

Original Article

Hormonal failure and osmoregulatory disruption in laboratory food-deprived Caspian kutum, *Rutilus frisii* larvae during brackish water challenge

Mohammad Mohiseni*

Department of Environmental Science and Fisheries, Lorestan University, Khorramabad, Iran.

Abstract: Caspian Kutum, *Rutilus frisii*, is a valuable species in the Caspian Sea basin. With the aim of restocking, the Iranian Fisheries Organization (IFO) annually released millions of Caspian kutum larvae into the estuaries of the Caspian Sea. This study was conducted to evaluate the effects of starvation on fish during Caspian seawater (CSW) adaptation. Caspian kutum larvae (0.5 ± 0.1 g) were divided into two groups; one was considered a control fed *ad libitum* during the experiment and another group was left food deprived. Both groups were continuously exposed to the CSW challenge (13 ppt) for 7 days and sampling was done on the second, third, fourth and seventh days after initiation of the CSW challenge. Different physiological factors, including hormones (cortisol, T3, and T4), gill Na^+/K^+ -ATPase activity, whole body glucose and protein, gill protein, body moisture, and seawater preferences, were analyzed in each sampling time. The results showed that although fed larvae can successfully overcome the physiological changes imposed by the CSW challenge, the starved fish indicated significant failures in the most measured parameters and eventually demonstrated significantly lower salinity preferences. Therefore, it can be concluded that starvation may negatively affect the success of CSW adaptation. Since physiological impairment during the CSW adaptation period is directly related to the effectiveness of the restocking program, more studies about the feeding condition of Caspian kutum larvae pre and post-release and the nutritional status of recipient rivers are suggested.

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Introduction

Rutilus frisii (Kamensky, 1901) is an important commercial fish in Iran, with a wide distribution from north to south, and its main population is on the southern coast of the Caspian Sea (Ebrahimi and Ouraji, 2012; Hasanpour et al., 2016; Eagderi et al., 2022). Having a migratory anadromous habit, Caspian kutum migrates to the rivers and lagoons of the southern Caspian Sea for spawning. The spawning season of Caspian kutum is from late March to mid-May. During reproduction, they spawn on aquatic weeds, gravel, and sandy substrates (Bastami et al., 2012). Because of the severe decline in the annual catch of this species during the 1970s and 1980s due to the demolition of their natural spawning substrates, overfishing, and other factors, IFO launched a restocking project in 1984 (Salehi, 2008). In the restocking centers, larval rearing is performed in the earthen ponds until a releasing weight is around 1 g. Then,

the fingerlings are released into the rivers that carry them toward the Caspian Sea (Jafari et al., 2009).

During downstream migration, changes in morphology and physiology will occur to successfully prepare the fish for seawater entry (Lerner et al., 2007). This transformation is driven by a change in environmental factors and mediated by significant alteration in specific hormones, including thyroid hormones, cortisol, growth hormone, and insulin-like growth factor I (McCormick, 2001). Cortisol has a critical role in hypoosmoregulatory capacity through the development and proliferation of gill chloride cells (mitochondria-rich cells) and upregulating of Na^+/K^+ -ATPase (NKA) expression (Madsen et al., 1995). The activation of these processes implies an increased energy requirement that eventually can alter gill energy metabolism and whole organism energy partitioning. Integral in the seawater adaptation is a reduction in

*Correspondence: Mohammad Mohiseni
E-mail: mohiseni.m@lu.ac.ir

glycogen supply, change in body lipids, and depletion of energy stores. This will make the juveniles vulnerable to starvation during the early phase of seawater adaptation (Stefansson et al., 2009). Starvation is known to affect hypo-osmoregulatory ability in many fish species, including rainbow trout (Jürss et al., 1983), Mozambique tilapia (Jürss et al., 1984), Arctic charr (Aas-Hansen et al., 2003), sea bream (Polakof et al., 2006) and Atlantic salmon (Stefansson et al., 2009).

There are several reports which investigated and described the important factors affecting the reproduction of the Caspian kutum broodstock (Nikoo et al., 2010; Shafiei Sabet et al., 2010), growth and production of fingerlings in the hatcheries (Afraei et al., 2010; Ouraji et al., 2011; Samarin et al., 2011). The effect of different salinities and fingerling sizes on the survival of the Caspian Kutum is also reported (Enayat Gholampoor et al., 2011; Hosseini et al., 2012). Despite the important role of normal and successful hypo-osmoregulation during the seawater adaptation in further survival and fitness of marine life stage, to the best of our knowledge, there is no information about the effects of starvation on salinity adaptation of Caspian kutum fingerling. The present study investigated the behavioral and physiological changes in the starved Caspian kutum larvae during Caspian seawater CSW challenges.

