Running title: Macronutriets impact on sturgeon physiology during starvation

Impact of starvation on digestive enzymes activities and plasma metabolites in Siberian sturgeon (*Acipenser baerii*, Brandt, 1869)

Sedigheh Babaei[1*], Abdolmohamad Abedian Kenari[2], Mahmood Naseri[1], Mohamad Ali Yazdani Sadati[3], Isidoro Metón[4]

[1]Department of Natural Resources and Environmental Engineering, School of Agriculture, Shiraz University, Shiraz, Fars, Iran.

[2]Department of Aquaculture, Faculty of Marine Sciences, Tarbiat Modares University, Noor, Iran.

[3]Agricultural Research, Education and Extension Organization (Sturgeon Institude), Rasht, Iran.

[4]Secció de Bioquímica i Biologia Molecular, Departament de Bioquímica i Fisiologia, Universitat de Barcelona, Barcelona, Spain.

***Correspondence**: S. Babaei, Department of Natural Resources and Environment, School of Agriculture, Shiraz University, P.O. BOX: 7194684334 Shiraz, Fars, Iran.

Tel: +987136138172; Fax: +987132287159

E-mail: Babaei.Sedigheh@gmail.com, S-babaei@shirazu.ac.ir

Abstract

To increase the current knowledge about the relationship between nutritional status and the digestive capacity of Siberian sturgeon (*Acipenser baerii*), we addressed the effect of starvation-refeeding and macronutrient composition on growth parameters and key digestive enzyme activities in *A. baerii*. *A. baerii* juveniles were fed four different diets for 3 weeks, then starved for 2 weeks and allowed to refed for 5 weeks with the same diets. Another group of fish were fed 10 weeks with the corresponding diets. Among 10-week fed fish, high protein diets promoted higher body weight values, while the lowest specific growth rate was observed in fish fed a low protein, medium carbohydrate, high lipid diet ($p < 0.05$). At the end of the experiment, in fish refed for 5 weeks following a feeding-starvation cycle and in 10-week fed animals, the higher levels of blood glucose, triglycerides and cholesterol were found in fish fed low protein diets ($p < 0.05$). In all treatments, 2 weeks of starvation decreased α-amylase activity in the intestine ($p < 0.05$), while 4 days of refeeding increased lipase ($p > 0.05$) and α-amylase activity in the intestine as well as pepsin in the stomach ($p < 0.05$). Our findings suggest that *A. baerii* maintains a high capacity to digest proteins and lipids after 2 weeks of starvation and that α-amylase can be used as an indicator of the nutritional status in fish submitted to starvation-refeeding cycles. Indeed, refeeding with high protein and CHO: L ratio diets after starvation could improve the growth rate of *A. baerii* in culture.

Keywords: Siberian sturgeon, Starvation, Digestive capacity, Plasma metabolite, Compensation growth.

Introduction

Fish can sustain long periods of feed intake deprivation resulting from reproduction, migration and climate changes. In many fish, starvation is a frequent process in their natural life cycle, in

which the metabolism is adapted for using fuel stores to survive and maintain body functions. Therefore, the rearing process could benefit from short-term fasting programs without adverse effects on growth. Aquaculture species might be subjected to feed deprivation for ameliorating poor water quality conditions, reducing transportation and handling stress, managing disease outbreaks, and reducing visceral fat and feed cost (Einen, Morkore, Bencze, & Thomassen, 1999; Davis & Gaylord, 2011).

During food deprivation periods, animals employ different behavioral, physiological and structural mechanisms to utilize fuel stores for covering metabolic needs. Depending on the species, these adjustments can include reduced movement and modified metabolic capacity of tissues (Machado et al., 1988; Navarro & Gutierrez, 1995; Mendez & Wieser, 1993). During starvation, the needs and physiological responses depend on the species, duration of starvation, body energy reserves, distinctive pathways for using metabolic fuel stores, and dietary nutrients consumed before food deprivation (Hilton, 1982). Products resulting from lipid and carbohydrate store degradation under starvation have a major impact on blood metabolites and key enzyme activities in metabolism (Chatzifotis, Papadaki, Despoti, Roufidou, & Antonopoulou, 2011).

Blood glucose concentration is maintained at a steady level during long periods of food deprivation in many fish species (Navarro & Gutierrez, 1995). This apparent resistance of blood glucose levels against fluctuations occurs largely at the expense of liver glycogen during the initial stage of starvation (Navarro & Gutierrez, 1995). Indeed, starvation frequently decreases triglycerides, and total cholesterol levels (Pérez-Jiménez, Guedes, Morales, & Oliva-Teles, 2007). However, some studies reported increased or steady levels of plasma triglycerides at the early stages of starvation in European sea bass (*Dicentrarchus labrax*, L) and rainbow trout (*Onchorhynchus mykiss*) (Echevarría, Martínez-Bebiá, & Zamora, 1997; Kirchner, Seixas, Kaushik, & Panserat, 2005).

In addition to measuring changes in plasma metabolites, assessing the activity of digestive enzymes under different nutritional conditions can considerably contribute to gain insight into

digestion and nutrition processes in fish. Responses of the digestive system are closely related to the amount and composition of the diet, irrespective of the intrinsic feeding habits of the fish (Furné et al., 2008; Pérez-Jimenez et al., 2009). The activity of pancreatic enzymes could be used to determine the critical periods and the nutritional status of fish during starvation and refeeding (Bolasina, Perez, & Yamashita, 2006). Indeed, changes in the activity of digestive enzymes reflect availability of energy and nutrients, and the analysis of digestive enzyme activities has contributed to optimizing the ratios of macronutrients in aquafeeds (Twining, Alexander, Huibregste, & Glick, 1983; Spannhof & Plantikow, 1983). For example, based on digestive enzyme activities, Divakaran, Kim, & Ostrowski (1999) suggested different ratios of macronutrients for optimal growth of Pacific threadfin (*Polydactylus sexfilis*) and Bluefin trevally (*Caranx melampygus*).

Although numerous studies addressed the effects of starvation and refeeding in fish, the importance of pre-starvation and post-feeding diets has not been extensively explored. Given the relatively long maturation period of sturgeons and its high production cost, better knowledge of sturgeon nutrition and physiology may improve rearing conditions and reduce the production cost (Yarmohammadi et al., 2013). In the present study, the effect of diet composition and a starvation-refeeding cycle was assessed on growth, plasma metabolites (glucose, cholesterol, triglycerides and total protein), and digestive enzyme activities (pepsin, lipase and amylase) in Siberian sturgeon (*Acipenser baerii*).

