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Effects of stocking density on blood cortisol, glucose and cholesterol levels of beluga juveniles (*Huso huso* Linnaeus, 1758) cultured in subtropical climate

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Abstract
The influence of stocking density (1.5, 3 and 6 kg.m^{-2}) was investigated on biochemical plasma including: Cortisol, Glucose and Cholesterol level of Beluga juveniles, for a period of 8 weeks. The mean weight at the start of trial was 143±0.29 g. Concentration of cortisol, glucose and plasma Cholesterol at the start trial was 8.57±1.1 ng.ml^{-1}, 70.12±3.6 mg.dl^{-1}, and 46±17.5mg.dl^{-1} respectively. Cortisol and glucose concentrations showed significantly difference between treatments 3 and 6 kg.m^{-2} on 30th day of rearing, but on 60th day of rearing cortisol and glucose concentrations had no significant difference among treatments. Cholesterol level had significant difference at treatment 1.5 kgm^{-2} on 30th and 60th day of rearing as compared to treatment 3 and 6 kg.m^{-2} and control group, Cholesterol concentration had no significant difference among treatments 3 and 6 kg.m^{-2}. Results showed that rearing density had no a major effect on the growth, biochemical parameters of Beluga.

Keywords: cortisol, great sturgeon, stress, biochemical parameters, stocking density

1. Introduction
This sturgeon species [Beluga (*Huso huso*)] is one of the largest anadromous fish and is found in the Caspian Sea. Currently, the native wild distribution of this species is only found in the Ural River. In the Caspian Sea, at least three beluga populations have been identified by microsatellite technique (Pourkazemi, 2008) [24]. Great sturgeon is primarily because of the highest market value of caviar and the depletion, or in some cases decimation, of wild sturgeon stocks throughout the world (Birstein *et al*. 1997) [9]. This species is suitable for aquaculture because of fast growth, reproduction in captivity, and tolerance of unfavourable rearing conditions (Vlasenko, 1994 [35]; Rafatnezhad and Falahatkar, 2011) [29].
In most forms of environmental and aquaculture-related stress, the hypothalamic-pituitary-interrenal (HPI) axis in fishes is stimulated, leading to a hormonal response, notably the secretion of corticosteroids into circulation (Barton, 2002 [3]; Iwama *et al*., 2006) [13]. Cortisol synthesis and its release from interrenal cells (i.e. adrenal homologue) can take a few minutes, whereas the release of catecholamines from chromaffin cells via the sympathetic nervous system-chromaffin axis occurs very quickly (e.g. within seconds) (Mazeaud *et al*., 1977 [19]; Barton, 2002) [3]. Therefore, serum or plasma cortisol concentration is typically used as an indicator in chondrosteans, as it is in teleosts, for determining the degree of stress experienced by the fishes (Barton *et al*., 1998 [5], 2000 [4]; Bayunova *et al*., 2002 [7]; Lankford *et al*., 2003 [15], 2005 [16]; Wuertz *et al*., 2006 [38]; Webb *et al*., 2007) [36]. The immediate release of catecholamines during stress stimulates the conversion of stored glycogen to glucose via glycogenolysis, resulting in elevations in circulating concentrations of glucose (Mazeaud *et al*., 1977) [19]. Thus, the change in serum or plasma glucose also provides a useful indicator of stress in fishes (Wedemeyer *et al*., 1990) [37]. Genetic, developmental and environmental factors influence the magnitude and duration of stress responses (Barton and Iwama, 1991 [2]; Barton, 2002 [3]; Iwama *et al*., 2006) [13]. Prior experience with chronic stressors, such as poor water equality or high stocking density, can alter the stress response to acute disturbance (Pickering and Pottinger, 1987 [26]; Ruane *et al*., 2002 [32]; Barton *et al*., 2005 [6]; Falahatkar, Poursaeid, Shakoorian and Barton, 2009) [10]. Stocking density is a key factor in determining the productivity and profitability of commercial fish farms. Knowledge of current stocking

density practices is vital to judge the impact of the importance of any density limits on economic sustainability (Rafatnezhad *et al*., 2008) [28].

The responses examined in this study included evaluation of cortisol concentrations and plasma glucose is very important because, both of them are commonly used to assess the degree of acute stress experienced by fishes (Wedemeyer *et al*., 1990 [37]; Falahatkar *et al*., 2009) [10]. Cholesterol to communicate with built cell membrane, external layer of lipoproteins plasma and quality feeding. A study determined that tank area is better criterion as compared to tank volume, because of the benthic orientation of lake sturgeon (Rafatnezhad *et al*., 2008) [28] as in other sturgeons.

