



Starvation and refeeding effects on pyloric caeca structure of Caspian salmon (*Salmo trutta caspius*, Kessler 1877) juvenile

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ABSTRACT

Effect of starvation and refeeding on the structure of pyloric caeca was studied in the juveniles of Caspian Sea salmon. Juveniles (average body weight 12 ± 0.1 g) were subjected to four levels of feeding: full-fed for 6 weeks (FFF), 3 weeks fed and 3 weeks following starvation (FS), 3 weeks starved and 3 weeks fed (SF), and full-starved (SSS) for 6 weeks. Light microscopic studies showed significant reduction ($p < 0.05$) in the enterocytes height and number, villus length, epithelial area and pyloric caeca total area in starved groups as compared to control group. These reductions were more significant ($p < 0.05$) in long term starved group (SSS) than short term starved group (FS). Additionally, refeeding increased pyloric caeca size and enterocyte's number in SF group whereas, the epithelial total area and villus length did not reach the same area and length as control group. Results indicated that in Caspian Sea salmon juveniles food deprivation and consuming of food source, adversely affected the tissue of pyloric caeca while refeeding can be effective on healing tissue damage.

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1. Introduction

Deliberate releases of artificially produced fish into wild populations have recently caused concern among fish biologists (Youngson and Verspoor, 1998; Einum and Fleming, 2001; Strand and Finstad, 2007). Releases are associated with a range of ecological problems including changes in the frequency of competitive interactions, levels of food availability, and a functional response of predators (Einum and Fleming, 2001). In addition to all these, various investigations revealed that farm fish also experience stress responses in relation to capture, handling and tank confinement which may lead to alterations in fish behavior and performance after release (Strand and Finstad, 2007). The fish may require minutes, days or weeks to return to their pre-stress state (Schreck et al., 1997). Starvation is an example of an adverse situation that released fish may be encountered in natural environment after releasing. In order to successfully release cultured fish into wild rivers, sufficient feed to grow the juveniles must be available in the river (Mizuno et al., 2002). The lack of food is a situation faced and tolerated by many fish species during their life cycle (McLeese and Moon, 1989; Navarro and Gutiérrez, 1995; Krogdahl and Bakke-McKellep, 2005). The digestive tract and its associated glands are the first organs

impaired by food deprivation (Theilacker, 1978). In both larval and adult fish, starvation can lead to atrophy of intestinal structures and the associated glands (Collins and Anderson, 1995; Green and McCormick, 1999; Gisbert and Doroshov, 2003; Rios et al., 2004; Ostaszewska et al., 2005). Pyloric caeca are blind diverticula of proximal intestine that possess a luminal epithelium and are structurally similar to other segments of intestine (Buddington and Diamond, 1986; Veillette and Young, 2005). Their number varies greatly with species and age (Bergot et al., 1981; Stevens, 1988), with as many as 200 in salmonids, accounting for roughly 70% of the nominal surface area of the foregut intestinal. They can increase the absorptive surface area of an otherwise comparatively short intestinal tract and are very important for enzymatic breakdown and absorption of feed constituents (Buddington and Diamond, 1986). It is reported that the proximal intestine and its associated caeca are the major sites for absorption of lipids in salmonids (Ostos Garrido et al., 1993).

A number of gut histopathological changes were reported in fish subjected to periods of starvation (McLeese and Moon, 1989; Rios et al., 2004; Ostaszewska et al., 2005; Krogdahl and Bakke-McKellep, 2005) which can have a negative effect on profitability during the course of a production.

The Caspian trout, *Salmo trutta caspius* is an anadromous subspecies of brown trout which is restricted to the western and southern coast of Caspian Sea (Rajabi and Khodabandeh, 2013). In the last decade, the populations of this economic species are declining as a result of overfishing, water pollution, habitat

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destruction and drought (Ramezani, 2009; Ghanizadeh Kazerouni and Khodabandeh, 2011). Hence the stock of this highly precious species has so drastically decreased that it has been listed as an endangered species (Ghanizadeh Kazerouni and Khodabandeh, 2010). Considering this, artificial reproduction and restocking has been introduced as a solution for enhancement and protection of wild populations. *S. trutta caspius* are reared in the culture centers in Iran, and are released to the rivers at the smolt size of 20 g (Rajabi and Khodabandeh, 2013). Released juveniles of salmon are at risk, envisage to food deprivation after releasing to the stream, which is due to their lack of skill in predation or environmental condition. Studies on Caspian trout nutrition and feeding are overwhelming, however according to the knowledge, there is a lack of information on their status during food deprivation. The current study was conducted to determine the effects of starvation and refeeding on the pyloric caeca structure, which is the main site for digestion in salmonids.