Materials and methods

Rearing procedures

A group of 360 Siberian sturgeon juveniles (initial body weight 30 ± 5 g), obtained from International Sturgeon Research Institute (Guilan, Iran), were randomly stocked in 24, 500-L circular fiberglass tanks (n=15 per tank) and fed on commercial pellets (BIOMAR, France, 1.9 mm) for a week for adapting to the experimental conditions. The outdoor tanks contained treated

river water and were constantly aerated in a flow-through system (4.5- 5 liter S^{-1}) with a natural photoperiod regime. They were cleaned and siphoned daily to remove debris. Temperature, dissolved O_2, pH, debit, and photoperiod were maintained at 22 ± 4 °C, 7.1 ± 1.5 mg L^{-1}, 7-8, 4.5 ± 0.5 L min^{-1} and 12L: 12D, respectively. Following acclimation to our facilities, four groups of fish were fed 3 weeks with the corresponding experimental diet supplied to satiation three times a day (8:30, 15:00 and 21:30 hours). Thereafter, fish were starved for 2 weeks and then refed to satiaition for 5 weeks with the same diets. Another four groups of fish were fed 10 weeks (non-starved control) to satiation with the corresponding experimental diet. The number of replicate tanks for all conditions tested were 3.

Four isoenergetic diets were formulated with different levels of protein, lipids, and carbohydrates. The experimental diets were named LP-C (protein, 38 %; carbohydrate: lipid (CHO: L) ratio= 3), HP-C (protein, 44 %; CHO: L ratio= 3), LP-L (protein, 38 %; CHO: L ratio= 1.4) and HP-L (protein, 44 %; CHO: L ratio= 1.4). Sunflower, fish oil, and lecithin were added to the dry ingredients and mixed until the dough was performed. Two mm pellets were prepared and dried until use. The chemical composition of experimental diets is shown in Table 1.

Proximate analysis of diets

Proximate analysis of each diet, including moisture, protein, ash and fat were performed according to the AOAC method (AOAC, 2005). Total carbohydrate content was calculated by difference. Carbohydrate = 100 – (crude protein + crude lipid + ash).

Sample preparation

Fish submitted to a starvation-refeeding cycle were sampled at five different time-points for enzymes analysis: days 21 (after 3 weeks of feeding), 24 (after 3 weeks of feeding and 3 days of starvation), 35 (after 3 weeks of feeding and 2 weeks of starvation), 39 (after 4 days of refeeding following starvation) and 70 (end of experiment). As a control, a group of fish were fed 70 days

(10 weeks). Fish were anesthetized with clove powder (500 mg L^{-1}) (Yarmohammadi, Shabani, Pourkazemi, Soltanloo, & Imanpour, 2012) and weighed (accuracy of 0.01 g) individually to determine final wet weight. The specific growth rate (SGR) and feed conversion ratio (FCR) were measured by the following equations (Mohanta *et al.* 2008):

SGR: $((\text{Ln } W_f - \text{Ln } W_0)/t) \times 100$, t= 70 days

FCR: Dry feed consumed (g)/ WG_t (g)

W_0: Initial body weight

W_f: Final weight at the end of the trial

$WG = W_f - W_0$

Blood samples were collected in days 21 (after 3 weeks of feeding), 35 (after 3 weeks of feeding and 2 weeks of starvation) and 70 from the caudal vessel of two fish per tank (6 per treatment). To this end, 2 ml heparinized syringes were used and blood samples were centrifuged at 3,000 g for 10 min to isolate plasma, which was stored at -20 °C for further analyses (Yarmohammadi et al., 2012). Fish were euthanized by a sharp blow in the head and dissected to remove liver and visceral tissues (using clean equipment on ice). The tissues were weighed, immediately frozen in liquid nitrogen and stored at -80ºC until further analysis. All experimental procedures were approved by the Tarbiat Modares University's Animal Welfare Committee in compliance with local legislation.

Biochemical analyses of plasma metabolites

Biochemical analyses were done on the samples collected at the end of each feeding period: 21 days (week 3), after two weeks of starvation (week 5) and following five weeks of refeeding (week 10). Plasma glucose (kit ref. number: 150017) concentration was measured with a kit based on a colorimetric glucose oxidase – peroxidase reaction (Pars Azmoon, Karaj, Iran). Cholesterol (kit ref. number: 150010) and triglycerides (kit ref. number: 1500032) were analyzed with Pars Azmoon Kits. Glucose, triglycerides, and cholesterol were determined using an automatic

148 analyzer (Hitachi 902, Japan). The OD_{546} of both standard and test solutions were measured
149 against a blank in a photometer (Clinic III, Tajhiz Sanjesh Co., Esfahan, Iran).

150 **Enzyme activity assays**

151 Frozen fish stomachs were partially thawed, weighed and homogenized on ice (1:10, w/v) in 10
152 mM HCl with electric homogenizer (WIGGEN, D500, Berlin, Germany) for 30 s (Rungruangsak-
153 Torrissen, Moss, Anderson, Berg, & Waagbo, 2006). The homogenates were centrifuged at 30,000
154 g for 30 min at 4 °C (Hermle Z36HK, Wehingen, Germany). After centrifugation, the supernatant
155 was collected and frozen at -80 °C (Furne et al. 2008) until measuring pepsin (E.C.3.4.23.1)
156 activity. Pepsin activity assay was based on Worthington's (1991) method. In brief, the enzyme
157 extract was mixed with the substrate (2 % hemoglobin solution in 0.3 N HCl at pH = 2.0) and
158 incubated for 10 min at 25 °C. The reaction was stopped with 5 % trichloroacetic acid (TCA), and
159 the assay tubes were centrifuged at 4,000 g for 6 min at 4°C. The absorbance of the supernatant
160 was recorded at 280 nm. One unit of pepsin activity was defined as μg of tyrosine released at 25
161 °C min^{-1}, considering the extinction coefficient (280 = 1250 M^{-1} cm^{-1}). Intestine extracts were
162 prepared as described by Harpaz, Eshel, & Lindner (1994) to assay lipase (E.C.3.1.1) and α-
163 amylase (E.C.3.2.1.1). After homogenization in 50 mM Tris–HCl buffer with 20 mM $CaCl_2$ and
164 50 mM KCl, pH 8.5 at a 1:5 ratio (1 g intestine in 5 mL of buffer), homogenates were centrifuged
165 at 17,000 g for 20 min at 4°C. The supernatants were transferred to new vials and stored at -80 °C
166 until use. Lipase activity was determined by hydrolysis of n-nitrophenyl myristate. Each assay
167 (0.5 mL) contained 0.53 mM n-nitrophenyl myristate, 0.25 mM 2-methoxyethanol, 5 mM sodium
168 cholate and 0.25 M Tris–HCl (pH 9.0). The reaction was terminated by adding 0.7 mL of acetone/
169 n-heptane (5:2, v/v) after incubating for 15 min at 25 °C. The reaction mixture was vigorously
170 mixed and centrifuged at 6,080 g for 2 min. The absorbance was read at 405 nm in the resulting
171 lower aqueous layer. The extinction coefficient of n-nitrophenol was 16,500 M^{-1} cm^{-1} L^{-1}. One

172 unit of enzyme activity was defined as 1 μmol of n-nitrophenol released per min (Iijima, Tanaka,
173 & Ota, 1998).
174 α-Amylase activity was assayed by starch-iodine detection (Zambonino Infante, Cahu, Péres,
175 Quazuguel, & Le Gall, 1996). In summary, 50 μL of enzymatic extract was mixed with the
176 substrate (3 g L^{-1} starch in 66 mM Na_2HPO_4) and incubated for 20 min at 25 °C. The reaction was
177 stopped with 20 μL of 1 N HCl. After the addition of 2 mL of 0.33 mM iodine solution, the
178 absorbance was determined at 580 nm. One unit of α-amylase activity was defined as the mg
179 starch hydrolyzed per min at 25 °C.
180 Total soluble protein was measured by the Bradford assay (1976) using bovine serum albumin as
181 the standard. Enzyme activities were expressed as specific activity (U mg^{-1} protein) and total
182 activity (tissue mg^{-1} protein). All the enzymatic assays were run for six samples per treatment.