2. Materials and Methods

2.1. Experimental design and maintenance of fish

This study was performed at hantooshzadeh warm water fish rearing complex company, Dezful, khouzestan, Iran (32°,22'N; 48°,24'E) with the participation of South of Iran Aquaculture Research Center (SIARC), from March to May 2015. Using great sturgeon juveniles, (*Huso huso*), with an initial weight of 143±0.29 g (Mean ± S.E) which were artificially propagated at the same hatchery and adapted to an artificial diet. Fish were randomly distributed into 9 small pond reinforced concrete at three different densities including 1.5, 3 and 6 kgm^{-2} with three replicates. The dimensions of the rearing small pond were (2×1×1m), with a flow-through rate of 36.2±1.0 $L.min^{-1}$ (using water from the deep well). Water depth in each pond was 70 cm. The water supply of each pond was independent of the others. Before the initiation of the experiment, fish were acclimatized to experimental conditions for 2 weeks. Feed was offered four times (08:00, 12:00, 16:00 and 20:00 hours) daily using a commercial diet (BioMar, no.3Nersac, France; 50% crude protein, 18% crude fat, 10% ash, 1.3% fibre) for 8 weeks. Food was supplied as 1.5-2 percentage body weight (BW) daily, according to water temperature during the experimental period (Rafatnezhad and Falahatkar. 2011) [29]. Uneaten pellets were drained off and counted every day to calculate the total feed consumption per pond using a mean dry pellet weight. There was no fighting among fish for obtaining food.

2.2. Sampling protocol and blood analysis

In each pond, 30% of the fish were randomly sampled at the start of trial, 30th day and 60th day in end of the experimental period in order to evaluate serum biochemical parameters. Animals were not anesthetized before blood sampling, as anaesthesia has been considered as a stressful action and unsuitable for cortisol measurement (Barton *et al*., 1998 [5]; Papoutsoglou *et al*., 2006) [23]. Fish were quickly captured and 3-ml blood samples were taken from the caudal vein using a non-heparinized 5ml syringe (Trenzado *et al*., 2006) [34]. Blood samples were stored in ice and transferred to the laboratory where plasma was separated by centrifugation at 3000*g* for 10 minutes based on Rotllant *et al*, (2001) [30]. A 100-µl blood plasma sample from each specimen (67 number of blood plasma sample per sampling time on the first day and 30th day and 60th day) was transferred to an eppendorf tube and stored at -20 °C for next analyses. Cortisol was measured by radioimmunoassay (Rotllant *et al*., 2001) [30], glucose by enzymatic method (Weil *et al*., 2001) [39] and cholesterol using a commercial kit (Pars Azmoon Co. Ltd., Tehran, Iran) (Yousefi *et al*., 2011 [40]; Hasanalipour *et al*., 2013) [13].

2.3. Water quality analysis

Throughout the experiment, the following water quality parameters were measured daily and weekly from the out flow of each experimental concreted pond, pH and temperature were measured, daily by a pH-meter (HANNA-HI-83141, South Korea). Ammonia, Nitrite and Nitrate using a photo-meter (Model Pc22; Tintometer, GmbH, Dortmund, Germany). Dissolved oxygen (DO) was measured daily by an Oxymeter (HANNA-HI-9142, South Korea). Average water temperature (°C), DO, pH, NH_3, NO_2 and NO_3 in all treatments were 23.3±1.48 °C, 6.81±0.98 $mg.L^{-1}$, 8.06±0.09, 0.35±0.26 $mg.L^{-1}$, 1.07±0.66 $mg.L^{-1}$ and 14.66±2.64 $mg.L^{-1}$ respectively. No effect of the three stocking densities was found on NH_3 concentrations when a continuous water flow 36.2±1.0 $L.min^{-1}$ was maintained through the tanks.

2.4. *Statistical analysis*

Results were analysed by analysis of variance using one-way ANOVA, and comparisons among treatment means were made by Tukey's test as a *post-hoc* test using SPSS software (SPSS® version 19. Chicago, IL, USA). Statistical significance was accepted at the $P<0.05$ level. All data in the text are presented as Mean ± S.E.

