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Research Article

Changes of Glucose and Cortisol during Stress in Fishes

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ABSTRACT

There is now extensive literature on the physiological and endocrine basis of stress in fish, largely constructed from studies of captive or cultured fish, and within this, largely examining the effects on teleosts fishes. The effects of stress resulting from aquaculture practices on fish and methods of minimizing such effects have received considerable attention through the years. Glucose is a carbohydrate that has a major role in the bioenergetics of animals, being transformed to chemical energy (ATP), which in turn can be expressed as mechanical energy. Cortisol is the principal glucocorticoid secreted by the inter-renal tissue (steroidogenic cells) located in the head-kidney of teleost fish. This hormone is released by the activation of the hypothalamus-pituitary-interrenal axis (HPI axis).

Key words: Salt stress, Glucose, Cortisol, Environment

INTRODUCTION

There is now extensive literature on the physiological and endocrine basis of stress in fish, largely constructed from studies of captive or cultured fish, and within this, largely examining the effects on teleosts fishes (see reviews by Barton and Iwama, 1991; Pickering, 1998; Sumpter, 1997; Wendelaar Bonga, 1997; Barton, 2002). Because of the potential difficulties associated with accessing and suitably sampling fish in their natural environment there is considerably less information on stress and its physiological and endocrine effects, in natural settings (Sharples, 1992).

Stress

The effects of stress resulting from aquaculture practices on fish and methods of minimizing such effects have received considerable attention through the years (wedemeyer, 1972; Mazeaud *et al.*, 1977; Nikinmaa *et al.*, 1983; Barton and Iwama, 1991; Mazik *et al.*, 1991; Cech *et al.*, 1996.). Stress induced by common practices such as handling, crowding, transport, or poor water conditions can increase the incidence of diseases and mortality, and is therefore an important factor affecting the economics of aquaculture.

Salt

Salt additives, particularly NaCl, can alleviate the severity of the stress response and improve survival during handling, transport and post-stress recovery in fish. The presence of salts apparently helps by reducing osmoregulatory dysfunction (Wedemeyer, 1972; Nikinmaa *et al.,* 1983; Carmichael *et al.*, 1984; Mazik *et al.,* 1991; Barton and Zitzow, 1995). Although the addition of NaCl to the water is usually beneficial, it did not improve survival during stocking and harvest of juvenile striped bass *Morone saxatilis* and *Morone* hybrid bass *Morone chrysops=M. saxatilis* (Grizzle *et al*., 1985). It also did not reduce the effects of capture and transport on fingerling rainbow trout *Salmo gairdneri* (Barton and Peter, 1982).. Therefore, the efficacy of salts to mitigate the effects of stress may vary between species and even stocks of the same species. It probably depends also on the severity of the stressor.

Glucose

Glucose is a carbohydrate that has a major role in the bioenergetics of animals, being transformed to chemical energy (ATP), which in turn can be expressed as mechanical energy (Lucas 1996). In suboptimum or stressful conditions (internal or external) the chromaffin cells release catecholamine hormones, adrenaline and noradrenaline toward blood circulation (Reid *et al.* 1998). Those stress hormones in conjunction with cortisol mobilize and elevate glucose production in fish through glucogenesis and glycogenolysis pathways (Iwama *et al.* 1999) to cope with the energy demand produced by the stressor for the "fight of flight" reaction. This glucose

