Effect of diet composition on growth performance, hepatic metabolism and antioxidant activities in Siberian sturgeon (*Acipenser baerii*, Brandt, 1869) submitted to starvation and refeeding

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Abstract

Many fish species undergo natural starvation periods. Adaptation to starvation is possible through the activation of behavioral, biochemical and physiological mechanisms. Knowledge of the effect of dietary nutrients on the intermediary metabolism during starvation and refeeding can be useful to improve fish health and optimize aquaculture production. To analyze the effect of dietary nutrients on liver metabolism of Siberian sturgeon (*Acipenser baerii*) submitted to starvation and refeeding, four isoenergetic diets differing in nutrient composition were designed: LP-St (38 % protein, 12 % lipid, 36 % carbohydrate), HP-St (44 % protein, 10 % lipid, 30 % carbohydrate), LP-L (38 % protein, 18 % lipid, 25 % carbohydrate) and HP-L (44 % protein, 16 % lipid, 22 % carbohydrate). Four groups of fish were fed 3 weeks to satiety with the corresponding diet, starved for 2 weeks and then refed 5 weeks to satiety on the same diet. Starvation mobilized the hepatic lipid store to a greater extent than glycogen. Starvation increased superoxide dismutase activity irrespective of the diet, while low protein diets (LP-St and LP-L) increased catalase activity. The oxidative damage decreased after 5 weeks of refeeding. Refeeding the starved fish on the HP-St diet promoted the greatest growth performance. In addition to report for the first time the effect of diet composition on growth, liver composition and antioxidant activities in Siberian sturgeon submitted to starvation and refeeding, our findings suggest that refeeding on HP-St diet stimulated the use of dietary carbohydrates and allowed a protein sparing effect in Siberian sturgeon.

Keywords: Siberian sturgeon, starvation, refeeding, oxidative stress, energy reserve, hepatosomatic index

Introduction

Starvation periods are common in fish species (Morales et al. 2004). Starvation refers to the biological condition wherein an animal, otherwise willing or able to eat, is unable to do so as a result of some extrinsic limitation on food resources (McCue 2010). It can be induced artificially in commercial fish farms for decreasing water pollution, disease management and optimizing the feeding strategy to reduce the production cost (Caruso et al. 2012).

Under fed conditions, fish grow and increase the store of energy reserves. On the contrary, fasting leads to the mobilization of fuel from the body store and mass loss (Power et al. 2000; Morales et al. 2004). The reduction rate of muscle mass is extremely variable due to different energy requirements depending on body weight and phylogenetic affiliation (Garland et al. 2005). Some organs, such as the liver, can tolerate large reductions in mass during the starvation period by controlling fuel storage and nutrient mobilization (Metón et al. 2003; Pérez-Jiménez et al. 2007).

During fasting, most species use liver glycogen as the first substrate to obtain energy (Viegas et al. 2012). In parallel with liver glycogen exhaustion, lipid reserves are also used as a fuel. When both glycogen and lipid supplies are nearly depleted, protein is mobilized (Navarro and Gutiérrez 1995; Metón et al. 2003). However, some fish species, such as *Salmo gairdneri* and *Notopterus notopterus*, use lipids and protein as energy substrates during starvation, without affecting significantly the hepatic glycogen store (Narasimhan and Sundararaj 1971; Leatherland and Nuti 1981; Pérez-jiménez et al. 2007). In fish, the use of body glycogen, lipid and protein to obtain energy during starvation varies according to the species, period of food deprivation and the diet composition prior to fasting (Hilton 1982).

It was reported that caloric restriction can induce oxidative stress in fish (Chatzifotis et al. 2011). Oxidative stress occurs when reactive oxygen species (ROS) generation exceeds its removal and may lead to cell death (Sies 1986). By catalyzing the conversion of superoxide anion into molecular oxygen and water, superoxide dismutase (SOD; EC 1.15.1.1) and catalase (CAT; EC 1.11.1.16) are key antioxidant enzymes that were previously shown to be present in the fish liver (Aras et al. 2009). Some studies addressed the effect of food deprivation on oxidative stress and antioxidant defenses (Feng et al. 2011; Bayir et al. 2011). However, the impact of diet composition on antioxidant activities in fish submitted to starvation-refeeding remains largely unknown.

Despite diet composition and feeding regimes may have a major impact on fish health and production, little is known regarding to optimization of feeding strategies in cultured fish species with a marked commercial interest, such as sturgeon, exposed to starvation and refeeding. To increase the current knowledge about the effect of diet composition and feeding regime on somatic and metabolic parameters of Siberian sturgeon (*Acipenser baerii*), in the present work we evaluated growth performance, liver composition and activity of liver antioxidant enzymes in Siberian sturgeon submitted to starvation and refeeding on various diets differing in nutrient composition.