2. Materials and methods

2.1. Fish and feeding trial

480 juveniles of Caspian salmon (average body weight 12 ± 0.1 g) were randomly collected from Shahid Bahonar reproduction and culture farm (Kelardasht, Iran) and allocated into 12 concrete ponds (40 fish per 1.5 m^3 water), in an open system. Water flow rate in each tank was approximately 20 L min^{-1} and water temperature was fluctuated from 9 to 11°C . Each treatment was conducted in triplicates (3 tanks/treatment). The fish were fed by a commercial, modified rainbow trout diet with four levels of feeding: full-fed for 6 weeks (FFF), 3 weeks fed and 3 weeks following starved (FS), 3 weeks starved and 3 weeks fed (SF), or full-starved (SSS) for 6 weeks.

2.2. Light microscopic study

The pyloric caeca were removed from five individuals from each tank. Samples were fixed for 24 h in Bouin's fixative. Once fixed, the samples were dehydrated through a graded series of ethanol solution up to 100%, followed by xylene prior to embedding in paraffin. Samples were sectioned longitudinally and horizontally by MICRODS 4055 microtome at $4 \mu\text{m}$ thickness. Hematoxylin and Eosin stained sections were prepared from each block. Stained slides were examined by using Nikon light microscope and Olympus video camera linked to a computer. Histological micrographs analyzed by Image Tools software (Martoja and Martoja-Pierson, 1967; Khodabandeh et al., 2005, 2009).

2.3. Pyloric caeca

Cross sections of 30 pyloric caeca from each sample were examined by Image Tools (2, 0) software to quantify the pyloric caeca mean total area, epithelial area and muscularis/serosa layer.

2.4. Villus length

Randomly selected villus of 30 pyloric caeca from each group were measured from basement to top of the villi by Image Tools (2, 0) software to investigate the response of villus length to feeding regime.

2.5. Enterocyte height and numbers

Enterocyte height was used as an indicator of starvation. Randomly selected cells from the pyloric caeca were measured from the top of the basement membrane to the top of the microvilli forming

the brush border. 100 cells were measured from each treatment. Enterocyte number counted per 1 mm^2 of epithelium by Image Tools (2, 0) software.

2.6. Statistical analysis

Data are expressed as means \pm standard deviation ($\pm\text{SD}$). Effects of starvation and refeeding were analyzed by one-way analysis of variance (ANOVA) followed by LSD test (Least Significant Differences Fisher's). All statistical analysis was performed using SPSS (Version 13). In all tests, a probability level of <0.05 was considered to estimate significant differences of data.

3. Result

During the course of the experiment, no mortality was observed in all experimental groups. Pyloric caeca histological micrographs of the control group showed that the mucosal surface of each pyloric caeca had numerous projections (villi), which lined by simple epithelium (Fig. 1A and B). The pyloric caeca wall consisted of four layers: serosa – the outermost one, muscularis (inner circular and outer longitudinal smooth muscles), submucosa, and the mucosal epithelium (Fig. 1C). Adjacent to the submucosa was the loose connective tissue named *lamina propria* (Fig. 1C and D). The intestinal mucosal epithelium of the juvenile *S. trutta caspius* consisted of enterocytes which revealed large oval nuclei. The apical part of enterocytes was covered by numerous microvilli, forming the brush border (Fig. 1C and D). Among the enterocytes, the goblet cells, which are mucous cells, were present (Fig. 1D).

3.1. Starvation

The results showed that the enterocytes of Caspian trout were tightly packed together, in disorder manner and their brush borders lost their smooth appearance as starvation progressed to 6 weeks (Fig. 2B and D). Aggregation of goblet cells among epithelial cells was remarkable in SSS group (Fig. 2B). The number and condition of the epithelium cell were significantly different between feeding treatments and decreased in both starved groups (FS, SSS) ($p < 0.05$) (Fig. 4) and their height reduced (Fig. 5).