Material and Methods

The Caspian kutum larvae ($n=180$, 0.5 ± 0.1 g body weight) were provided from Shahid Rajaei, the center of fish reproduction (Sari, Iran) and transferred to the 300-L tank and maintained in normal condition (pH=7.3; temperature=18-20°C and oxygen around saturation level) for two weeks (Mohiseni et al., 2017). The larvae were fed 3% of their body weight three times a day on the starter diet for rainbow trout (BioMar, France). Daily water exchange during adaptation and also experimental periods was 30%. After the acclimation period, larvae were divided into fed and feed-deprived groups (with three replications), and both groups were transferred to the brackish water (13 ppt) simultaneously. The first group was fed *ad libitum* during the CSW challenge. The experimental salinity was made by mixing evaporated full-strength Caspian Seawater with dechlorinated tap water. The salinity challenge was done (randomly) for both groups for 7 days continuously, and sampling was done on the second, third, fourth, and seventh days after initiation of the CSW challenge.

The whole-body cortisol was measured according to Peterson and Booth (2010) with minor modifications. Briefly, Caspian kutum larvae were dried with a paper towel and weighed before extraction. The sample was then homogenized (Buch and Holm homogenizer, Denmark) in PBS, and aliquots of ethyl ether were added and vortex for 1 min. The samples were then centrifuged and frozen immediately at -20°C, and an unfrozen portion was transferred to a fresh tube for ethyl ether evaporation under nitrogen. The remaining extract was stored at -20°C until ready for Enzyme-linked immunosorbent assay (ELISA). Cortisol was measured with Monobind, a cortisol assay kit (USA). Thyroid hormone extraction was done based on Mukhi et al. (2005). Triiodothyronine (T3) and thyroxine (T4) were measured using Pishtazteb, ELISA assay kits (Iran).

Gill arches were collected for Gill Na^+/K^+ -ATPase (NKA) activity. It was determined following McCormick (1993) developed for microplates. The ouabain-sensitive hydrolysis of adenosine triphosphate is enzymatically coupled to the oxidation of nicotinamide adenine dinucleotide, which is directly measured in a microplate reader. Glucose was determined by enzymatic-colorimetric test (Moss and Henderson, 1999). Total protein concentrations were analyzed using the Bradford method with bovine serum albumin as standard (Bradford, 1976). To determine whole-body moisture, 5 larvae were dried with a paper towel and then weighed (based on mg) precisely. Afterward, the larvae were dried until full dehydration at 60°C (approximately 72 h) and then weighed. The difference between wet and dried mass was considered whole-body moisture and reported as a percentage (Moustakas et al., 2004).

The seawater (SW) preferences were evaluated based on Lerner et al. (2007). Briefly, the SW preference tank was constructed as two parallel chambers of Styrofoam connected by a PVC bridge. Two chambers were filled with Freshwater (FW) and CSW (13 ppt) separately. Each chamber was filled just below the bridge. 10 fish from both groups were transferred into the FW chamber and allowed to acclimate for 2 h. The level of FW was then elevated until the connection between the two chambers was formed. Afterward, the fish activity was videotaped from above the chambers for 1 h. The videotape was analyzed for the presence of fish in CSW at 30 s intervals for 45 min and presented at the percent of fish in CSW. Trials were conducted in three replications from each experimental group.