Materials and methods

Rearing procedures

A group of 180 Siberian sturgeon juveniles (initial body weight 30 ± 5 g) were obtained from International Sturgeon Research Institute (Gilan, Iran) and randomly supplied in 12, 500-L circular fiberglass tanks (n=15 per tank) in a flow through system containing treated river water with continuous aeration. Fish were fed on commercial pellets (BIOMAR, France, 1.9 mm) for one week while they acclimated to the experimental conditions (Bagherzadeh Lakani et al. 2013). Tanks were located outdoors and subjected to natural photoperiod of approximately 12 h:12 h (light: dark) cycle. Every day, all tanks were cleaned and siphoned to remove debris. Temperature, dissolved O_2, pH-value and flow rate were maintained at 22 ± 4 °C, 7.1 ± 1.5 mg L^{-1}, 7-8 and 4.5 ± 0.5 L min^{-1}, respectively. Four groups of fish were fed manually to satiety with the corresponding experimental diet three times a day (8:30, 15:00 and 21:30 hours) for 3 weeks, starved for 2 weeks and then refed for 5 weeks on the same diet and conditions. Three tanks were used for each condition.

Feeding trial

Ingredients and chemical composition of experimental diets used in the present study are shown in Table 1. Four isoenergetic diets (gross energy 19.9 ± 0.4 kJ g^{-1} dm) were formulated with different levels of protein, lipids and carbohydrates. Fishmeal was used as protein source. Diets were named LP-St (low protein, 38 % - high carbohydrate, 36 %), HP-St (high protein, 44 % - high carbohydrate, 30 %), LP-L (low protein, 38 % - high lipid, 18 %) and HP-L (high protein, 44 % - high lipid, 16 %). Dry ingredients were weighed, ground and mixed thoroughly. Fish oil, sunflower oil, lecithin and water were added to the

dry ingredients and mixed again, until dough was formed. Dough was pelleted in 2 millimeter and dried in a hot air oven (Hootakhsh, Tehran, Iran) at 60 °C for 5–6 h. The diets were broken up and sieved into proper pellet size, packed and stored at -20°C until used.

Sample preparation

Sampling was performed at week 3 (end of feeding period), 5 (after 2 weeks of starvation) and 10 (after 5 weeks of refeeding). Two animals of each tank (6 per dietary treatment) were anaesthetized with clove powder (500 mg L^{-1}) (Yarmohammadi et al. 2012) and then killed by a sharp blow in the head (Pérez-Jiménez et al. 2009). Liver tissue was dissected using clean equipment on ice (0 ºC), weighted, washed, immediately frozen in liquid nitrogen and kept at -80 ºC until further analysis. Fish hepatosomatic index (HSI) was measured by the following equation (Higgs et al. 2009):

HSI = [liver weight (g)/ wet body weight (g)] ×100)

Treatment of the samples

Liver tissues were homogenized (1:10, w/v) in homogenization buffer containing 100 mM potassium phosphate buffer (pH 7.4), 100 mM KCl and 1 mM EDTA at 0 – 4°C using an electric homogenizer (WIGGEN, D500, Germany) for 1.5 min. Homogenates were centrifuged at 10,000 g using a Hermle Z36HK centrifuge (Hermle Labortechnik, Germany) for 35 min at 4 °C. Supernatants were used to determine glycogen and measure enzyme activity (Atli and Canli 2010). All chemicals used in this study were obtained from Sigma-Aldrich (USA) and Merck (Germany).

Chemical analysis

Chemical composition (crude protein, lipid and moisture) of the experimental diets and fish livers was determined using the following (AOAC 2005) procedures: total protein content (N × 6.25) using an automatic Kjeldahl system (230-Hjeltec Analyser; Foss Tecator, Hoganas, Sweden) and total lipid with an automatic Soxtec system (2050-FOSS; Sweden). Moisture was determined by drying at 105 °C for 24 h in an oven (D-63450; Heraeus, Hanau, Germany), and ash by burning in a muffle furnace (Isuzu, Tokyo, Japan) at 550°C for 6 h. Glycogen was assayed using the BDU-GLY96 ELISA kit (Zellbio, Germany). In brief, the assay is based on glycogen hydrolyzation into glucose. Glucose oxidation forms an intermediate that reduces a colorless robe to a colored product with strong absorbance at 620 nm. The glycogen content is expressed as milligrams of glucose equivalents per gram of fresh liver tissue.

Determination of enzyme activities

SOD and CAT activities were determined using spectrophotometric methods. SOD was assayed with the ZB-SOD96 kit (ZellBio GmbH, Germany). SOD activity unit was considered as the amount of the sample that catalyzed decomposition of 1 μmol of O^{2-} into H_2O_2 and O_2 per minute. Absorbance was recorded at 550 nm.

CAT was assayed using the ZB-CAT96 kit (ZellBio GmbH, Germany). CAT activity unit was considered as the amount of the sample that catalyzed decomposition of 1 μmol of H_2O_2 into H_2O and O_2 per minute. Absorbance was recorded at 405 nm.

Total soluble protein was measured by the Bradford method (1976) using bovine serum albumin as a standard. Enzyme activities were expressed as specific activity (U mg^{-1} protein). All the enzymatic assays were run in triplicate.

Statistical analysis

Data were checked for normality (Kolmogorov-Smirnov test) and homogeneity of variances prior to their comparison. Data were analysed by one-way and two-way (diet and condition as the main factors) ANOVA using a computer program (IBM SPSS Statistics version 22, Armonk, NY, USA). Statistical differences among mean values with one independent variable were analyzed by one-way ANOVA performing mean comparisons with Duncan's test at a reliability level of 0.05. To determine homogeneous subsets of values with two independent variables, two-way ANOVA was performed using the Scheffé post hoc test ($P < 0.05$).