183 **Statistical analysis**

184 Data were checked for normality (Kolmogorov-Smirnov test) and homogeneity of variances
185 before comparison. One-way ANOVA was used to analyze differences between sampling
186 conditions for growth parameters, plasma metabolites, and enzyme activities. When statistical
187 significance was found for ANOVA, the Scheffé post-hoc test was used to determine differences
188 among treatments ($p < 0.05$). Potential interactions between dietary protein, dietary CHO: L ratio
189 and the nutritional status (feeding, starvation) were analyzed by three-way ANOVA using the
190 general linear model (GLM) procedure ($p < 0.05$). Data analysis was performed by using IBM
191 SPSS Statistics software.
192

193 **Results**

194 **Growth performance**

195 Growth parameters of Siberian sturgeon during the starvation-refeeding cycle and in fed fish are

196 shown in Table 2. At the end of the trial, fish submitted to starvation-refeeding exhibited lower
197 SGR than control fed animals irrespective of the diet (Table 3). In regard of fish fed 10 weeks,
198 those supplied with the LP-L diet showed significant lower SGR values than other fish. In fish
199 submitted to a starvation-refeeding cycle, the highest and lowest FCR values were found in fish
200 fed diets LP-C (1.5 ± 0.1) and HP-C (1.2 ± 0.1), respectively. There were no significant differences
201 in survival among treatments in any condition studied.
202 Dietary protein and starvation had a significant effect on growth parameters (SGR and FCR).
203 Indeed, the CHO: L ratio of the diet significantly affected Siberian sturgeon growth. Interaction
204 between macronutrient dietary composition and feeding conditions were significant on the final
205 weight and SGR (Table 3).

206 **Biochemical plasma parameters**

207 Two weeks of starvation produced a general decline in circulating glucose and triglyceride levels
208 with the exception of fish fed diet HP-L for glucose and diet LP-C for triglycerides. Refeeding
209 increased glucose, cholesterol and triglycerides in all treatments. Higher levels of circulating
210 glucose, triglycerides and cholesterol were found in fish fed low protein diets (LP-C and LP-L)
211 both after 5 weeks of refeeding and in 10-week fed animals (Table 4). At the end of a 2-week
212 starvation period, the CHO: L ratio of the diet significantly affected blood glucose levels. After
213 refeeding, the level of dietary protein significantly decreased blood glucose, cholesterol and
214 triglycerides (Table 5).

215 **Activities of digestive enzymes**

216 Total and specific activity of gastric and pancreatic enzymes are shown in Figures 1 to 3.
217 Irrespective of the diet, three days of starvation decreased both specific and total activity of pepsin
218 in the stomach of *A. baerii*. The values of total pepsin activity remained low after long-term
219 starvation (2 weeks) in all experimental conditions assayed, while significantly decreased levels
220 of specific activity was found in fish fed diets LP-C and HP-L. After 4 days of refeeding, total

pepsin activity was completely recovered while the specific activity was 2.2- to 4.4-fold higher than in 21-day fed animals. After 5 weeks of refeeding, the specific and total activity of pepsin dropped back to levels similar to those observed before starvation. After 10 weeks of feeding, the specific activity of pepsin was significantly lower in fish fed diet HP-L, with values ranging from 53.1 % to 60.7 % compared to those observed with other diets (Fig. 1A-B).

Two weeks of starvation significantly increased lipase specific activity in the intestine of *A. baerii* fed high carbohydrate diets (LP-C and HP-C) (Fig. 2A), while decreased total lipase activity when feeding low CHO: L diets (Fig. 2B). Specific and total lipase activity increased in all treatments after 4 days of refeeding. Both returned to values similar to those found in 21-day fed animals after five weeks of refeeding. In 10-week fed fish, diets LP-C and LP-L promoted the highest and the lowest specific activity of lipase, respectively.

Specific and total activity of α-amylase in the intestine after 21 days of feeding were stimulated in treatments with increased CHO: L ratio. Two weeks of starvation significantly declined total and specific activity of α-amylase in all treatments. After 4 days of refeeding, specific and total α-amylase activity significantly increased 4.9- to 11.7-fold over the values found in 2-week starved fish. At the end of the refeeding period, the highest α-amylase activity was observed in fish fed diet LP-L, similarly as with 70-day fed control fish (Figs. 3A-B).

Dietary protein content, CHO: L ratio and starvation had no significant effect on pepsin total activity (Table 6). However, the same conditions significantly altered α-amylase and lipase activity. During starvation, increased levels of CHO: L ratio led to higher lipase activity and decreased α-amylase activity. After refeeding, diet composition significantly affected the activity of the three digestive enzymes analyzed, even though the rearing condition did not altered α-amylase activity when compared to control fed fish.

Discussion

Due to the decline of native sturgeon population (IUCN Red Data List, 2018), aquaculture programs are being developed for different species of sturgeons, such as *Acipenser baerii*. A

number of studies addressed the effect of diet composition on growth performance in *A. baerii* (Kaushik, Luquet, Blanc, & Paba, 1989; Babaei, Abedian-Kenari, Hedayati, & Yazdani-Sadati, 2016a). However, knowledge about the effect of starvation-refeeding cycles on the digestive capacity of *A. baerii* remains unknown. The present study confirmed that high protein diets showed a tendency to induce higher final body weight values in *A. baerii* both in 10-week fed and starved-refed animals. After submitting fish to a starvation-refeeding cycle, HP-C diet promoted the highest SGR. Our findings suggest that inclusion of dietary carbohydrates to starved *A. baerii* may improve fish growth and reduce the production cost. Consistent with our results, dietary supplementation with cereals was reported to improve protein efficiency and energy in *A. baerii* (Medale, Blanc, & Kaushik, 1991).

The ultimate goal of performing starvation-refeeding cycles in aquaculture is to maximize fish growth. However, in the present study *A. baerii* juveniles were not able to fully compensate their weight during refeeding with any of the experimental diets assayed. Morshedi et al. (2013) reported that 8 days of starvation and 32 days of refeeding (4 times the period of hunger) completely compensates growth in *A. baerii*. Considering that in the present study fish were starved 2 weeks and refed up to 5 weeks, it is conceivable that a shorter period of starvation or a longer refeeding period could improve compensatory growth in *A. baerii*.