Both starved groups (FS, SSS) had significant decreases ($p < 0.05$) in pyloric caeca area, epithelial area, villus length, compared to FFF group (Figs. 1–7). In contrast, the area of the muscularis/serosa layer was not altered significantly in the starved groups with feeding level. Some of the changes in long term starvation group (SSS), were more significant than short term group (FS) (Figs. 6 and 7).

3.2. Refeeding

With feeding, the pyloric caeca enlarged in size (Fig. 3A). enterocytes were partially ordered and resulting in tidy layer of cells in villus (Fig. 3B and C). Microscopical observations showed that the enterocyte number and height (Figs. 4 and 5), villus length (Fig. 6), epithelial area and pyloric caeca total area were recovered and increased in refed group (SF) (Fig. 7), as compared to starved groups (FS, SSS) ($p < 0.05$) although, the epithelial total area and villus length did not reach to the same area as control group (FFF) ($p < 0.05$), after 3 weeks refeeding.

4. Discussion

Starvation period for many species is a part of their life cycle. In response to starvation, several physiological changes have been observed in fish (McLeese and Moon, 1989; Navarro and Gutiérrez, 1995; Gisbert and Doroshov, 2003; Rios et al., 2004; Krogdahl and

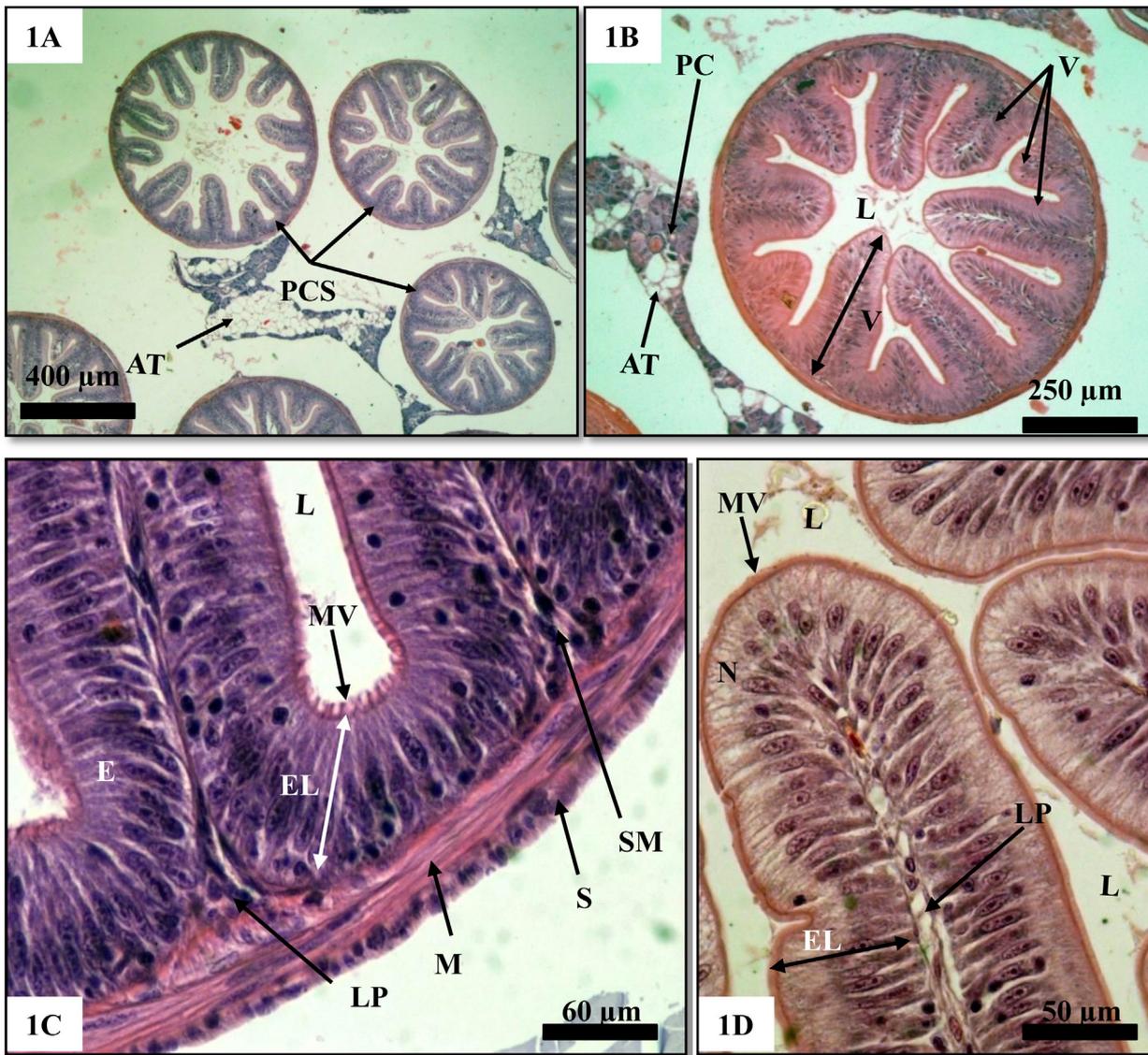


Fig. 1. Horizontal section of pyloric caeca in juvenile of *Salmo trutta caspius* (control group) (stained by H&E). (A and B) Normal pyloric caeca with adipose tissue, (C) showing the various layers of intestinal wall, (D) Enterocyte arranged in ordered manner. AT, adipose tissue; E, enterocyte; EL, enterocyte layer; L, lumen; LP, lamina propria; ML, muscularis layer; MV, microvilli; PCS, pyloric caeca sections; PC, pancreatic cells; S, serosa; SM, submucosa; V, villi.

Bakke-McKellep, 2005; Ostaszewska et al., 2005; Caruso et al., 2010, 2011, 2012). Fish mobilize their energy reserves to survive this food restriction. The chronological sequence of energy resources that can be mobilized in fish during starvation is as follows: carbohydrates, lipids and finally mobilization and catabolism of proteins in order to survive long periods of starvation (Smutna et al., 2002). Morphological changes, observed in many tissues during starvation, are generally attributed to the consumption of lipid, protein and glycogen (Collins and Anderson, 1995).