Results

Growth performance and HSI

The growth performance of Siberian sturgeon juveniles was affected by diet composition in fish submitted to starvation and refeeding (Table 2). Feeding on HP-St and LP-St resulted in the highest (182±7.9 g) and lowest (158±10 g) final body weight, respectively. Accordingly, feeding on HP-St promoted significantly higher weight gain values than in the group of fish fed diet LP-St during 5 weeks of refeeding. The highest and lowest FCR values were presented by fish fed diets LP-St and HP-St, respectively. Albeit not significant, it was observed a tendency to present higher specific growth rate (SGR) values in the fish supplied with high protein diets during refeeding.

153 Two weeks of starvation significantly decreased HSI irrespective of the diet. After 3 weeks of feeding and
154 5 weeks of refeeding, fish fed with high carbohydrate diets (HP-St and LP-St) presented the highest HSI
155 values. In all treatments, the lowest HSI value was found in the group of fish fed diet HP-L (Fig. 1).
156 *Liver composition*
157 Liver composition of Siberian sturgeon was significantly affected by diet composition and nutritional
158 status (Fig. 2). After 3 weeks of feeding, a trend to present higher hepatic glycogen levels was found in
159 the fish fed high carbohydrate diets (LP-St and HP-St). Starvation for 2 weeks significantly decreased
160 liver glycogen reserves, reaching similar values irrespective of the diet, about 13-14 mg g^{-1} liver. Five
161 weeks of refeeding were not enough to recover hepatic glycogen levels similar to those observed previous
162 to starvation, and no significant differences in liver glycogen content were found among the groups of
163 fish fed different diets.
164 Two weeks of starvation significantly fell down hepatic lipid reserves except in the group LP-L. Five
165 weeks of refeeding led to recover liver lipid reserves in all treatments to levels higher than in 3 week fed-
166 fish. After both 3 weeks of feeding and 5 weeks of refeeding, the supply of low protein diets (LP-St and
167 LP-L) significantly increased liver fat compared to fish fed high protein diets (HP-St and HP-L).
168 Concerning the hepatic protein content, no significant effect was observed between 3 week fed-fish and 2
169 week starved-animals. However, refeeding significantly increased liver protein irrespective of the diet. In
170 all conditions, the fish fed diet LP-L exhibited the higher hepatic protein content.
171 *CAT and SOD specific activities in liver*
172 The nutritional status and diet composition significantly affected liver CAT and SOD activities (Fig. 3A
173 and 3B). A trend to increase the specific activity of CAT in juveniles fed with LP-L and LP-St diets was
174 observed after 2 weeks of starvation, while almost no change was observed in the fish fed high protein
175 diets (HP-St and HP-L) (Fig. 4A). Compared to starved fish, 5 weeks of refeeding with low protein diets
176 (LP-St and LP-L) significantly decreased CAT activity. The lower CAT activity levels after refeeding
177 were found in the fish fed diet HP-L.
178 Starvation significantly increased SOD activity in the liver of fish fed the four experimental diets. After 5
179 weeks of refeeding, a trend to recover the values found in 3 week-fed fish was observed in all treatments
180 with the exception of fish fed diet HP-St (Fig. 3B).
181

182 **Discussion**

183 In recent years, some wild populations of Caspian Sea sturgeons are among critical endangered fish
184 species (IUCN Red Data List, 2015), because of overfishing for meat and caviar production, destruction
185 of their spawning grounds and water pollution (Babaei et al. 2011). Therefore, research efforts have
186 focused on Siberian sturgeon for developing aquaculture programs and reducing overfishing of native
187 sturgeons. The aim of this study was to determine the appropriate feeding schedule when using starvation
188 periods in order to improve production, maximize growth and produce less oxidative damage to Siberian
189 sturgeon in culture.

190 Previous studies reported influence of diet composition on growth performance in Siberian sturgeon
191 (Rónyai et al. 2002; Guo et al. 2012) and other sturgeons (Abedian Kenari et al. 2009, Hosseini et al.
192 2010). However, it remains largely unknown the effect of the nutrient composition of the diet on
193 physiological responses during starvation and refeeding in this species. In the present study, the higher
194 WG after refeeding was found in the group of fish fed on the high protein/high carbohydrate diet (HP-St).
195 This finding suggests that dietary carbohydrate may promote a faster recovery of BW than dietary lipids
196 after a food deprivation period in Siberian sturgeon. Concerning the protein content of the diet, our
197 findings are consistent with the optimal dietary protein level reported for maximal growth for *Acipenser*
198 *sinensis* (about 40–45 %; Xiao et al. 1999) and *A. persicus* (40 %; Mohseni et al. 2007). In hybrid
199 sturgeon (*Acipenser baerii* ♀ × *A. gueldenstaedtii* ♂) optimal dietary protein was estimated at 37 % (Guo
200 et al. 2012). However, refed fish show a fast weight recovery, mainly supported by the rapid restoration
201 of their metabolic profile (Furne et al. 2012).