Since glucose is an essential fuel for a number of tissues such as the brain, it is particularly important to preserve blood glucose levels during starvation (Gillis & Ballantyne, 1996). In the present study, two weeks of starvation decreased glucose plasma levels in *A. baerii* fed low protein diets. Consistent with the results reported by Furne et al. (2012) in *Acipenser naccarii* after 10 and 40 days of starvation, although glycogenolysis occurs in sturgeon, glucose demands and a minor content of hepatic glycogen (Babaei, Abedian-Kenari, Hedayati, Yazdani-Sadati, & Meton, 2016b) would explain the notable decrease of glycemia in starved *A. baerii*. Accordingly, significant reduction in plasma glucose levels also occurred in white sturgeon (*Acipenser transmontanus*) after 8 weeks of starvation (Hung, Liu, Li, Storebakken, & Cui, 1997).

In the present study, fasting did not significantly affect plasma triglycerides. Similarly, steady plasma triglyceride levels were reported by Kirchner et al. (2005) in food-deprived rainbow trout, and a slight increase of cholesterol was reported in starved striped bass (*Morone saxatilis*) (Mac Farlane, Harvey, Bowers, & Patton, 1990). In the present study, a relatively stable level of triglycerides and high cholesterol suggest that lipids were used preferentially in *A. baerii* during starvation. Our results seem to reflect a stimulatory role of high protein/high lipid diets (HP-L) on blood glucose, triglycerides and cholesterol during starvation. Furthermore, refeeding after fasting increased triglycerides. However, diet composition did not promote significant effects on plasma triglycerides and cholesterol after refeeding. Hyperphagia is considered a major mechanism leading to compensatory growth or body reserves of fish during refeeding (Kim & Lovell, 1995; Jobling & Koskela, 1996; Ali, Nicieza, & Wootton, 2003). Therefore, hyperphagia may overcompensate and increase plasma metabolites such as cholesterol and triglycerides due to enhanced body fat deposition. In this regard, a similar overcompensating process was reported by Furne et al. (2012) in Adriatic sturgeon for plasma levels of lipids, triglycerides and protein.

According to Buddington & Doroshov (1986), the activity of digestive enzymes in sturgeons is affected by growth, diet composition, natural feeding habits and genetics. Therefore, assessment of the activity of digestive enzymes may reflect piscine digestion physiology (Bolasina et al., 2006), and adaptation to nutritional changes in the environment (Sunde et al., 2004). In the present study, diet composition (protein content and CHO: L ratio) altered the activity of digestive enzymes of *A. baerii*. Similarly as previously reported by Furné et al. (2008), we found higher specific activity for α-amylase than for lipase, which may be related with omnivorous feeding habits of *A. baerii* (Fountoulaki, Alexis, Nengas, & Venou, 2005). Considering the higher values of α-amylase activity and SGR of *A. baerii* fed the HP-C diet after refeeding, the implementation of partial substitution of dietary protein by carbohydrates would reduce the production cost of feedstuffs, which in turn could promote a protein-sparing effect and decrease nitrogen waste in sturgeon rearing systems (Simon & Jeffs, 2013). Nevertheless, the inhibitory effect of high

299 carbohydrate intake on α-amylase activity reported for other species (Simon & Jeffs, 2013;
300 Krogdahl, Hemre, & Mommsen, 2005a), should be considered. In addition, high levels of dietary
301 fat in fed and starved *A. baerii* appeared to negatively affect pepsin and lipase activity. In this
302 regard, Fountoulaki et al. (2005) also reported a negative effect of dietary fat on protein and starch
303 digestibility in *Sparus aurata*. Similarly, as for *A. baerii*, dietary lipids stimulate lipolytic enzymes
304 in mammals (Spannagel et al., 1996) and other fish species (Zambonino Infante & Cahu, 1999).
305 A sharp reduction in the activity of α-amylase (more than 50 %) in the gut of *A. baerii* subjected
306 to starvation was observed in the present study. Consistent with our findings, Furné et al. (2008)
307 reported more than 50 % and 80 % reduction of α-amylase activity in Rainbow trout and Adriatic
308 sturgeon after 10 and 72 days of starvation, respectively. Similarly, Albentosa & Moyano (2008)
309 showed reduced activity of carbohydrases in *Ruditapes decussatus* and *Prenerupis pullastra* after
310 2 weeks of starvation. In both species, α-amylase activity increased to values similar to non-
311 stressed fish after refeeding. This phenomenon was also observed in the present study for *A. baerii*.
312 Therefore, the activity of digestive enzymes, in particular, α-amylase, can be used as biomarkers
313 of the nutritional status.
314 In this study, specific lipase activity of *A. baerii* significantly increased in response to starvation
315 when feeding diets with high CHO: L ratio (LP-C and HP-C) (Fig. 2A), whereas total pepsin and
316 lipase activity decreased or remained unaffected (Fig. 2B). In white shrimp *Litopenaeus*
317 *vannamei*, starvation triggered amino acid catabolism in digestive gland cells of hepatopancreas
318 and increased the specific activity of amylase, protease, trypsin and chymotrypsin. However, it
319 led to decreased total activity of the mentioned enzymes (Comoglio, Gaxiola, Roque, Cuzon, &
320 Amin, 2004). The reduction of the protein content in the digestive tract may be then followed by
321 increased specific activity of digestive enzymes and decreased total activity. Our findings point to
322 the convenience of reporting the activity of gastrointestinal enzymes as total activity during
323 starvation due to changes in the protein content of the intestine.
324 During starvation, rapid degradation of visceral protein occurs in the intestine before the visceral

fat is entirely consumed (Abolfathi, Hajimoradloo, Ghorbani, & Zamani, 2012). In long-term starved Atlantic salmon, the intestinal soluble protein content dropped by about 75 % (Krogdahl & Bakke-McKellep, 2005b). Similarly, starvation decreased pepsin activity in the stomach of Swamp eel (*Monopterus albus*) (Yang et al., 2007). Reduction in the activity of digestive enzymes under starvation may be an adaptation to feed scarcity and energy maintainance (Comoglio et al., 2004). However, increased or unaffected digestive enzyme activities under starvation have been reported as well. For instance, pepsin activity did not change after 25 days of starvation in Atlantic cod (*Gadus morhua*) (Gildberg, 2004). In Adriatic sturgeon, pepsin activity remained unaltered up to 20 days following starvation, and lipase activity slightly increased in 40-day starved fish (Furné et al., 2008). Maintainance of high levels of digestive enzyme activity even after long-term starvation may assure a relatively quick metabolic recovery when food becomes available, thus representing a nutritional and digestive advantage under changing conditions (Furné et al., 2008). Zeng et al. (2012) reported that lipase activity decreases in the proximal or distal parts of the intestine after 8 days of starvation in Southern catfish (*Silurus meridionalis*). This activity rose in the proximal or distal parts after 16 days of starvation, while it dropped in the medial part. Hence, unaltered or increased digestive enzyme activities during starvation may also result from distinct adaptative responses in different parts of the intestine.

In the present study, a huge increase of digestive enzyme activities, particularly α-amylase and pepsin, was observed in *A. baerii* after 4 days of refeeding. Thereafter, long-term refeeding decreased digestive enzyme activities to levels similar to those found in 10-week fed animals. Similarly, Comoglio et al. (2004) reported that long-term refeeding and reconstitution of soluble gut protein leads to decreased activity of digestive enzymes.