Intestinal tract of salmonid is a dynamic structure which can react rapidly to changes in environmental condition, dietary inputs and pathogenic organisms (Jutfelt, 2006). Additionally intestine presents some main functions in fish that includes water and electrolyte balance, immunity, nutrient absorption and regulation of digestion and metabolism. Therefore it could be a critical interest to ecologists and great importance to culturists (Rios et al., 2004).

The histological observations of the pyloric caeca in the current study indicated that, starvation would cause histopathological changes in the pyloric caeca. Many of the observed changes resemble those found in other studies of malnourished fish including

reduction in: (a) enterocyte number and height (Hall and Bellwood, 1995; Rios et al., 2004; Ostaszewska et al., 2005); (b) villus length followed by reduction of epithelial layer (Hall and Bellwood, 1995; McLeese and Moon, 1989); (c) pyloric caeca total size (McLeese and Moon, 1989; Rios et al., 2004).

Gut epithelium has continuous cell proliferation which depends costly in terms of energy (Buddington et al., 1997). As a result of the reduction in energy reserves during starvation period, cell component turnover is reduced to save energy (Jobling, 1994). In this study, the enterocyte was particularly sensitive to low food levels, with a reduction in number and height. A decrease in the number of epithelial cell could result from either a reduction in cell proliferation rate or an increase in the extrusion rate of either old or dead cell (Hall and Bellwood, 1995).

Hence, renewal rate adjustment in intestine during starvation period could lead to tissue degeneration which was observed in *Salmo trutta* and other starved fish (McFadzen et al., 1994; Hall and Bellwood, 1995; Ostaszewska et al., 2005). A decrease in cell proliferation rate appears to be a result of the increased cell cycle time and slower movement of intestinal cells up the villi which was