202 In Siberian sturgeon, the higher HSI and hepatic glycogen values were found in fish fed high
203 carbohydrate diets (HP-St and LP-St). Positive correlation between HSI and liver glycogen content with
204 dietary carbohydrate levels has been well documented in *Acipenser baerii* (fed on gelatinized starch)
205 (Médale et al. 1991), white sturgeon (*Acipenser transmontanus*) (fed on high D-glucose diet) (Fynn-
206 Aikins et al. 1992) and other fish species, such as *Sparus aurata* (Metón et al. 1999). Similarly as in the
207 present study, a significant reduction in HSI of Siberian sturgeon during starvation was observed
208 previously when feeding on commercial pellets before fasting (Ashouri et al. 2013). Decreased HSI was
209 also observed in starved white sturgeon (Hung et al. 1997b), brown trout, *Salmo trutta* (Bayir et al. 2011)
210 and gilthead sea bream (Metón et al. 1999). Refeeding increased HSI in all groups of fish. However, the

211 higher HSI values were observed in Siberian sturgeon fed high carbohydrate diets, while feeding a high
212 protein/high lipid diet (HP-L) resulted in the lowest HSI levels. During refeeding, hyperphagia can
213 prompted some metabolic pathways to recover the metabolic profile and reestablish the tissue reserves
214 (Furne et al. 2012), probably resulting in increased HSI and body weight.
215 In many fish species, glycaemia maintenance during food deprivation is directly related to the ability to
216 mobilize liver glycogen, at least during the initial stages of starvation (Perez-Jimenez et al. 2007). Hepatic
217 glycogen, when required, is enzymatically broken down to glucose and transported to peripheral tissues.
218 Our findings indicate that after starvation, hepatic glycogen modestly decreased in all treatments (notably
219 in the fish fed low carbohydrate diets). In contrast, liver glycogen is mobilized as early as after 5 – 20
220 days of fasting in *A. naccarii* (Furne et al. 2012) and white sturgeon (Hung et al. 1997b).
221 More than 60 % of liver dry mass (230 - 330 mg g^{-1} liver) of Siberian sturgeon liver are lipids. Similar
222 liver fat content is found in white sturgeon (300 - 370 mg g^{-1} liver) (Fynn-Aikins et al. 1992). After 3
223 week feeding, the lowest lipid content was present in the liver of Siberian sturgeon fed the high
224 protein/high carbohydrate diet (HP-St). Consistently, there is convincing evidence that high protein diets
225 increase fat loss compared to diets with lower protein content (Halton and Hu 2004), as it is the case in
226 hybrid sturgeon (Guo et al. 2012). Lipids have a major role in fish that do not mobilize significant levels
227 of liver glycogen during starvation (Sheridan and Mommsen 1991). Most animals are able to tolerate a 20
228 – 70 % loss of total body lipid content during starvation (McCue 2010). The use of the lipid store during
229 food deprivation depends on the species, the lipid-reserve tissue and mobilization of other energy supplies
230 such as glycogen (Furné et al. 2012). Starvation significantly decreased liver fat in Siberian sturgeon.
231 Besides, our findings argue for the mobilization of lipid store and to a lesser extend glycogen in the liver
232 of 2 week-starved Siberian sturgeon. These results suggest that the Siberian sturgeon liver may
233 preferentially utilize lipids as an energy resource. Similar results were reported for channel catfish (Kim
234 and Lovell 1995) and Adriatic sturgeon *A. naccarii*, where liver lipid also decreased more importantly
235 than hepatic glycogen and protein (Furné et al. 2012). Furthermore, a greater utilization of sturgeon
236 hepatic lipids for energy purposes during fasting may result from the high hepatic lipid content in this
237 species (Furné et al. 2012). The time-course of recovery in the liver differed for glycogen and fat after
238 refeeding: liver lipid was significantly higher after 5 weeks of refeeding compared to the values observed
239 before food deprivation, while the refeeding period was not enough to recover liver glycogen.

240 The hepatic protein content was less affected than liver fat by the nutritional condition and diet
241 composition in Siberian sturgeon. Similarly, white sturgeon (Hung et al. 1997a) and Persian sturgeon
242 (Mohseni et al. 2007) fed with diets differing in nutrient composition were reported to keep body protein
243 relatively constant. Indeed, reduction in carcass protein content of white sturgeon after 10 weeks of
244 fasting was much lower (9 %) than that of the lipid content (84 %) (Hung et al. 1997 b), suggesting that
245 sturgeon preferentially conserve muscle protein over lipids during food deprivation (Falahatkar et al.
246 2013).

247 Over the past few decades, the stress response of fish has been extensively investigated. However, the
248 relationship between diet composition, fish stress and immune response as well as between feeding
249 regime and immune response have received little attention (Caruso et al. 2011; Li et al. 2012). High lipid
250 storage in sturgeon is rich in unsaturated fatty acids (García-Gallego et al. 1999), which exhibit a very
251 strong tendency towards oxidation (Fang et al. 2003). The specific activities of antioxidant enzymes in
252 Siberian sturgeon were low compared with other species. The lower oxygen consumption by sturgeon and
253 its phylogenetic position (ancestral species exhibit less antioxidant activity) (Tappel et al. 1982) may
254 explain these findings.
255 As with Siberian sturgeon, increased SOD activity has been described during starvation in *Pseudo*
256 *sciaenacrocea* (Zhang et al. 2008) and brown trout (Bayir et al. 2011). These results suggest that the rate
257 of $O^{2\bullet-}$ generation increases during starvation.
258 CAT activity is associated with elevated concentrations of H_2O_2, which in turn is produced by SOD
259 reaction. In the present study, starvation increased SOD activity irrespective of the diet, while low protein
260 diets (LP-St and LP-L) resulted in high CAT activity values. Compared with starvation, our results
261 indicate that the oxidative damage decreased after 5 weeks of refeeding. In contrast, Furne et al. (2009)
262 reported that oxidative stress remained after 60 days of refeeding in liver and red blood cells of rainbow
263 trout and sturgeon. The low antioxidant activities in the fish fed diet HP-L after refeeding may be related
264 to the low HSI and low hepatic lipid content (as a free radical production inducer) observed in this group
265 of fish.
266 In conclusion, our findings show for the first time that Siberian sturgeon juveniles experience metabolic
267 adjustments to both starvation and refeeding, and that diet composition has a major impact on the
268 metabolic responses to nutritional status. Growth performance and liver composition suggest that