In conclusion, our findings indicate that *A. baerii* maintains a reliable capacity for digesting proteins and lipids after two weeks of starvation, and the activity of digestive enzymes, in particular α-amylase, can be used as indicator of the nutritional status in *A. baerii* submitted to starvation-refeeding cycles. According to our results, the supply of diets similar to HP-C (high

351 protein content and CHO: L ratio) after starvation periods could improve the growth rate of *A.*
352 *baerii*.
353
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359
360 **Conflict of interests**
361 The authors declare that there is no conflict of interests regarding the publication of this paper.
362
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548

549 **Figure legends:**

550 **Figure 1. A**) Specific activity (U mg protein $^{-1}$) of pepsin in *A. baerii* in fish submitted to a
551 starvation-refeeding cycle or fed the experimental diets during 70 days; **B**) Total activity (U Fish^{-}
552 ^{1}) of pepsin in fish submitted to a starvation-refeeding cycle or fed the experimental diets during
553 70 days. Capital letters indicate statistical differences between fish fed different diets for a given
554 time period and small letters indicate statistical differences among time periods for each diet ($p <$
555 0.05). Values are shown as mean ± SD (n = 6). 21F: 21 days of feeding; 3S: 3 days of starvation
556 after the feeding period, 14S: 14 days of starvation after the feeding period; 4R: 4 days of refeeding
557 after starvation; 35R: 35 days of refeeding after starvation; 70F: 70 days of feeding.

558

559 **Figure 2. A**) Specific activity (U mg protein $^{-1}$) of lipase in *A. baerii* in fish submitted to a
560 starvation-refeeding cycle or fed the experimental diets during 70 days; **B**) Total activity (U Fish^{-}
561 ^{1}) of lipase in fish submitted to a starvation-refeeding cycle or fed the experimental diets during
562 70 days. Capital letters indicate statistical differences between fish fed different diets for a given
563 time period and small letters indicate statistical differences among time periods for each diet ($p <$
564 0.05). Values are shown as mean ± SD (n = 6). 21F: 21 days of feeding; 3S: 3 days of starvation
565 after the feeding period, 14S: 14 days of starvation after the feeding period; 4R: 4 days of refeeding
566 after starvation; 35R: 35 days of refeeding after starvation; 70F: 70 days of feeding.

567

568 **Figure 3. A**) Specific activity (U mg protein $^{-1}$) of □-amylase in *A. baerii* in fish submitted to a
569 starvation-refeeding cycle or fed the experimental diets during 70 days; **B**) Total activity (U Fish^{-}
570 ^{1}) of α-amylase in fish submitted to a starvation-refeeding cycle or fed the experimental diets
571 during 70 days. Capital letters indicate statistical differences between fish fed different diets for a
572 given time period and small letters indicate statistical differences among time periods for each
573 diet ($p < 0.05$). Values are shown as mean ± SD (n = 6). 21F: 21 days of feeding; 3S: 3 days of

574 starvation after the feeding period, 14S: 14 days of starvation after the feeding period; 4R: 4 days
575 of refeeding after starvation; 35R: 35 days of refeeding after starvation; 70F: 70 days of feeding.

Table 1. Formulated Siberian sturgeon diets and chemical composition (g/ kg diet).

Ingredientes	LP-C	HP-C	LP-L	HP-L
	38P11L36C	**44P10L30C**	**38P18L25C**	**44P16L22C**
Fish meal*	462.4	571	493	588.6
Wheat meal	398.8	329.6	212	179.8
Fish oil‡	32.2	17.4	62.2	43.5
Sunflower oil†	32.2	17.4	62.2	43.5
Soy lecithin§	5	5	5	5
Mono calcium phosphate	5	5	5	5
Mineral mix¶	20	20	20	20
Vitamin mixǀ	15	15	15	15
Anti fungi	2.5	2.5	2.5	2.5
Anti-oxidant‡‡	0.2	0.2	0.2	0.2
Filler♦	11.6	1.7	107.9	81.9
Binder◊	15	15	15	15
TOTAL	1000	1000	1000	1000
Analysed proximate composition (g/ kg in dry matter (dm))				
moisture	50 ± 0.9	50 ± 9	58 ± 3	59 ± 8
Crude protein	379 ± 4	441 ± 3	382 ± 3	439 ± 5
Crude lipid	115 ± 11	100 ± 7	175 ± 21	157 ± 9
Carbohydrates	359 ± 14	302 ± 8	249 ± 28	219 ± 4
ash	97 ± 0.4	107 ± 6	135 ± 5	126 ± 6
CHO: L	31	30	14	14
Gross energy (Kj g^{-1} dm) ††	19.7	19.6	20.2	20.3

* Clopeonella meal (Mazandaran Animal & Aquatic feed (Manaqua) Co. and Pars kilka Co. Iran)

‡ Kilka oil (Manaqua Co. Iran)

† Sunflower oil (Ladan Co. Iran)

§ Soybean lecithin with phosphatidylcholine (Behpak company, Iran)

¶ Mineral mix provided (mg Kg^{-1}): Fe: 6000, Cu: 600, Mn: 5000, Zn: 10000, I: 600, Se: 20, Co: 100, choline chloride: 6000, Career up to 1 kg.

ǀ Vitamin mix provided (Unit Kg^{-1}): A: 1200000 IU, D3: 400000 IU, E: 50000 mg, K3: 800 mg, B9: 1000 mg, C: 30000 mg, B1: 2500 mg, B2: 4000 mg, B6: 25000 mg, B12: 8 mg, Biotin: 150 mg, Niacin: 35000 mg and Inositol: 50000 mg Career up to 1 kg.

‡‡ Antioxidant (Gluba Tiox, French)

♦ Carboxymethyl Cellulose (DAEJUNG Co. Korea)

◊ Amet binder (Afraz mehrtaban company, Iran)

†† Estimated energy was calculated based on 1 g crude protein being 23.6 kJ, 1 g crude fat being 39.5 kJ and 1 g carbohydrate being 17.2 kJ (NRC 1993). See Fig. 1 for diet abbreviations.

Table 2. Growth parameters of *A. baerii* fed the experimental diets in starved-refed fish and 70-day fed animals (control).

Growth parameters	LP-C		HP-C		LP-L		HP-L	
	Control*	Starved	Control	Starved	Control	Starved	Control	Starved
W_0 (g)	29.1±1.4	29.2±0.6	30.1±1.6	30.0±0.5	30.7±2.1	30.2±0.1	30.1±0.4	29.8±0.8
W_f (g)	$235±14^{AB}$	$158±10^{b}$	$238±9^{AB}$	$182±8^{a}$	$221±14^{B}$	$165±12^{ab}$	$246±10^{A}$	$169±7^{ab}$
SGR (% day $^{-1}$)	$3.0±0.0^{A}$	$2.4±0.1^{b}$	$3.1±0.1^{A}$	$2.6±0.1^{a}$	$2.8±0.0^{B}$	$2.4±0.1^{b}$	$3.2±0.0^{A}$	$2.5±0.1^{ab}$
FCR	$1.2±0.1^{AB}$	$1.5±0.1^{a}$	$1.2±0.1^{AB}$	$1.2±0.1^{b}$	$1.3±0.1^{A}$	$1.4±0.1^{ab}$	$1.1±0.1^{B}$	$1.4±0.1^{ab}$
Survival (%)	100	100	100	100	100	100	100	100

* Groups fed for 10 weeks.