3. Results

Changes in the average weight of great sturgeon (*Huso huso*) juveniles reared at different densities after 8 weeks of rearing, the mean weight was 527.27±6.82 g, 467.91±18.81 g and 431.02±24.25 g (Mean ± S.E) in densities 1.5, 3, and 6 $kg.m^{-2}$ respectively. And were no significantly difference among treatments ($P>0.05$).Concentrations of cortisol, glucose and Cholesterol serum at the start trial were 8.57±1.1 $ng.ml^{-1}$, 70.12±3.6 $mg.dl^{-1}$, 46±17.5 $mg.dl^{-1}$ respectively. The results of the present study showed that plasma Cortisol and glucose concentration were significantly different between treatments, only in 30th day of rearing at treatment $3kg.m^{-2}$($P<0.05$), but in 60th day had no significant difference among treatments-time ($P>0.05$). Cholesterol plasma level has significant difference at treatment 1.5 $kg.m^{-2}$ in 30th and 60th day of rearing as compared to densities 3 and 6 $kg.m^{-2}$($P<0.05$), but in treatments 3 and 6 $kg.m^{-2}$ Cholesterol concentration had no significant difference among treatments ($P>0.05$) (Table 1; Figures 1, 2, 3).

Table 1: concentration of cortisol, Cholesterol and glucose in plasma at great sturgeon juveniles in during culture (mean ± S.E)

Density ($kg.m^{-2}$)	Day	Cortisol ($ng.ml^{-1}$)	Glucose ($mg.dl^{-1}$)	Cholesterol ($mg.dl^{-1}$)
Control	0	$8.57±1.1^{ab}$	$70.12±3.6^{ab}$	$46±17.5^{a}$
1.5	30	$9.65±0.76^{ab}$	$73.77±2.93^{ab}$	$68±18.1^{b}$
3	30	$12.77±1.88^{a}$	$85.00±6.00^{a}$	$51±14^{a}$
6	30	$9.77±0.88^{b}$	$63.33±3.89^{b}$	$49±16^{a}$
1.5	60	$9.84±0.89^{ab}$	$75.66±3.65^{ab}$	$75±20.5^{b}$
3	60	$11.71±1.49^{ab}$	$70.77±3.24^{ab}$	$48±16.5^{a}$
6	60	$10.11±0.78^{ab}$	$66.55±1.97^{ab}$	$52±14.7^{a}$

Means bearing different superscripts in a column differ significantly ($P<0.05$).

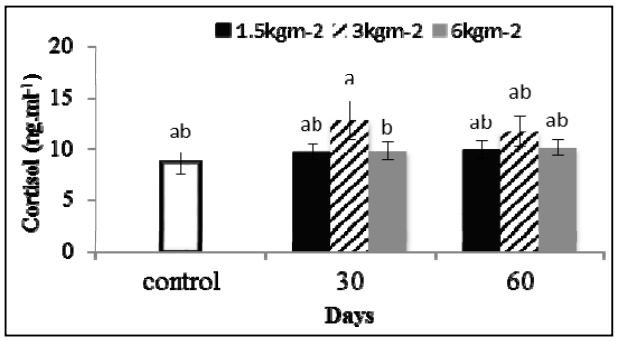

Fig 1: Changes of cortisol concentration (mean ± SE) of the blood plasma in great sturgeon juveniles

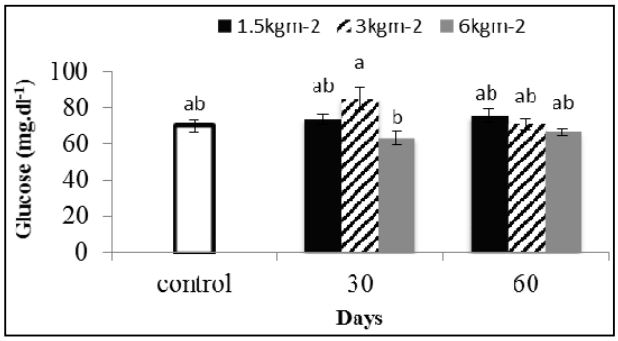

Fig 2: Changes of glucose concentration (mean ± SE) of the blood plasma in great sturgeon juveniles

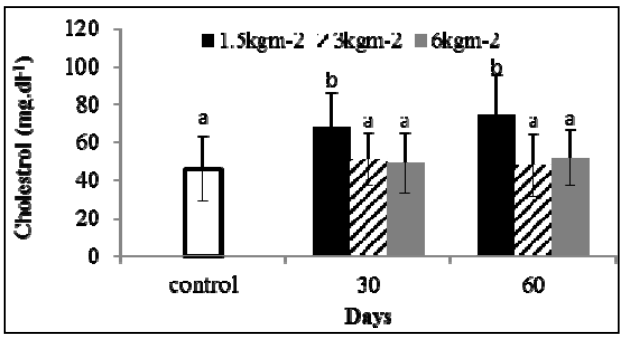

Fig 3: Changes of Cholesterol concentration (mean ± SE) of the blood plasma in great sturgeon juveniles

4. Discussion

During the present experiment, fish showed stocking density had no dramatically effect on plasma glucose, cortisol concentration and cholesterol level in great sturgeon (*Huso huso*) juveniles. Rafatnezhad, Falahatkar and Tolouei (2008) [28] have found that stocking density does not affect at plasma glucose and cortisol in great sturgeon juveniles (*Huso huso*) at densities (1-8kg.m^{-2}). Ruane, Carballo and Komen (2002) [32] have found that density does not affect at plasma glucose in common carp (*Cyprinus carpio*). Increased blood glucose is a characteristic response of fish subjected to an acute or chronic stressor.