refeeding with a high protein/high carbohydrate diet stimulates the use of dietary carbohydrates, while allows sparing protein in Siberian sturgeon. Given that supply of diets with a significant amount of carbohydrates to sturgeon can diminish feeding costs and allow sparing protein without decreasing the growth performance after food deprivation periods, the results of the present study may be useful to improve feed management to achieve better nutrition efficiency and fish health.

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Legends

Table 1. Formulation and chemical composition of experimental diets for Siberian sturgeon juveniles (g 100 g^{-1} diet).

Table 2. Growth performance of Siberian sturgeon juveniles fed the experimental diets LP-St, HP-St, LP-L and HP-L.

Figure 1. Hepatosomatic Index (HSI) of Siberian sturgeon submitted to starvation and refeeding with diets differing in nutrient composition. Sampling was performed after 3 weeks of feeding (3 F), 2 weeks of starvation (2 S) and 5 weeks of refeeding (5 R). Values are expressed as means ± SD (n=3 tanks). Statistical significance for independent variables (Diet and Treatment) and the interaction between them are indicated as follows: *$P < 0.05$; ***$P < 0.001$. Homogeneous subsets for the independent variables (Diet and Condition) are indicated with different letters (small and capital, respectively).

Figure 2. Liver composition (glycogen, lipid and protein) of Siberian sturgeon submitted to starvation and refeeding with diets differing in nutrient composition. Sampling was performed after 3 weeks of feeding (3 F), 2 weeks of starvation (2 S) and 5 weeks of refeeding (5 R). Values are expressed mg g^{-1} liver. Statistical significance for independent variables (Diet and Treatment) and the interaction between them are indicated as follows: **$P < 0.01$; ***$P < 0.001$; NS, not significant. Homogeneous subsets for the independent variables (Diet and Condition) are indicated with different letters (small and capital, respectively).

Figure 3. Specific activity of antioxidant enzymes in the liver of Siberian sturgeon submitted to starvation and refeeding with diets differing in nutrient composition. CAT (A) and SOD (B) activities were assayed in liver of fish after 3 weeks of feeding (3 F), 2 weeks of starvation (2 S) and 5 weeks of refeeding (5 R). Values are expressed as means (U mg $protein^{-1}$) ± SD (n= 3 tanks). Statistical significance for independent variables (Diet and Treatment) and the interaction between them are indicated as follows: ***$P < 0.001$. Homogeneous subsets for the independent variables (Diet and Condition) are indicated with different letters (small and capital, respectively).

466 **Table 1.**

Ingredientes	LP-St	HP-St	LP-L	HP-L
	38P11L36C	**44P10L30C**	**38P18L25C**	**44P16L22C**
Fish meal*	46.24	57.1	49.3	58.86
Wheat meal	39.88	32.96	21.2	17.98
Fish oil‡	3.22	1.74	6.22	4.35
Sunflower oil†	3.22	1.74	6.22	4.35
Soy lecithin§	0.5	0.5	0.5	0.5
Mono calcium phosphate	0.5	0.5	0.5	0.5
Mineral mix¶	2	2	2	2
Vitamin mix‖	1.5	1.5	1.5	1.5
Anti fungi	0.25	0.25	0.25	0.25
Anti-oxidant‡‡	0.02	0.02	0.02	0.02
Filler♦	1.16	0.17	10.79	8.19
Binder◊	1.5	1.5	1.5	1.5
TOTAL	100	100	100	100
Chemical analysis (%)				
moisture	5.0 ± 0.0	5 ± 0.9	5.8 ± 0.3	5.9 ± 0.8
Crude protein	37.9 ± 0.4	44.1 ± 0.3	38.2 ± 0.3	43.9 ± 0.5
Crude lipid	11.5 ± 1.1	10± 0.7	17.5 ± 2.1	15.7 ± 0.9
Carbohydrates**	35.9 ± 1.4	30.2 ± 0.8	24.9 ± 2.8	21.9 ± 0.4
ash	9.7 ± 0.04	10.7 ± 0.6	13.5 ± 0.5	12.6 ± 0.6
CHO: L	3.1	3	1.4	1.4
Gross energy (kJ g^{-1} dm) ††	19.7	19.6	20.2	20.3

Diets were named LP-St (low protein- high carbohydrate), HP-St (high protein- high carbohydrate), LP-L (low protein- high lipid) and HP-L (high protein- high lipid).