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production is mostly mediated by the action of cortisol which stimulates liver gluconeogenesis and also halts peripheral sugar uptake (Wedemeyer *et al.* 1990). Glucose is then released (from liver and muscle) toward blood circulation and enters into cells through the insulin action (Nelson & Cox 2005). Regardless of the wide use of glucose as an indicator of stress, some authors (Mommsen *et al.* 1999, Flodmark *et al.* 2001) emphasized that care has to be taken when using plasma glucose as the only indicator. It has been reported that glucose content is a less precise indicator of stress than cortisol (Wedemeyer *et al.* 1990, Pottinger 1998). Mommsen *et al.* (1999) were skeptical about the use of glucose as a stress indicator, whereas Simontacchi *et al.* (2008) stated that glucose and cortisol "cannot be considered itself as reliable stress indicators". Factors that affect the intensity of response. Similar to cortisol, some factors can indirectly alter the response of glucose levels in blood. Vijayan & Moon (1994) suggests that "the rearing history including nutritional status may affect the stress response and glucose clearance rates". That affirmation is supported by other authors who concluded that blood glucose results have to be interpreted with care, taking into account extrinsic factors such as diet, life stage, time since last feeding and season of the year, etc., because they may affect liver glycogen stores (Nakano & Tomlinson 1967, Barton *et al.* 1988, McLeay 1977, Wedemeyer *et al.* 1990). Nutritional status is a factor that can have an effect in the glucose response. The intake of diets with different lipid and protein content resulted in different responses of blood glucose of the orangespotted Grouper (*Epinephelus coioides*) when it was exposed to cold stress (Cheng *et al.* 2006). The channel catfish under fasting conditions evidenced hyperglycemia after 30 days of experiment (22.8 versus 4.7 ng·ml-1 in the control group) (Peterson & Small 2004).

Hematocrit values

Decreased hematocrit values with increasing salinity, as observed, have been also reported for chinook salmon *Oncorhynchus tshawytscha* fry (Morgan and Iwama, 1991).

Stress responses in the natural environment

A primary question in the examination of environmental stress is whether animals in the wild typically experience stress over the normal range of activity and environmental conditions. The best field evidence is from studies on birds, and these suggest that probably they don't. Studies on free living populations show that quite harsh environmental conditions and the rigors of reproduction are not necessarily stressful if they are predictable (Wingfield, 1994). Increasing corticosteroid concentrations when conditions do become sufficiently adverse typically modulate behaviour (eg. a shift from nesting to foraging or even refuge seeking) both to ameliorate the stress but also to potentiate recovery and resumption of reproductive behaviour when conditions permit. Events capable of stimulating increases in plasma corticosterone levels include storms and extreme temperatures (Romero et al., 2000). However, even here the relationship may not be straightforward with Arctic passerines being able to cope with storm events during the breeding season but showing stress responses only during the more energetically demanding period of molting (Romero et al. 2000). This has lead to the view that events such as migration and reproduction are demanding but not necessarily stressful. Extreme events such as storms encapsulate an 'emergency life history stage' (ELHS) as a response to the unpredictable or extreme event. The ELHS is temporary and maximises survival chances through the associated stress response, only becoming maladaptive if the ELHS persists for too long (Wingfield and Ramenofsky, 1999). The phase of negative energy balance at which an ELHS is triggered is in turn thought likely to be a function of individual body condition. Cockrem et al. (2009) suggest on the basis of observed correlation between behaviour and stress responsiveness, that low responders (which tend to have proactive 'personalities') do best under predictable environmental conditions, whereas high responders (which tend to have reactive personalities) are best adapted for responding to unpredictable events. This is similar to the view expressed by Øverli et al., (2002) that a spread of stress responsiveness in fish populations allows for a range of adaptive or coping strategies in the face of environmental stress. In an examination of studies of 53 species (37 avian, 7 mammalian, 7, reptilian and 2 piscine) where some measure of fitness was correlated with basal corticosteroid concentrations, (Bonier *et al.*, 2009) concluded that there was not a consistent relationship between the two. This was despite a prediction based on the relationship in many species whereby basal corticosteroid levels, and fitness increase and decrease, respectively in the face of environmental challenge, that fitness would decline with increasing basal corticosteroid levels. This further emphasizes that the use of corticosteroid levels as predictors of relative fitness requires careful validation in relation to the particular species, population and situation (Bonier et al., 2009). The level to which natural populations of fish experience stress is difficult to gauge for two reasons. Firstly, there is a very limited number of field studies where free ranging fish have been sampled in ways that allow correlation with pre-capture behaviour and activity, and secondly, the events that might equate to extreme conditions in terrestrial systems (storms and floods) largely preclude sampling, or observation of behaviour at these times. A possible exception to this might be stress associated with reduced flow rates and, or water levels in riverine systems. A series of field studies on the tropical spiny damselfish *Acanthochromis polyacanthus* does offer some insight of the corticosteroid dynamics in free living fish. Plasma cortisol levels in territorial adult fish captured underwater and sampled immediately, ranged between <1 and 42 ng mL-1, and there was no apparent relationship between cortisol level and the time a diver had been in close proximity to the territory (Pankhurst, 2001). Neither was there any relationship between baseline cortisol levels and plasma levels of testosterone (T) and 17β-estradiol in females, or T and 11-ketotestosterone (11KT) in males, despite laboratory experiments showing stress-suppression of sex steroids in both sexes. Earlier studies showed that there was some variation in plasma cortisol levels with behaviour in females but not males, with highest cortisol levels occurring when fish were paired but not yet protecting broods (both sexes of spiny damselfish