In many studies, fishes reared at high densities had no significant difference in plasma cortisol level compared with those reared at lower densities (Kebus *et al*., 1992 [14]; Rotllant and Tort, 1997 [31]; Procarione *et al*., 1999 [27]; Martins da Rocha *et al*., 2004 [17]; Falahatkar *et al*., 2008) [10]. A previous exposure to a chronic stressor, such as low water quality or high stocking density, however, can alter the cortisol response to a subsequent acute disturbance (Pickering and Pottinger, 1989 [25]; Ruane *et al*., 2002 [32]; Barton *et al*., 2005) [6].

Bayunova *et al*. (2002) [7] have revealed that high loading density of stellate sturgeon (*Acipenser stellatus Pallas*) during holding in the transport tank showed increased cortisol and glucose concentrations. Similarly, Montero *et al*. (1999) [21] have found that high stocking density in gilthead seabream juveniles (*Sparus aurata*) resulted in plasma cortisol concentrations four-fold higher than those in fish held under LD ambient conditions. But in present study, Cortisol and glucose concentrations showed significantly difference only between treatments 3 and 6 kg.m^{-2} on 30th day of rearing, but on 60th day of rearing cortisol and glucose concentrations had no significant difference among treatments, and This comparison showed stressor factors in our experiment is the least rate.

Generally, but with some exceptions (Belanger *et al*., 2001 [8]; Bayunova *et al*., 2002 [7]; Lankford *et al*., 2005) [16], the corticosteroid response of chondrostean fishes to stress is relatively low in magnitude in comparison with those of teleosts (Barton *et al*., 1998 [5], 2000 [4]; Barton, 2002 [3]; Webb *et al*., 2007) [36], and this is further substantiated by these experiments. For example, Falahatkar *et al*., 2007 [10] have reported a small elevation in serum cortisol but significant from 10.8 to 14.6 ng.ml^{-1} when juvenile (*Huso huso*) were subjected to handling and confinement. In contrast, teleosts respond to a single acute stressor with cortisol elevations that can exceed 200 ng.ml^{-1} (Barton, 2002) [3] and to multiple and chronic stressors with cortisol responses much higher (Maule *et al*., 1988 [18]; Mazik *et al*., 1991 [20]; Noga *et al*., 1994) [22]. The reasons for low corticosteroid responses of some sturgeon species to stress compared with teleosts may result from a variety of factors. These include differences in neuroendocrine events that occur in the brain between sensory perception of the stressor and activation of the HPI axis, anatomical differences in interrenal tissue (adrenal homologue) and differences in interrenal sensitivity and physiological response capacity (Barton *et al*., 2000) [4].

In other investigation, Falahatkar *et al*., (2009) [10] in juvenile great sturgeon (*Huso huso*) to find out, concentration of plasma cortisol and glucose in high (HD) and low (LD) densities at 9 hour after handling, had no significant different among treatment and it was similar with present study.

Our results for cholesterol were similar of those of Asadi *et al*. (2009) [1] and Gessner *et al*. (2009) [11]. In the present study, mean values of cortisol, glucose and cholesterol in specimens that experienced moderate and high densities were comparable to those of other studies reared in low density or were well adapted to rearing condition, i.e. for cortisol and glucose (Barton, 2002 [3]; Rafatnezhad *et al*., 2008) [28], and for cholesterol (Shahsavani *et al*., 2010) [33], showing that animals were not stressed. Concentration cholesterol at investigation Hasanalipour *et al*. (2013) [12] in Siberian sturgeon (*Acipenser baerii*) was nearly two-fold as compared to present study. Therefore, a high concentration of blood cholesterol may suggest the dietary lipid imbalance and to be different fish species (Wedemeyer *et al*., 1990) [37]. At the end of the experiment, results showed unlike many other fish, great sturgeon (*Huso huso*) exhibited lower stress responses to high stocking density. This indicates the hardy nature of this species and its tolerability to rearing conditions in the high stocking densities in Torrid Zone.

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