* Clopeonella meal (Mazandaran Animal and 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.

‖ 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)

** Carbohydrates were calculated by difference. Carbohydrate = 100 – (crude protein + crude lipid + ash + moisture) (Azarm et al. 2013).

†† 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).

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468 **Table 2.**

Growth parameters	LP-St	HP-St	LP-L	HP-L
BW_0 (g)[‡]	29.2±0.6	30.0±0.5	30.2±0.1	29.8±0.8
BW_1 (g)[†]	64±3.6	62±5.6	63±5.9	62±1.1
BW_2 (g)[‖]	61±1.3[b]	65±2.1[a]	63±1.3[ab]	63±2.5[ab]
BW_3 (g)[¶]	158±10[b]	182±7.9[a]	165±12[ab]	169±7.3[ab]
WG_r (g)[#]	97 ±9.2[b]	117±8.2[a]	101±11.3[ab]	106±4.9[ab]
WG_t (g)[**]	129±11[b]	152±8[a]	134±12[ab]	139±7[ab]
SGR_r (% day $^{-1}$)[◊]	3.0±0.1	3.2±0.2	3.0±0.2	3.1±0.0
SGR_t (% day $^{-1}$)[♦]	2.4±0.1	2.6±0.1	2.4±0.1	2.5±0.1
FCR (g g^{-1})[§]	1.5±0.1[a]	1.2±0.1[b]	1.4±0.1[ab]	1.4±0.1[ab]
Survival (%)[††]	100	100	100	100

Diets were named LP-St (low protein- high carbohydrate), HP-St (high protein- high carbohydrate), LP-L (low protein- high lipid) and HP-L (high protein- high lipid).

[‡] BW_0: initial Body Weight.

[†] BW_1: Body Weight at week 3 (after 3 weeks of feeding)

[‖] BW_2: Body Weight at week 5 (after 2 weeks of starvation)

[¶] BW_3: Body Weight at week 10 (after 5 weeks of refeeding)

[#] WG_r: Weight Gain (r) = BW_3 (g) – BW_2 (g) during the last 5 weeks of experimentation (refeeding period)

[**] WG_t: Weight Gain (t) = BW_3 (g) – BW_0 (g) during the total experimental period (10 weeks)

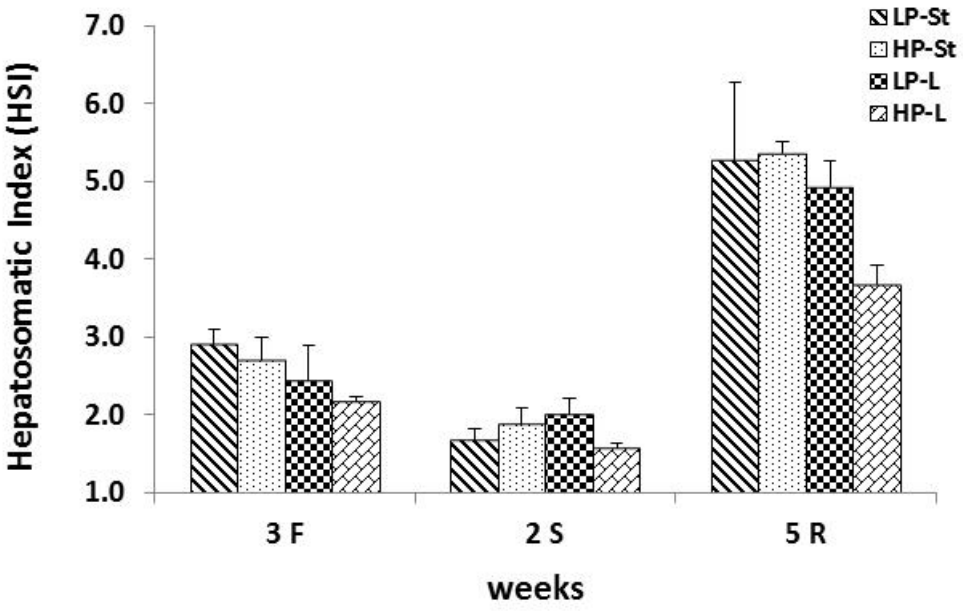
[◊] SGR_r: Specific Growth Rate (r) = (Ln BW_3−Ln BW_2) × 100; during the last 5 weeks of experimentation (refeeding period) (Mohanta et al. 2008).

[♦] SGR_t: Specific Growth Rate (t) = (Ln BW_3−Ln BW_0) × 100; during the total experimental period (10 weeks)

[§] FCR: Feed Conversion Ratio = Dry feed consumed (g)/ WG_t (g) (Mohanta et al. 2008)

[††] Survival (%) = (Number of fish in each group remaining in end of experiment/ initial number of fish) × 100 (Hamza et al. 2008).

The small letters indicate statistical differences between groups ($P<0.05$) (one-way ANOVA). Values are means ± S.D. (n=3; number of tanks per treatment).