tend the eggs and then juveniles for several months) (Pankhurst *et al*., 1999). Treatment of territorial adults with exogenous cortisol had no short-term effect on territorial or guarding behavior, but both control (saline-injected) and cortisol treated fish had become diver negative to the extent that recapture was not possible. Laboratory experiments indicated that the cortisol treatment probably elevated plasma levels to ~100 ng mL-1 (Pankhurst, 2001). Free ranging wild bluegill sunfish show elevated cortisol levels in males engaged in parental care (as noted earlier, up to 125 ng mL-1) but maintain regular cycles of plasma T and 11KT in association with spawning and egg protection (Magee *et al*., 2006).

Cortisol

Cortisol is the principal glucocorticoid secreted by the interrenal tissue (steroidogenic cells) located in the head-kidney of teleost fish (Iwama *et al.* 1999). This hormone is released by the activation of the hypothalamus-pituitary-interrenal axis (HPI axis) (Mommsen *et al.* 1999). When an organism undergoes stress conditions, the hypothalamus releases corticotropin-releasing factor (CRF) toward blood circulation. This polypeptide further stimulates secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland (Fryer & Lederis, 1986) which finally activates the release of cortisol by the interrenal tissue (Mommsen *et al.,* 1999). Cholesterol is the precursor of cortisol; this sterol is transformed to pregnenolone by the action of the enzyme P450 side chain cleavage (P450SCC) in the inner mitochondrial membrane. Then pregnenolone is further converted into 11- deoxycortisol by steroidogenic enzymes and this product is finally converted to cortisol by enzyme 11b-hydroxylase (Miller, 1998; Castillo *et al.,* 2008). The secretion of cortisol is slower than catecholamines, but its effects are more prolonged (Gamperl *et al.,* 1994a, b; Waring *et al.,* 1996), combining mineral and glucocorticoid actions to restore homeostasis (Wendelaar-Bonga, 1997; Maule *et al.,* 1993; Colombe *et al.,* 2000). Cortisol activates glycogenolysis and gluconeogenesis processes in fish; but also causes that chromaffin cells increase the release of catecholamines which further increase glycogenolysis and modulate cardiovascular and respiratory function (Reid *et al.,* 1992; Reid *et al.,* 1998). This whole process increases the substrate levels (glucose) to produce enough energy according with the demand.