Capital letters indicate significant differences among 10-week fed fish with different diets (controls) and small letters indicate significant differences among starved-refed fish with different diets ($p<0.05$). Values are shown as mean ± SD (n = 3; number of tanks per treatment).

Table 3. GLM univariate analysis of variance to analyze interactions between dietary macronutrients (protein content and CHO: L ratio) and condition (starvation and feeding) on growth performance of *A. baerii* (3-Factorial, $p < 0.05$).

	Mean Square table		
Factors	**FBW**	**SGR**	**FCR**
Protein	1123**	0.07 **	0.06 *
CHO: L ratio	56 ns	0.02 ns	0.02 ns
Condition (Control, starved)	26680**	1.55 **	0.14 **
P * CHO:L	4.00 ns	0.00 ns	0.00 ns
P * condition	0.20 ns	0.00 ns	0.00 ns
CHO:L * condition	0.02 ns	0.00 ns	0.00 ns
P*CHO:L*condition	614 *	0.03 *	0.03 ns
Error	118	0.01	0.01

* $0.01 < p$ value < 0.05 ** p value < 0.01 ns: not significant

Table 4. Effect of starvation and refeeding on the plasma levels of glucose, cholesterol and triglycerides of *A. baerii* fed the experimental diets.

Plasma metabolites	Diet			
	LP-C	**HP-C**	**LP-L**	**HP-L**
After 21 days of feeding				
Glucose (mg dl^{-1})	59 ± 13^{ABb}	45 ± 10^{Bc}	65 ± 9^{Ab}	47 ± 8^{ABc}
Cholesterol (mg dl^{-1})	73 ± 8^{ABb}	50 ± 25^{Bb}	79 ± 3^{Ab}	60 ± 5^{ABc}
Triglycerides (mg dl^{-1})	197 ± 46^{Ab}	160 ± 107^{Ab}	248 ± 49^{Ab}	235 ± 23^{Ab}
After 14 days of starvation				
Glucose (mg dl^{-1})	42 ± 4^{BCc}	41 ± 1^{Cc}	47 ± 0^{Bc}	59 ± 4^{Ab}
Cholesterol (mg dl^{-1})	112 ± 26^{Bab}	108 ± 1^{Ba}	81 ± 17^{Bb}	168 ± 40^{Aa}
Triglycerides (mg dl^{-1})	197 ± 25^{Ab}	122 ± 1^{Bb}	220 ± 13^{Ab}	213 ± 4^{Ab}
After 35 days of refeeding				
Glucose (mg dl^{-1})	92 ± 9^{Aa}	92 ± 2^{Aa}	94 ± 9^{Aa}	86 ± 7^{Aa}
Cholesterol (mg dl^{-1})	141 ± 42^{Aa}	115 ± 3^{Aa}	120 ± 2^{Aa}	114 ± 7^{Ab}
Triglycerides (mg dl^{-1})	648 ± 192^{Aa}	588 ± 119^{Aa}	650 ± 25^{Aa}	646 ± 143^{Aa}
After 70 days of feeding (control fed fish)				
Glucose (mg dl^{-1})	91 ± 5^{Aa}	82 ± 3^{Bb}	83 ± 4^{Ba}	78 ± 1^{Ba}
Cholesterol (mg dl^{-1})	119 ± 6^{Aab}	109 ± 19^{Aa}	111 ± 2^{Aa}	87 ± 12^{Bbc}
Triglycerides (mg dl^{-1})	671 ± 30^{Aa}	461 ± 64^{Ba}	667 ± 21^{Aa}	512 ± 77^{Ba}

Capital letters indicate significant differences between fish fed different diets for a given time period and small letters indicate statistical differences among time periods for each diet ($p < 0.05$). Values are shown as mean ± SD (n = 3 tanks).

Table 5. GLM univariate analysis of variance to analyze interactions between dietary macronutrients (protein content and CHO: L ratio) and condition (starvation and feeding) on plasma metabolites of *A. baerii* (3-Factorial, $p < 0.05$).

	Mean Square table					
Factors	**After starvation**			**After refeeding**		
	Triglycerides	**Cholesterol**	**Glucose**	**Triglycerides**	**Cholesterol**	**Glucose**
Protein	7776ns	2109*	30ns	69337*	1633*	182*
CHO: L ratio	7141ns	925ns	135*	4266ns	962ns	96ns
Condition (Control, starved)	146953**	805ns	5612**	18370ns	1536*	337**
P * CHO:L	8214ns	3927**	345**	4704ns	14ns	8ns
P * condition	150ns	3015*	63ns	34050ns	2ns	16ns
CHO:L * condition	3037ns	26ns	273**	60ns	24ns	20ns
P*CHO:L*condition	42ns	2380*	7ns	2ns	433ns	54ns
Error	2113	433	16	10403	288	29

* 0.01 <*p* value < 0.05 ** *p* value < 0.01 ns: not significant

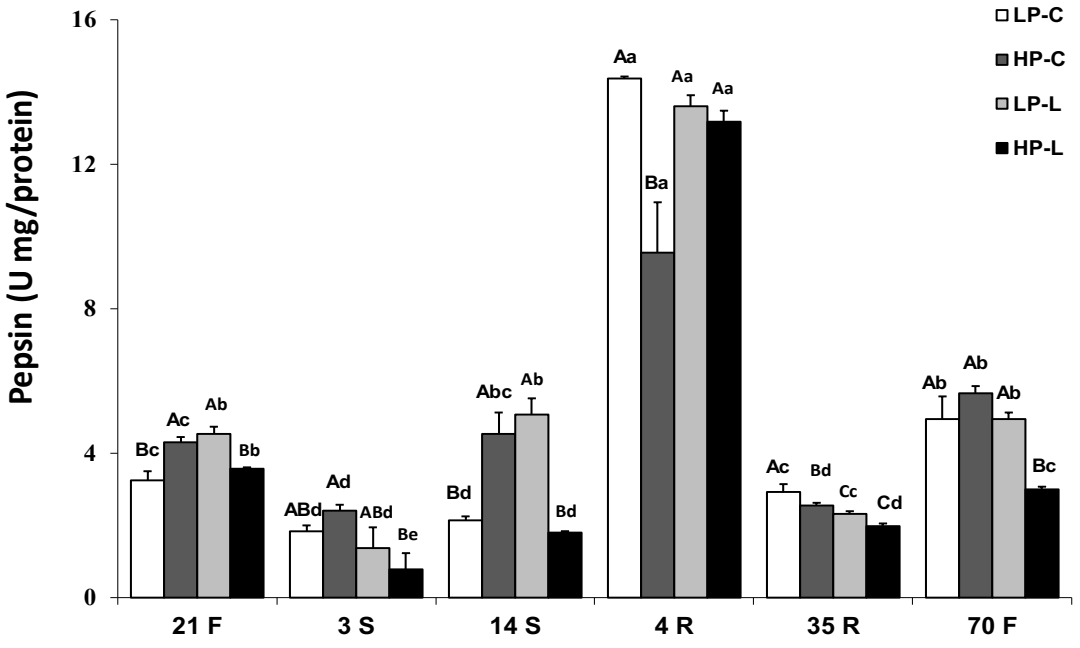
Table 6. GLM univariate analysis of variance to analyze interactions between dietary macronutrients (protein content and CHO: L ratio) and condition (starvation and feeding) on digestive enzymes activities of *A. baerii* (3-Factorial, $p < 0.05$).