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Two-way ANOVA

Dependent variable	Interaction	Diet	Condition	Diet				Condition		
				LP-St	HP-St	LP-L	HP-L	3F	2S	5R
HSI	*	***	***	b	b	b	a	B	A	C

472
473 **Fig. 1.**
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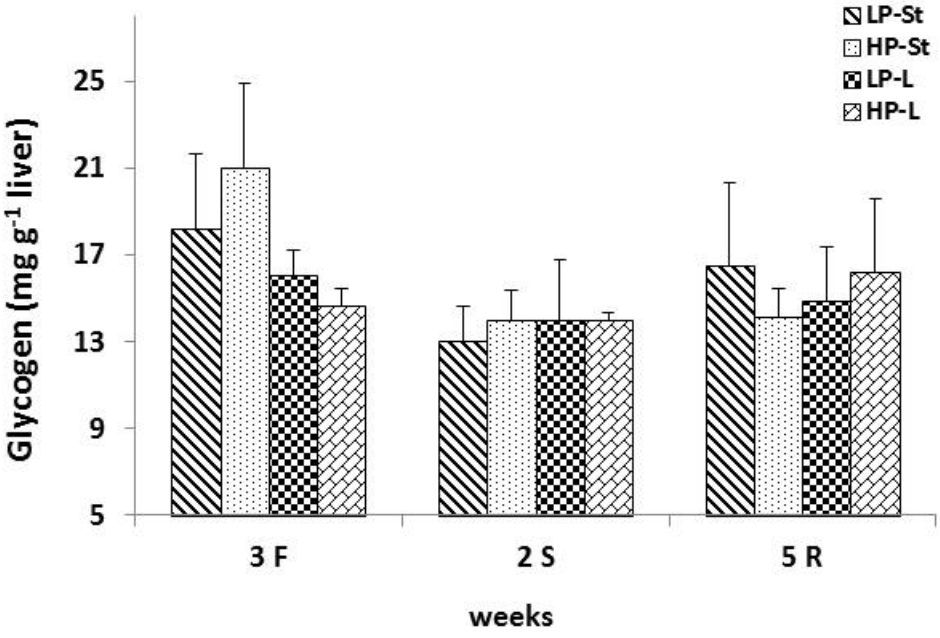
476 **A**



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479 **B**

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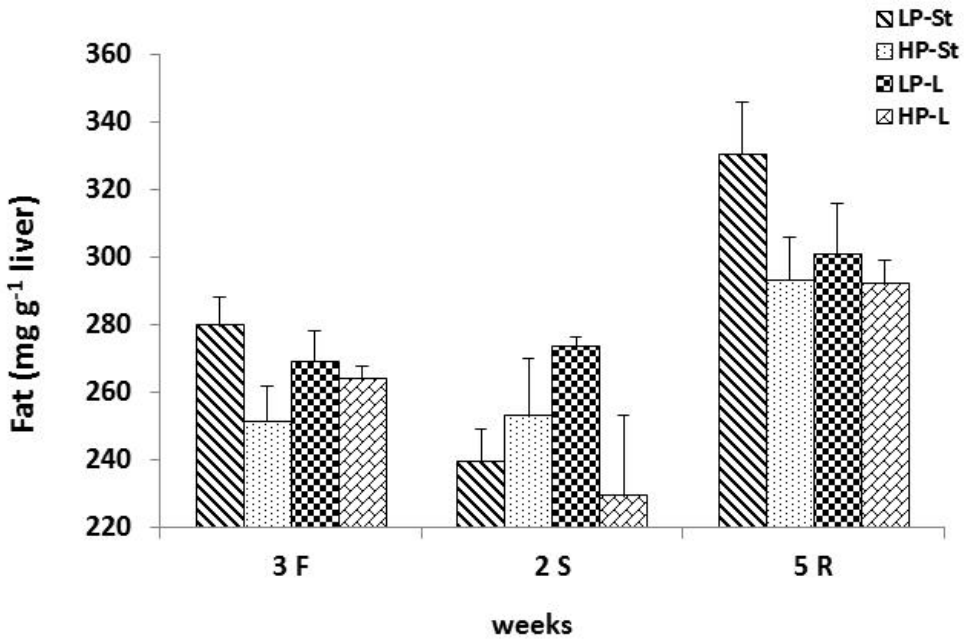

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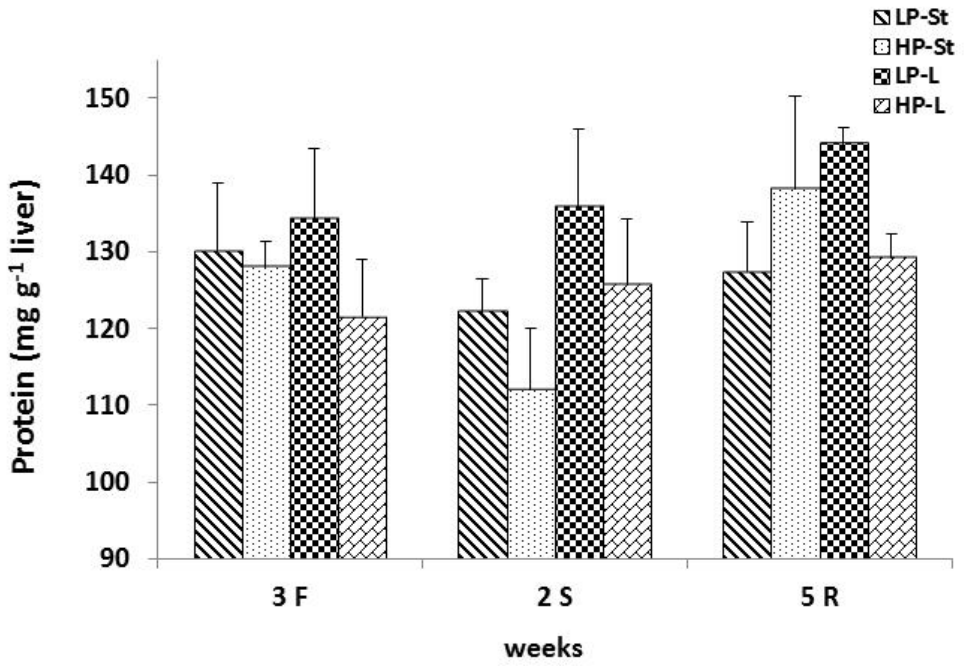
483 **C**