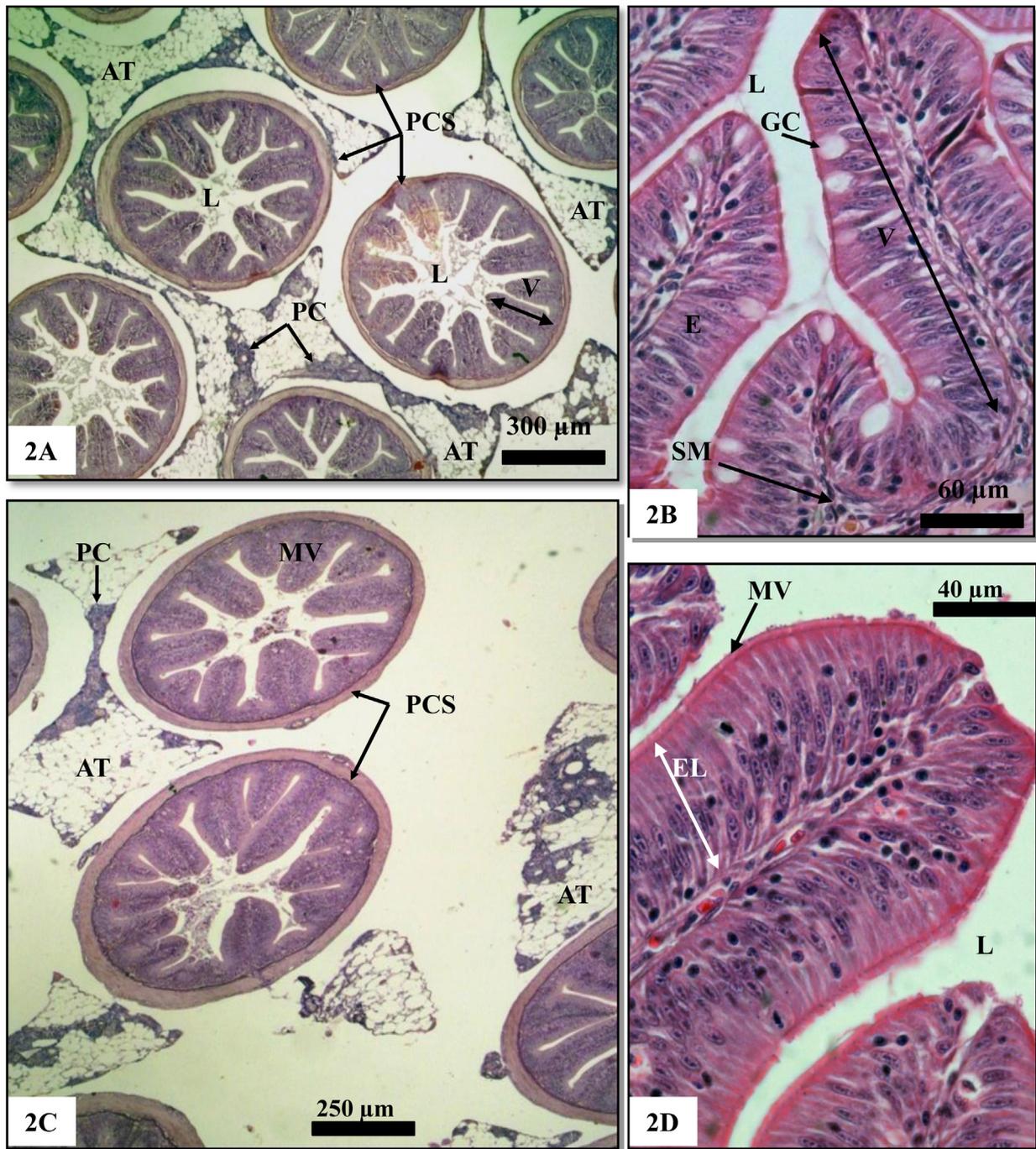


Fig. 2. Horizontal section of pyloric caeca in juvenile of *Salmo trutta caspius* (starved groups) (stained by H&E). (A and B) 6 weeks starved group (SSS), (B) Aggregation of goblet cell in top of the villus. (C and D) 3 weeks starved group (FS). AT, adipose tissue; E, enterocyte, EL, enterocyte layer; GC, goblet cells; L, lumen; MV, microvilli; PCS, pyloric caeca sections; PC, pancreatic cells; SM, submucosa; V, villi.

observed in other vertebrates (Hopper et al., 1972; Holt et al., 1988) and not clearly in fish. Current observations demonstrated that a reduction of intestinal cell generation is parallel with the rate of villus epithelial cell loss during starvation in juveniles of trout.