	Mean Square table					
Factors	**After starvation**			**After refeeding**		
	Pepsin	**Lipase**	**Amylase**	**Pepsin**	**Lipase**	**Amylase**
Protein	0.001ns	4.1**	12*	0.024**	1.4**	209**
CHO: L ratio	0.000ns	3.9**	60**	0.066**	0.0**	67**
Condition (Control, starved)	0.007ns	7.3**	729**	0.621**	3.3**	2.2ns
P * CHO:L	0.113**	3.6**	28**	0.015**	6.4*	33**
P * condition	0.004ns	5.8**	0.89ns	6.6ns	4.3ns	1.78ns
CHO:L * condition	0.069**	1.7**	0.16ns	0.006ns	8.4*	25**
P*CHO:L*condition	0.088**	9.6ns	8.64*	0.024**	4ns	33**
Error	0.002	3.5	1.92	0.002	1.1	1.12

* 0.01 <*p* value < 0.05 ** *p* value < 0.01 ns: not significant

Figure 1

A


Pepsin (U mg/protein)
16
12
8
4
0
LP-C
HP-C
LP-L
HP-L
Bc Ac Ab Bb
ABd Ad ABd Be
Bd Abc Ab Bd
Aa Ba Aa Aa
Ac Bd Cc Cd
Ab Ab Ab Bc
21 F
3 S
14 S
4 R
35 R
70 F

B

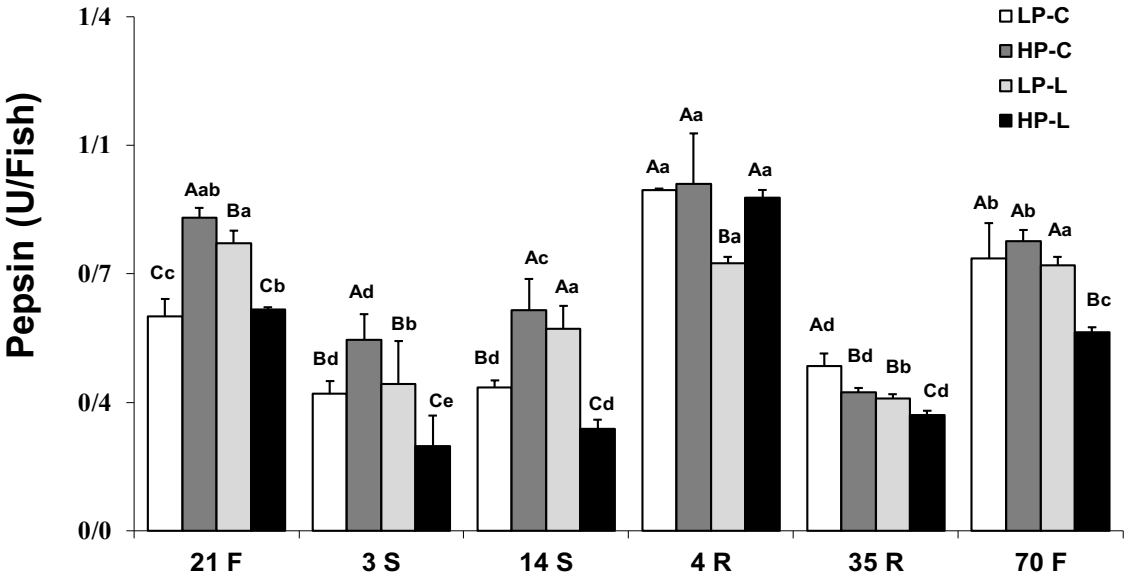

Pepsin (U/Fish)
1/4
1/1
0/7
0/4
0/0
LP-C
HP-C
LP-L
HP-L
Cc Aab Ba Cb
Bd Ad Bb Ce
Bd Ac Aa Cd
Aa Aa Ba Aa
Ad Bd Bb Cd
Ab Ab Aa Bc
21 F
3 S
14 S
4 R
35 R
70 F