MATERIALS AND METHODS

This article is review and the aims of Influence of frozen storage of fish on changes in lipids and fatty acids. In the experiment 1 was conducted by Erich et al (2003). Juvenile 0-group turbot (*Scophthalmus maximus* Rafinesque), which were the offspring of wildcaught parents of Norwegian origin, were obtained from Stolt Seafarm in Kvinesdal, Norway. The animals had an initial body mass approximately 10 g and were kept in 1-m2 square, grey, covered fiberglass tanks (400-l water volume, oxygen saturatio)80%, 62–63 fish per tank) at the High Technology Center in Bergen, Norway, with flowthrough seawater and a light regime of 18 h light and 6 h darkness (L:Ds18:6). Prior to the stress experiments described here, the fish were used in a 3-month growth study. The fish were reared at temperatures of 10"0.2, 18"0.3 and 22"0.2 8C (means"S.D.), and salinities of 15"0.4and 33.5"0.1‰ at each temperature. All treatment groups consisted of two replicate tanks. In this period, water flow was initially set to 4l ymin for all experimental tanks and increased gradually up to 10 lymin at the end of the experiment. Throughout the growth period the fish were left undisturbed apart from daily routine husbandry and monthly weightings to monitor the growth. During the growth period the fish were fed ad libitum with a commercial formulated feed (Supra Marin, Nor- Aqua AS, Bergen, Norway) containing 55% protein, 12% fat and 15% carbohydrate. Prior to the start of the stress experiment, prestress (PS) samples were taken from 8 fish at each temperature salinity combination. At the start of the experiment, all fish in one of each replicate tank were forced to swim actively in their tank for 10 min by chasing them manually with a dip net. At the end of this period all the fish had lost equilibrium and did not longer respond to the manual chasing. The fish from the other replicate tank of each temperature salinity combination were used as non-stressed controls. In line with studies, the amount of work done by the exercised fish was not quantified and this may have been influenced by temperature and or salinity. Instead, we have exercised turbot to a behavioral state of exhaustion at each temperature salinity combination, and examined response magnitudes and recovery of physiological disturbances. Samples were taken from both control (CO) and stressed (EX) fish at four subsequent time-points: *t*s0.5, 2, 5 and 24h (*n*s 6–8), in order to measure plasma cortisol, glucose, lactate, osmolality, Cly-ions, and at 10 and 22 8C also Naq and Kq-ions. Survival of the remaining fish was monitored until 4days post-stress. The animals were not fed 24h prior to and during the stress experiments. Fish were removed from the tanks and immediately killed with a blow to the head before sampling. Blood samples were collected from the caudodorsal blood vessels, just below the spine at the right (dorsal) side, with cooled 1-ml syringes flushed with heparin (Leo 5000 IE). Plasma samples were obtained by centrifugation of the blood for 10 min at 3000 rpm and 5 8C, and stored at y80 8C until further analysis. Plasma cortisol concentrations were measured with a specific rabbit- anti-cortisol antibody (Klinger, St. Albans, Herts., England). Radioactivity of the w3Hx-labeled cortisol tracer was quantified using a Wallac 1410 Liquid Scintillation Counter (Pharmacia). Plasma glucose and lactate were measured with commercial enzymatic assays from Sigma. Plasma osmolality levels were measured with an Osmomat 030 osmometer (Gonotech, Germany). Plasma Naqand Kq-levels were measured with an IL 943 flame photometer (Instrumentation Laboratories, Italy), whereas Cly-levels were measured with a CMT10 chloride meter (Radiometer, Denmark).

In the experiment 2 was conducted by Fakharzadeh (2011). Research was conducted using a 2 × 3 factorial experimental design (Table 1). Juvenile (2±0.6 g) of Persian sturgeon (A. Persicus) was obtained from the Shahid Beheshti Breeding Center (Rasht, Iran). Fish were then transferred to the University of Tehran and kept in a recirculating system with the average temperature of 19