It was also observed that starvation would cause a significant reduction of enterocyte height in Caspian trout. Regarding to the effects of starvation on intestinal structure, the shortening of enterocytes is also reported in other studies on both juvenile and adult fish (Rios et al., 2004; Ostaszewska et al., 2005). The decrease in height of epithelial cells corresponded to reduced feeding levels, may result from a production of shorter cells (Hall and Bellwood, 1995) or through the reduction of lipid vacuoles in enterocytes,

being energy reserves turnover a first consequence of food restriction. These energy resources have an important role in energy provision when food is limited (Ince and Thorpe, 1976). Although the production of shorter cell was not clearly demonstrated in starved fish but the presence of lipid vacuoles within enterocytes has been reported for recently fed fish (Noaillac-Depeyre and Gas, 1974, 1979) and would support this hypothesis. Gisbert and Doroshov (2003) pointed that, at the onset of feeding, the epithelial cells are filled with large lipid deposits that gradually disappeared during low feeding. When lipid reserves were exhausted, the body tissues of starved fish were catabolized and food deprivation caused degeneration of digestive tract and accessory organs. Therefore,

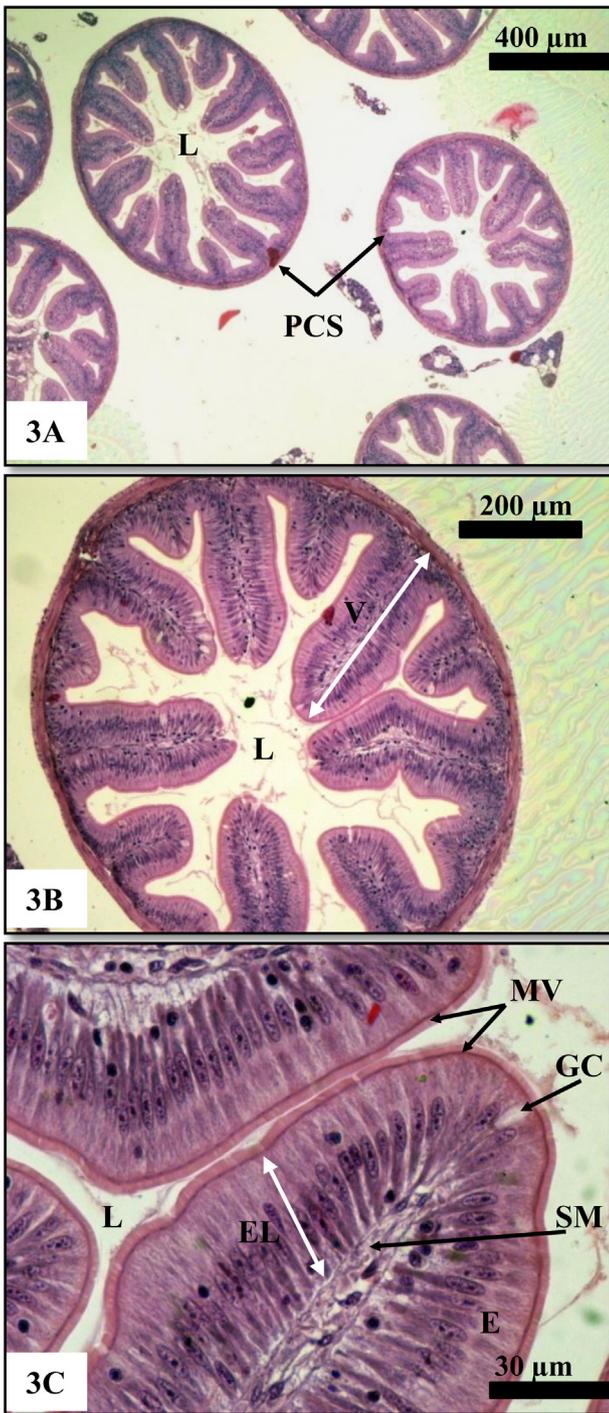


Fig. 3. Horizontal section of pyloric caeca in juvenile of *Salmo trutta* (refed groups = SF) (stained by H&E). (A and B) Short villus have been forming adjacent large ones and caused wide pyloric caeca lumen. (C) Enterocyte were partially ordered and not overlapping each others. E, enterocyte; EL, enterocyte layer; GC, goblet cells; L, lumen; MV, microvilli; PCS, pyloric caeca sections; SM, submucosa; V, villi.

enterocyte height could be a good reliable indicator of starvation (Theilacker, 1978; Theilacker and Watanabe, 1989; Theilacker et al., 1996; Gisbert and Doroshov, 2003; Rios et al., 2004; Ostaszewska et al., 2005).