Figure 2

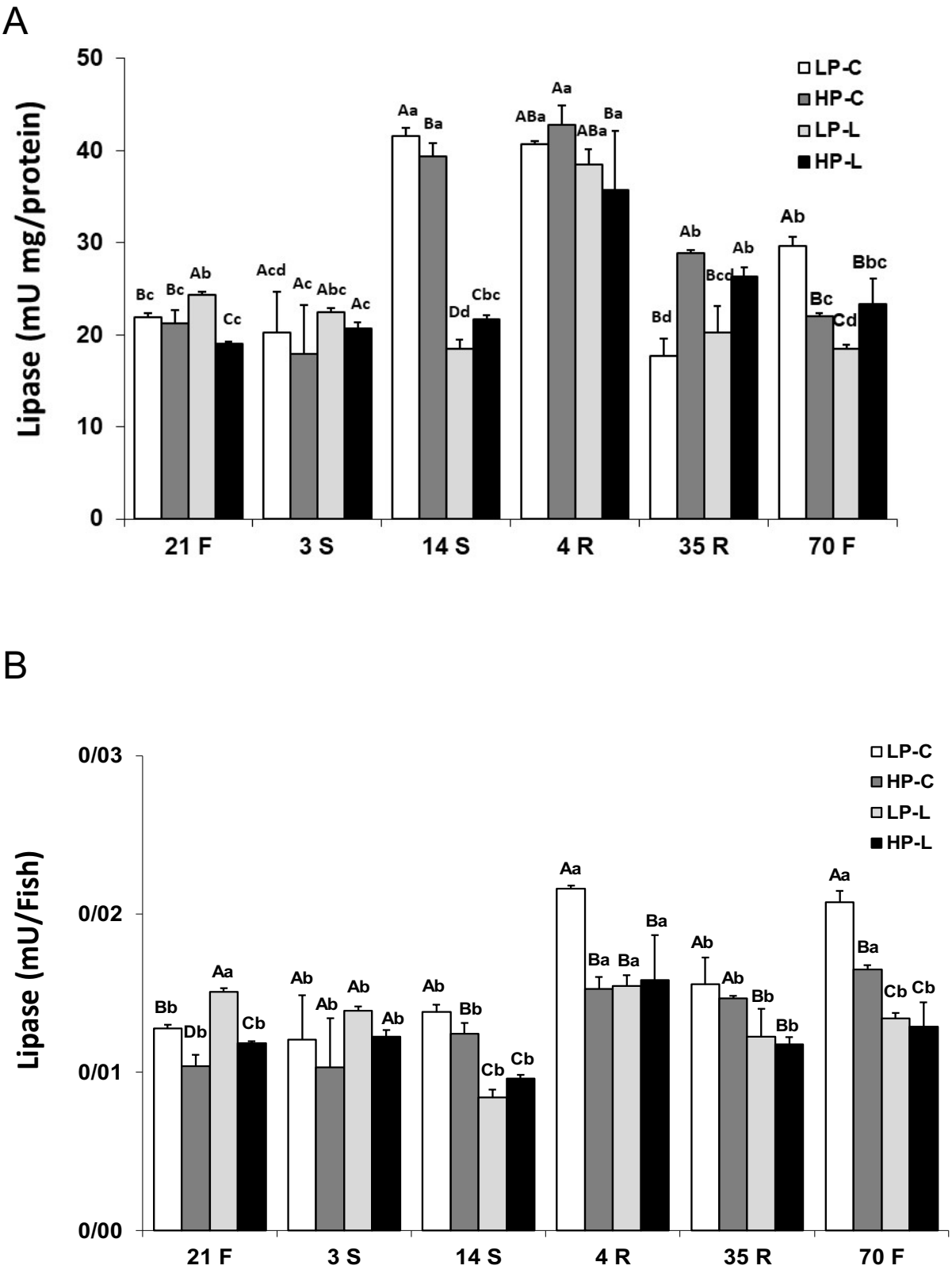
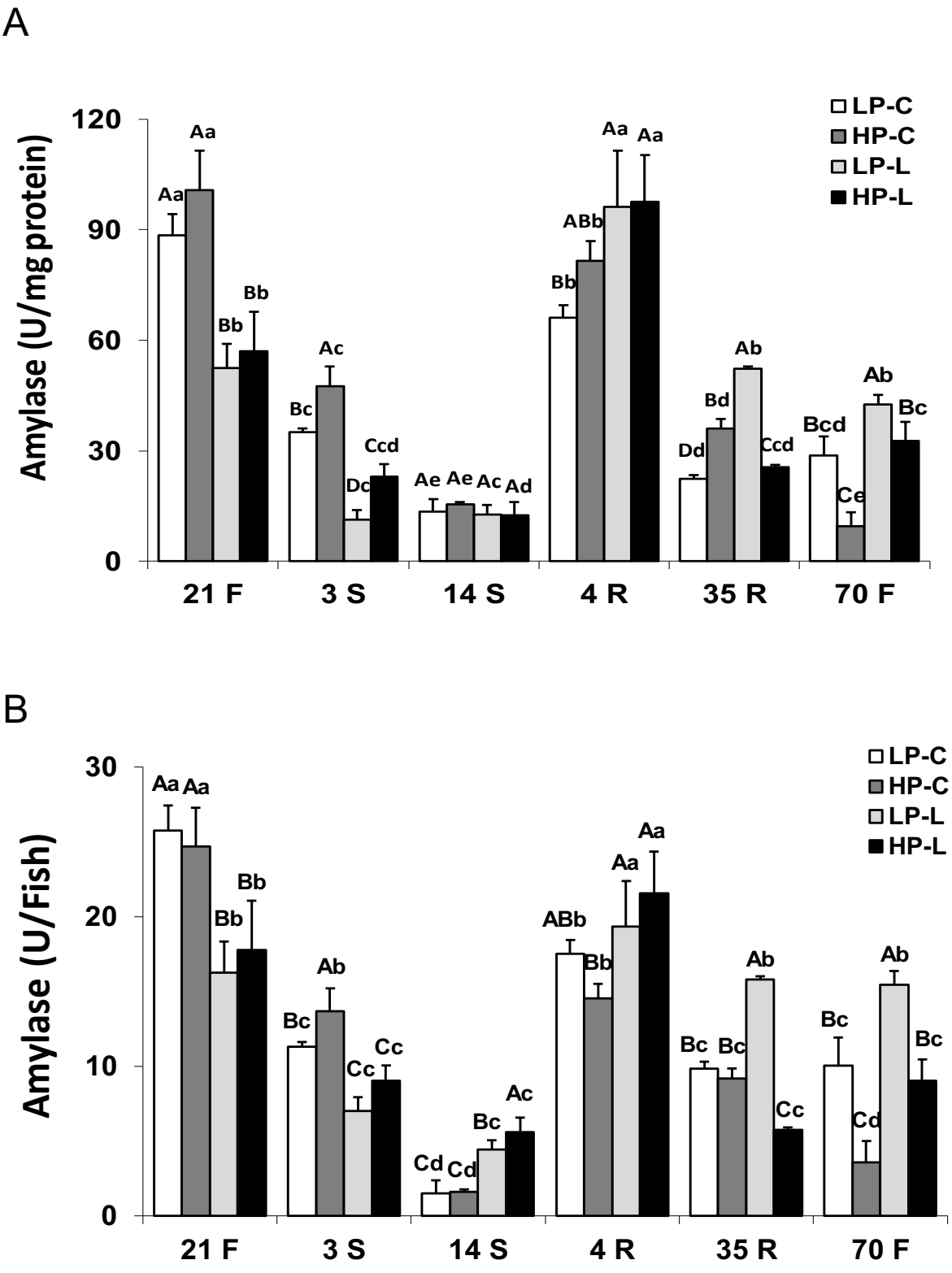

A
Lipase (mU mg/protein)
50
40
30
20
10
0
LP-C
HP-C
LP-L
HP-L
Bc Bc Ab Cc
Acd Ac Abc Ac
Aa Ba Dd Cbc
ABa Aa ABa Ba
Bd Ab Bcd Ab
Ab Bc Cd Bbc
21 F
3 S
14 S
4 R
35 R
70 F
B
Lipase (mU/Fish)
0/03
0/02
0/01
0/00
LP-C
HP-C
LP-L
HP-L
Bb Db Aa Cb
Ab Ab Ab Ab
Ab Bb Cb Cb
Aa Ba Ba Ba
Ab Ab Bb Bb
Aa Ba Cb Cb
21 F
3 S
14 S
4 R
35 R
70 F

Figure 3


A
Amylase (U/mg protein)
120
90
60
30
0
LP-C
HP-C
LP-L
HP-L
Aa
Aa
Bb
Bb
Bc
Ac
Dc
Ccd
Ae
Ae
Ac
Ad
Bb
ABb
Aa
Aa
Dd
Bd
Ab
Ccd
Bcd
Ce
Ab
Bc
21 F
3 S
14 S
4 R
35 R
70 F
B
Amylase (U/Fish)
30
20
10
0
LP-C
HP-C
LP-L
HP-L
Aa
Aa
Bb
Bb
Bc
Ab
Cc
Cc
Cd
Cd
Bc
Ac
ABb
Bb
Aa
Aa
Bc
Bc
Ab
Cc
Bc
Cd
Ab
Bc
21 F
3 S
14 S
4 R
35 R
70 F