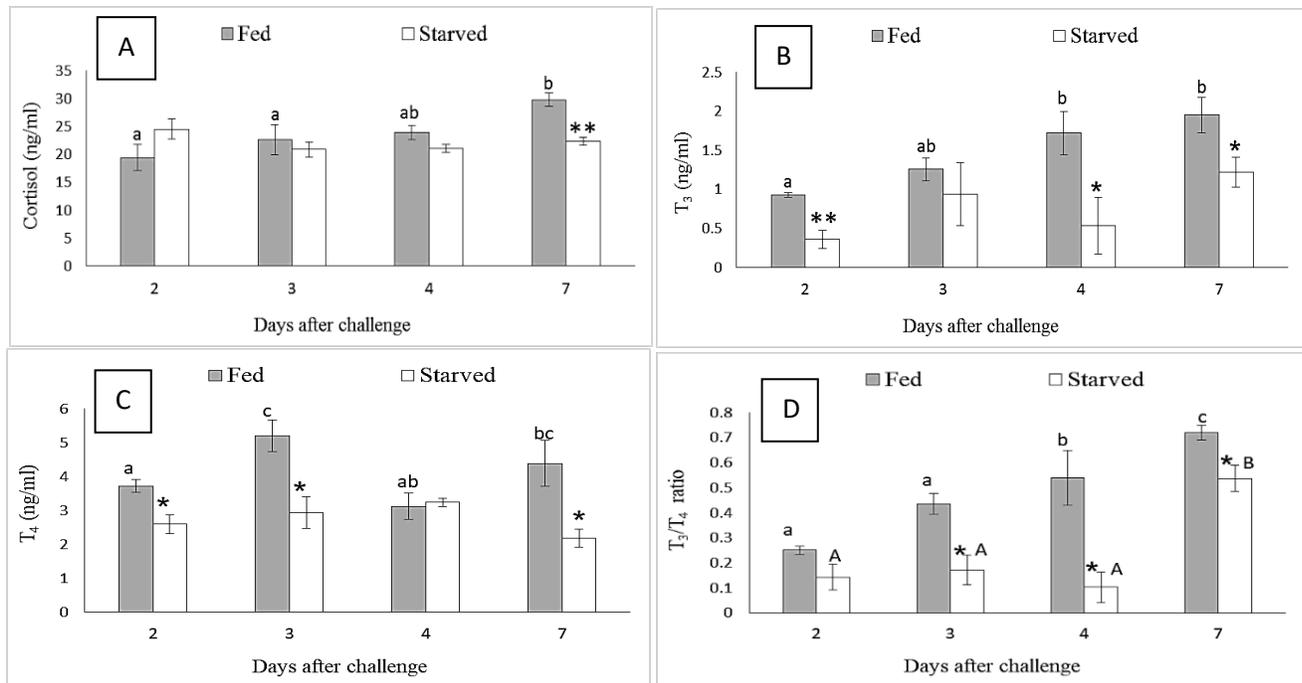


Figure 1. Hormonal change (A: Cortisol, B: T₃, C: T₄ and D: T₃/T₄ ratio) during seawater challenge in fed and starved Caspian kutum. (*) and (**) show a significant difference between fed and starved fish at the same time at $P<0.05$ and $P<0.01$, respectively. Dissimilar small letters show the differences among different times for Fed and dissimilar capital letters show differences among different times for the Starved groups ($P<0.05$).

All datasets were statistically analyzed by Independent Sample t-test. Pearson correlation was also used to determine correlated factors. All statistical analyses were performed using IBM SPSS Statistics for Windows (Version 19) at the significance level of $P<0.05$. The results were reported as mean \pm SE.

Results

Hormonal changes in both fed and starved groups are illustrated in Figure 1. Cortisol levels tend to increase over time and reach its maximum level at the end of the experiment in fed fish, while the pattern of the hormonal change in starved fish showed a significant reduction 7 days after the salinity challenge ($P<0.05$). Similarly, T₃ was also increased during the salinity challenge in fed fish and showed a significant difference with starved fish most of the time ($P<0.05$). The level of T₄ in starved fish remained almost unchanged during the experiment and showed significant reduction at 2, 3, and 7 days after the challenge ($P<0.05$). T₃/T₄ ratio was consistently higher in fed treatment throughout the experiment, with significant differences on 3, 4, and 7 days after the challenge ($P<0.05$).

Gill NKA activity was altered in response to the salinity challenge in both groups. The enzyme activity was consistently increased for fed fish over time, despite

the transient elevation of enzyme level in starved fish until the 4th day. The enzyme activity dropped significantly at the end of the experiment (Table 1). A similar trend was observed for glucose in both treatments, with a recorded significant decrease at 2, 4, and 7 days of salinity challenge for the starved group ($P<0.05$). Although the whole-body protein was approximately unchanged in starved fish, the recorded values for the fed group indicated a slight increment and were higher than the starved group all the time, with a significant difference on the 7th day ($P<0.05$).

The gill protein was elevated to the maximum level in both groups 3 days after the challenge, but the recorded value for fed was higher ($P<0.05$). The gill protein decreased in both groups, but the reduction rate for starved fish was significantly higher than for fed fish ($P<0.05$). The whole-body moisture in the starved group tended to decrease over time, but it remained largely stable in fed fish throughout the experiment, resulting in these fish having significantly higher levels on 3, 4, and 7 days after the challenge than those in starved fish ($P<0.05$).

