

[a] Murashita et al., 2007

[b] Furutani et al., 2012

[c] Murashita et al., 2006

Table 3

Growth performance of yellowtail fed the experimental diets for 6 weeks.[a]

	FM	LFM	*P*
Initial BW (g)	2.6 ± 0.1	2.6 ± 0.1	0.93
Final BW (g)	48.7 ± 2.4	28.6 ± 0.3*	< 0.01
Weight gain (%)[b]	1755 ± 55	995 ± 63*	< 0.01
Specific growth rate (% day^{-1})[c]	7.0 ± 0.1	5.7 ± 0.1*	< 0.01
Feed efficiency ratio[d]	1.2 ± 0.0	1.0 ± 0.0*	< 0.01
Feed intake (% BW day^{-1})[e]	3.7 ± 0.0	4.1 ± 0.1*	< 0.01

[a]Values are means ± SD (n = 3). * Signicicantly different from the Control diet. ($P >$ 0.05).

[b]Weight (%) = 100 × (final BW-initial BW)/initial BW.

[c]Specific growth rate (% day^{-1}) = 100 × (ln final BW - ln initial BW)/trial days.

[d]Feed efficiency ratio = BW gain/food intake.

[e]Feed intake (% BW day^{-1}) = 100 × food intake/[(initial fish number + final fish number)/2 × (initial BW + final BW)/2]/feeding days.

Table 4

Biliary bile acid characteristics of yellowtail fed the experimental diets for 6 weeks.[a]

	Control	LFM	*P*
Total bile acid concentration (mM)	431 ± 5.9	380±9.3*	0.003
Total bile acid content (nmol g BW^{-1})	624 ± 32	706±86	0.299
C-tau/CDC-tau	3.5 ± 0.2	7.0±1.9*	0.037

[a]Values are means ± SD ($n = 9$). * Signicicantly different from the Control diet. ($P < 0.05$).

Supplementary table 1

Blood hemoglobin and plasma parameters in yellowtail fed the experimental diets for 6 weeks.[a]

	Control	LFM	*P*
Alkaline phosphatase (IU l^{-1})	83±13	79±10	0.692
Hemoglobin (g dl^{-1})	9.5±1.1	8.2±0.9*	0.024
Total protein (g dl^{-1})	3.3±0.3	2.5±0.2*	0.022
Triacylglycerol (mg dl^{-1})	62±6.6	56±5.7	0.321
Total cholesterol (mg dl^{-1})	268±21	181±14*	0.004
Glucose (mg dl^{-1})	201±15	139±24*	0.021
Inorganic phosphorus (mg dl^{-1})	6.7±0.6	5.6±0.1	0.052
Calcium (mg dl^{-1})	16.9±1.1	15.1±0.9	0.092

[a]Values are means ± SD ($n = 3$). * Signicicantly different from the Control diet. ($P > 0.05$).