Due to decreased numbers and heights of epithelial cells, corresponding reduction of villus length followed by reduction of epithelial area occurred in Caspian trout pyloric caeca. This change implies a reduction of the absorption surface area, particularly in

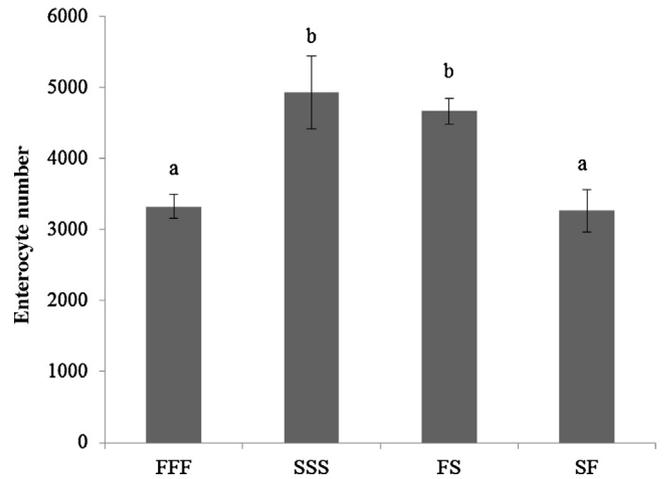


Fig. 4. Mean epithelium enterocyte number (per 1 mm²) in different feeding groups. Different letters indicate significant differences.

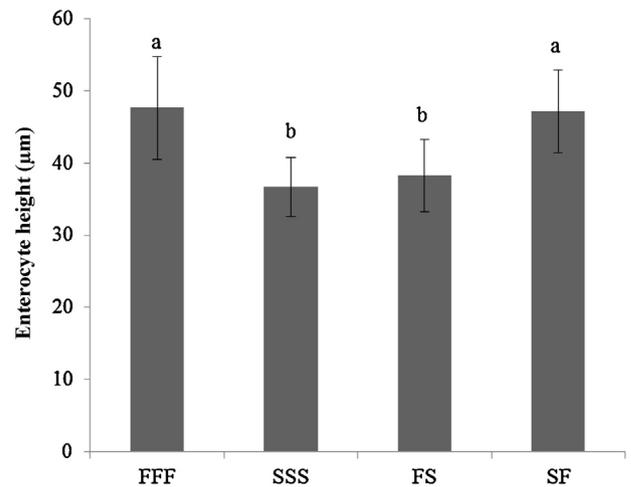


Fig. 5. Mean enterocyte height (±S.D.) in different feeding groups. Height of the epithelial cells showed significant differences due to starvation are indicated by different letters.

the pyloric caeca, which is the main site of nutrient absorption in salmonids. Reduction in gut size is often observed in fish in status of food deprivation (Collins and Anderson, 1995; Rios et al., 2004; Krogdahl and Bakke-McKellep, 2005). In our study pyloric caeca total area was decreased in 3 weeks starved groups and it was more

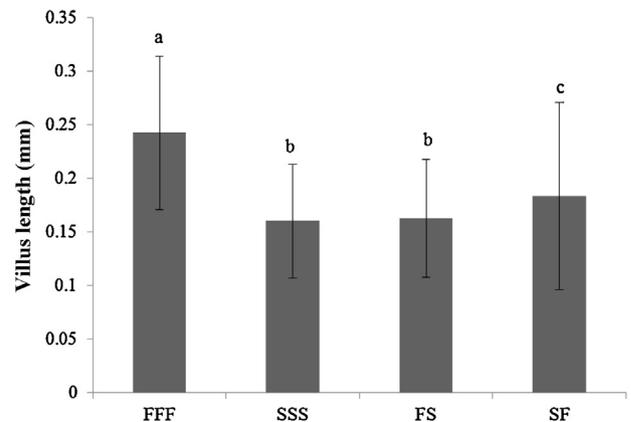


Fig. 6. Mean villus length of pyloric caeca in different feeding groups of Caspian salmon.