The starved fish were first observed in seawater immediately after the formation of the aqueous bridge, but with time advancement, the percentage of fish in seawater significantly decreased (about 92%) and

Table 1. Gill and whole-body parameters change after different times of salinity challenge in fed and starved Caspian kutum.

Factor	Time (day)	Fed	Starved	P-value
Gill Na ⁺ K ⁺ ATPase (NKA) (μmol ADP/mgPr/h)	2	26.97±3.02 a	27.99±3.42	0.834
	3	43.33±3.55 a	33.32±1.51	0.06
	4	43.41±4.84 a	41.94±5.72	0.854
	7	70.88±10.85 b	38.61±2.06	0.043*
Whole body Glucose (mg/dl)	2	72.95±4.75	56.93±2.26 B	0.048*
	3	81.5±26.91	41.21±13.3 AB	0.15
	4	105.01±16.06	57.9±9.98 AB	0.03*
	7	108.88±0.71	39.89±17.31 A	0.025*
Whole body protein (mg/dl)	2	3.08±0.17 a	3.11±0.13	0.88
	3	3.84±0.97 a	2.89±0.11	0.39
	4	5.04±0.38 b	3.67±0.42	0.072
	7	4.69±0.57 ab	3.03±0.11	0.047*
Gill protein (mg/dl)	2	2.19±0.21	1.61±0.08 A	0.062
	3	3.34±0.24	2.17±0.17 B	0.018*
	4	2.87±0.16	1.48±0.08 A	0.002**
	7	2.84±0.13	1.21±0.07 A	0.033*
Whole body moisture (%)	2	69.53±8.02	62.14±2.76 B	0.433
	3	66.93±2.05	53.13±1.51 A	0.006**
	4	73.28±2.65	60.99±1.85 B	0.046*
	7	70.98±2.95	52.33±0.97 A	0.004**

Values are means±standard error; (*) shows a significant difference between two groups at $P<0.05$ and (**) shows a significant difference at $P<0.01$. Dissimilar small letters show the differences among different times for Fed and dissimilar capital letters show differences among different times for the Starved groups ($P<0.05$).

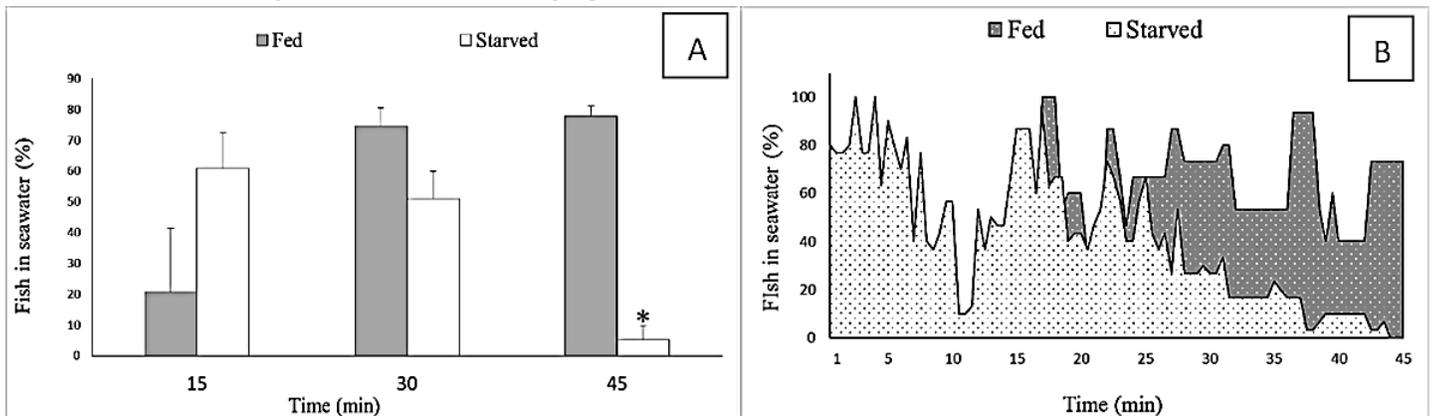


Figure 2. Seawater preferences quantity (A) and pattern (B) for fed and starved fish during seawater challenge. (*) indicate significant differences from the fed group at the same time.

reached the minimum (5.22%) at the end of the experiment (Fig. 2A, B). On the other hand, fed fish displayed obvious latency (about 12.5 min) to enter CSW, but their CSW preference behavior was increased until 30 min and remained stable toward 45 min. Furthermore, the results exhibited that the most measured osmoregulatory parameters were significantly correlated ($P<0.05$) in fed fish, whereas a few correlated parameters were found for starved fish (Table 2).