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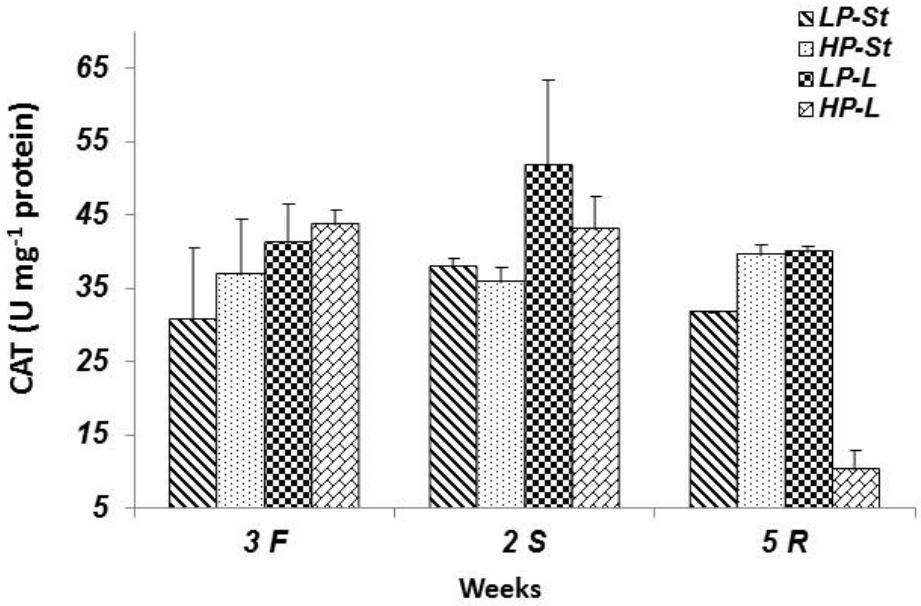
Two-way ANOVA

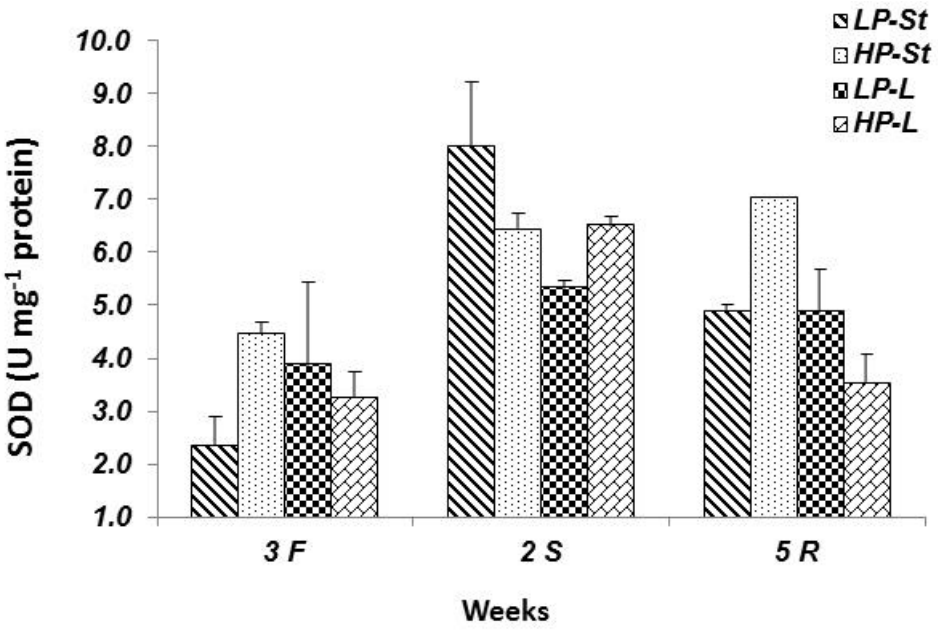
Dependent variable	Interaction	Diet	Condition	Diet				Condition		
				LP-St	HP-St	LP-L	HP-L	3F	2S	5R
Glycogen	NS	NS	**	-	-	-	-	B	A	AB
Fat	**	**	***	b	a	b	a	B	A	C
Protein	NS	**	**	a	a	b	a	A	A	B

Fig. 2.

A



B



Two-way ANOVA

Dependent variable	Interaction	Diet	Condition	Diet				Condition		
				LP-St	HP-St	LP-L	HP-L	3F	2S	5R
Catalase	***	***	***	a	a	b	a	B	B	A
SOD	***	***	***	a	b	a	a	A	C	B

Fig. 3.