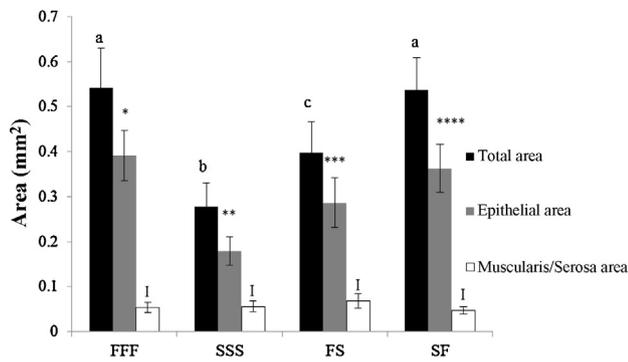


Fig. 7. Mean pyloric caeca total area, epithelial area and muscularis/serosa layer (\pm S.D.) in different feeding groups of Caspian salmon. Note the decreased epithelial layer and caeca total area in both starved groups (SSS, FS). The recovered area in sf group could explain the response to refeeding in trout juveniles. Significant differences due to starvation are indicated by different letters.

significant in 6 weeks starved groups. In previous studies, the size of caeca of starved *Pseudopleuronectes americanus* (McLeese and Moon, 1989) and the pyloric caeca thickness of *Hoplias malabaricus* (Rios et al., 2004) also decreased in response to food deprivation. Additionally in several teleosts the responses to starvation include the decline in the number of goblet cells (McLeese and Moon, 1989; Ba-Omar and Victor, 2000) which is not in complete agreement with current observations. In contemporary with epithelium atrophy in caeca of 6 weeks starved trout, the presence of numerous goblet cells among enterocytes was notable which suggests that gastric glands were still functional during starvation period. The same phenomena have been reported in pyloric region of food deprived larvae of *Acipenser medirostris* (Gisbert and Doroshov, 2003). The secretion of neutral and acidic mucosubstances, may serve to protect the digestive mucosa from auto-digestion during food deprivation (Gisbert et al., 1998).

Within 3 weeks refeeding, the villus cell numbers in refed trout returned to the same value as the control group. This could result from shortening of cell cycle time and their movement rate to villus, under steady-state conditions. The reduction of villus cell number was also reversed in refed rats (Holt et al., 1988). In addition, enterocytes also swell in size after 3 weeks feeding. Although enterocyte hypertrophy was not directly measured in fish, the presence of lipid droplet which was reported in refed fish could justify this phenomenon. However it must be noted that similar results have been reported in other vertebrates. Starck and Beese (2001), observed the incorporation of lipids into the intestinal tissue of small intestine soon after refeeding, in the refed Burmese python.

Regarding to current observation, the entrance of lipid droplets to intestinal lumen of *S. trutta caspius* lead us to suppose enterocytes hypertrophy to result from lipid droplet accumulation. Fish, reptile and mammals, although distinct phylogenetically and ecologically, could resemble each others in their physiological and anatomical responses to feeding with some little differences.

In the case of trout pyloric caeca structure regeneration, the responses to refeeding of the examined fish were documented by increase not only in enterocyte size and number, but also in villus length and epithelial area, which could result in both increment of intestinal cell number together with enterocyte hypertrophy. The epithelial surface enlargement as a reflex to nutrient entrance to intestinal lumen was anticipated by refeeding. Feeding obviously reverses the starvation response in Caspian trout caeca as the pyloric caeca total size was recovered and enlarged in refed *S. trutta caspius*. The size of caeca of starved *H. malabaricus* also recovered even after 240 days starvation (Rios et al., 2004). The expected metabolic response of refed fish involves the rapid growth of many organs that have atrophied during the fasting period of

low metabolic activity. The first organs to respond with significant increases in size are intestine and associated pyloric caeca, the organs most immediately involved in digesting the nutrient. Despite the observed fact that the pyloric caeca area, enterocyte height and number recovered in refed group, in comparison to the starved groups, the epithelial total area and villus length did not reach to the same area as the control group by 3 weeks refeeding. According to the observation, this period of refeeding, is insufficient to lead to full recovery and activation of intestinal epithelium in trout after 3 weeks starvation.

Based on obtained results, the histological changes of digestive system in *S. trutta caspius* follows the similar patterns reported for other malnourished fish. Both periods of starvation caused substantial rapid decreases in enterocyte numbers and height, villus length, epithelial surface and in pyloric caeca size. Furthermore, re-feeding after starvation could be effective on healing tissue damage. However it must be noted that these physical and structural changes might face released juveniles to some problems in natural habitat during starvation period. Thus, enterocyte atrophy could affect their efficiency in absorption nutrients and also impair functional role in osmoregulation. On the other hand reduced feeding can also influence swimming abilities and less success in searching and capturing prey (Margulies, 1993). Studies on starvation and refeeding period in fish are over whelming. However more information is required about cultured juvenile condition after releasing, to produce more practical feeding treatments appropriate to the digestive ability of Caspian trout.

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