Discussion

This study revealed that starvation may lead to endocrine

disruption in the CSW adaptation period in Caspian kutum. Cortisol is a mineralocorticoid hormone in teleost and has a direct role in osmoregulatory change and seawater adaptation in anadromous, downstream migratory juvenile fish (McCormick, 2001; Nemova et al., 2021). Cortisol is also participating in energy-providing through glycogenolysis for normal metabolism and stress response (Laiz-Carrión et al., 2002). In the current study, whole-body cortisol levels significantly increased over time in fed fish. A prolonged cortisol increase is reported during smolting and successful seawater adaptation in anadromous fish (McCormick,

Table 2. Pearson correlation among different osmoregulatory factors during seawater challenge in fed and starved Caspian kutum.

		Cortisol	T ₃	T ₄	T ₃ /T ₄	Na ⁺ K ⁺	Glucose	Body	Gill	Body	Salinity
Cortisol	Fed	1	.663*	.177	.764**	.675*	.626*	.319	-.330	.719**	.178
	Starved	1	-.019	-.328	.105	-.186	-.514	-.004	-.354	.489	.327
T ₃	Fed	.663*	1	.101	.857**	.673*	.430	.764**	.034	.696*	.718*
	Starved	-.019	1	.317	.817**	.052	-.155	-.194	.063	-.502	.100
T ₄	Fed	.177	.101	1	.043	.128	.213	.094	.819**	.312	.436
	Starved	-.328	.317	1	-.112	.223	.587*	.223	-.147	-.179	-.187
T ₃ /T ₄	Fed	.764**	.857**	.043	1	.839**	.488	.487	.004	.685*	.697*
	Starved	.105	.817**	-.112	1	.085	-.336	-.270	-.070	-.421	.217
Na ⁺ K ⁺ ATPase	Fed	.675*	.673*	.128	.839**	1	.372	.366	.013	.477	.818**
	Starved	-.186	.052	.223	.085	1	.370	.510	-.108	.058	-.508
Glucose	Fed	.626*	.430	.213	.488	.372	1	.131	-.032	.295	.004
	Starved	-.514	-.155	.587*	-.336	.370	1	.331	-.281	-.024	-.597
Body protein	Fed	.319	.764**	.094	.487	.366	.131	1	.176	.486	.691*
	Starved	-.004	-.194	.223	-.270	.510	.331	1	-.321	.313	-.445
Gill protein	Fed	-.330	.034	.819**	.004	.013	-.032	.176	1	-.182	.800**
	Starved	-.354	.063	-.147	-.070	-.108	-.281	-.321	1	-.249	.197
Body moisture	Fed	.719**	.696*	.312	.685*	.477	.295	.486	-.182	1	.674*
	Starved	.489	-.502	-.179	-.421	.058	-.024	.313	-.249	1	-.313
Salinity preference	Fed	.178	.718*	.436	.697*	.818**	.004	.691*	.800**	.674*	1
	Starved	.327	.100	-.187	.217	-.508	-.597	-.445	.197	-.313	1

*. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed).

2001; McCormick et al., 2005; Lerner et al., 2007; Mancera and McCormick, 2019). Cortisol also has a promotive effect on the development and proliferation of gill chloride cells, which is directly connected to an increase in gill NKA activity (Madsen et al., 1995; McCormick et al., 2008). The activation of this process involves an elevation in energy requirement that apparently could alter the gill energy metabolism (Laiz-Carrión et al., 2005). There was a significantly positive correlation between cortisol levels and NKA activity in the fed group. Therefore, the significant decrease in cortisol levels due to starvation may explain this study's significant reduction in gill NKA activity.

Thyroid hormones failed to increase during the CSW challenge in the starved Caspian kutum fish. Thyroxine (T₄) and triiodothyronine (T₃) are the principal thyroid hormones involved in the development and growth of fishes (Peter and Peter, 2009). T₄ is the main secreted prohormone by the thyroid gland in teleost. Further enzymatic outer ring deiodination will transform the less potent T₄ into the bioactive thyroid hormone (T₃). In fishes, thyroid hormones have a fundamental role in different physiological processes, including somatic growth, metamorphosis, parr-smolt transformation, bioenergetics, and reproduction (Arjona et al., 2010). Several studies have shown that thyroid hormones

regulate basal and active metabolic rates in different tissues of teleost (Narayansingh and Eales, 1975; Pavlidis et al., 1997; Aas-Hansen et al., 2003; López-Bojórquez et al., 2007; Jarque and Piña, 2014; Tovo-Neto et al., 2018; Deal and Volkoff, 2020). Several studies have also reported the involvement of thyroid hormones during salinity acclimation. Prolonged T₄ treatment led to an increase in the number of chloride cells and gill NKA activity in Atlantic salmon (Madsen and Korsgaard, 1989). Physiological levels of T₃ and T₄ have also increased chloride cell size and gill NKA activity in Mozambique tilapia (Peter et al., 2000). Accordingly, we found a significant positive correlation between NKA activity and T₃ levels only for the fed group.

The liver metabolism may be enhanced during CSW adaptation because of its direct involvement in glycogen/glucose turnover in fish. This process will make the glucose available to provide energy requirements for the osmoregulatory phenomenon in different tissues, especially the gill and kidney (Sangiao-Alvarellos et al., 2003). Food deprivation resulted in changes in hepatic energy metabolism, as reported in several teleosts (Sangiao-Alvarellos et al., 2005; Stefansson et al., 2009; Costas et al., 2011). These included: (1) elevation of glycogenolysis and gluconeogenesis rate that can be attributed to the increased plasma cortisol concentration,

(2) increased liver capacity for glucose transferring, and (3) decreased concentration of the plasma triglyceride and protein. Cortisol is one of the most important factors during salinity adaptation and has a special role in chloride cell proliferation and development. Chloride cells are the main site of NKA that makes a driving force for monovalent ion secretion via gill arcs (Madsen et al., 1995; Evans et al., 2005). According to the results, the cortisol and NKA levels were increased a time-dependent and correlated manner, whereas both factors finally failed to surge in the fasting group during the seawater challenge i.e. gluconeogenesis is the common role of cortisol action in the liver. Cortisol is responsible for glycogen fraction and glucose production. Accordingly, we found a significant correlation between body cortisol and glucose of Caspian kutum fish only in the fed group.

Osmoregulatory adaptation during the CSW challenge is one of the most energy-consuming processes in aquatic animals (Madsen et al., 2015). Various tissues prefer specific energy resources to overcome the major changes due to the CSW challenges. The excess energy requirement of the liver and brain is mainly based on carbohydrates, while amino acids and lactate are more important in the gills and kidneys. Therefore, a significant decrease in the whole body and gills protein content in food-deprived fish in the current study was in agreement with the previous studies (Sangiao-Alvarellos et al., 2005; Polakof et al., 2006). On the other hand, the protein content of the body and gills of fed larvae remained approximately unchanged during different times after the CSW challenge.

During the first hours after CSW challenges, the drinking rate is elevated constantly due to the water loss via gills epithelia. In this process, the water is absorbed from the intestine along with excess divalent ion secretions. Excess monovalent ions will further be secreted via gills through the efficient actions of chloride cells. This process keeps the hydromineral balance and blood osmolality within the normal range (Marshall and Bryson, 1998; Webb et al., 2001; Grosell, 2010). It seems that fed larvae successfully kept their body moisture during the CSW adapting periods while starved larvae, probably due to the failures of different osmoregulatory factors, would not be able to ameliorate water loss during the salinity challenge. The body moisture was, therefore, constantly decreased over time and reached the lowest level at the end of the experiment.

Voluntary movement into CSW is a complete

organism response dependent on the exact integration between physiological and developmental cues. The external environment can alter the timing and quality of response (Lerner et al., 2007). Based on the results, there was a behavioral instability in starved larvae, they were observed in CSW immediately after the formation of the aqueous bridge, but their presence in CSW was subsequently reduced and reached the minimum at the end of the recording. Fed larvae showed more stability in CSW entrance, and their presence in CSW gradually increased and reached the maximum. Disruption of CSW preference is probably related to the physiological failure of the starved larvae in CSW adaptation. Several studies have also emphasized on negative effects of food deprivation on the seawater adaptation phase for different species (Aas-Hansen et al., 2003; Taylor and Grosell, 2006; Stefansson et al., 2009; Costas et al., 2011).

Conclusion

This study showed that starving had potentially impaired effects on the osmoregulatory fitness of Caspian kutum larvae. Key factors related to the CSW adaptation and cortisol, NKA, and thyroid hormones failed due to starvation, and larvae could not overcome imposed changes during the CSW challenge. Since the Caspian kutum larvae were released mainly in the small size (0.5-1 g) by IFO through an annual restocking program, the feeding condition of larvae should be considered and monitored before and after their release into the rivers.

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