°C and the minimum oxygen level of 6 ppm and a daily water change of 30%. After 72 h adaptation period, fish were transferred to the experimental tanks. Daphnia magna were fed with the emulsion of sheep manure with the daily rate of 10 ml per liter (the emulsion were prepared by adding 1 kg of clean sheep manure in 4 liter fresh water and then filtered) in a 400 liter tank. The temperature varied between 24 to 27 °C and the photoperiod was set at 14 h light/10 h dark. Rearing tanks were well aerated over the culture period (with a dissolved oxygen rate of 5±0.50 mg/liter). After blooming of daphnia in the rearing tanks, 12 polyethylene containers (1.5 liter) were used for enrichment procedure. Then 50 adult daphnia were cultured in each well aerated enrichment container for 24 h. In order to do so, 10 ml of manure emulsion was mixed with the total amount of hormone (Hydro cortisone sodium phosphate/ MERC) needed for the final enrichment concentrations. The daphnia were fed with the enrichment media four times per day within six hours intervals. Unenriched daphnia were only fed with sheep manure emulsion, without hormonal treatment. At the first series of tanks, bathing method was employed using three concentrations (3, 5 and 7 ppm) of resolved hydrocortisone hormone. In the second series of tanks, enriched daphnia with 3 enrichment hormone concentration of (3, 5 and 7 ppm) were employed. Then fish were daily fed by enriched daphnia up to 1.5-2% of their body weight. At the same time in the bath treatment tanks, unenriched daphnia were used to feed the fish with the same rate explained for the enriched daphnia treatment. At the end, the treated fish faced salinity stress (with concentration of 7 ppt) for 24 h and then the physiological responses and mortality rate of fish were measured. Three fish per tank were anesthetized using clove oil (1 ml diluted in 40 liters of water). Blood samples were taken via tail fin and a drop of EDTA solution was added to each sample to prevent coagulation. Blood sampling was taken as follows: before transferring fish to the experiment tanks, right after the hormonal treatments, 30 min after salinity stress, 60 min after salinity stress, and at the end of the experiment (24 h after the salinity stress). The mortality rate of fish was evaluated: exactly after stress, 12 h after stress, and 24 h after stress. Data were analyzed in two stages. At the first stage the effects of stress inducing method and hormonal concentrations was separately investigated and then the possible interactions between two main factors (type of hormonal treatment and hormonal concentration) were studied using two-way ANOVA. Duncan's test was employed to compare significant differences among treatments ($P<0.05$). In the second stage, each combination of hormonal treatment and hormonal concentration were considered as an individual treatment and then their mean were statistically considered through employing one-way ANOVA. SAS statistical program (version 9.0) was used for statistical analysis and EXCELL software was employed for making the graphs ($P<0.05$).

RESULTS AND DISCUSSION

In the experiment 1 was conducted by Erich et al (2003). No mortalities occurred at any temperature salinity combination during the experiment. Mean plasma cortisol values before stress (PS) ranged from 7 to 16 ngyml and were not significantly different between groups (*P*) 0.3, Fig. 1). Levels in CO-fish did not change significantly over time at any temperatureysalinity combination. In EX-fish, plasma cortisol increased transiently between 2 and 5 h at 10 8C-33.5‰. No significant changes were found at 10 8C-15‰ (Erich et al., 2003). At 18 and 22 8C, cortisol levels already increased at *t*s0.5 h, and declined between *t*s2 and 5 h. Similar trends were found at both salinities, but at 18 8C-33.5‰ changes were not significant. Statistical analyses for the interaction between temperature and salinity in EX-fish revealed significant temperature (*P*-0.0001) and salinity (*P*-0.05) effects at *t*s 0.5 h, an interaction effect at *t*s2 h (*P*-0.001), and temperature (*P*-0.0001) and interaction (*P*-0.05) effects at *t*s24h, reflecting the overall differences in cortisol responses at 10 8C compared to 18 and 22 8C.

Mean plasma glucose levels before stress (PS) ranged between 2.1 and 2.5 mmolyl and were not significantly different between groups (P) 0.5, Fig. 2) (Erich et al., 2003).. Levels in CO fish did not change signifi cantly over time at any temperatureysalinity combination. In EX-fish, plasma glucose increased significantly at 10 8C-33.5‰, whereas at 10 8C- 15‰ no significant changes occurred. At 18 8C, plasma glucose increased transiently at both salinities. Glucose levels in EX-fish at 22 8C showed similar patterns, with significant increases at *t*s 0.5 h, and at 15‰ also at *t*s2 h. Statistical analyses revealed a significant temperature effect on plasma glucose in EX-fish at *t*s0.5 h (*P*- 0.001).

Mean plasma lactate levels before stress (PS) were between 0.2 and 0.5 mmolyl and not significantly different between groups (P) 0.2,. Levels in CO fish did not change significantly over time at any temperature or salinity(Erich et al., 2003).. In EXfish at both salinities at 10 8C, levels were significantly higher at ts0.5 and 2 h. Similar but more pronounced elevations were found in EX-fish at both salinities at 18 8C, with higher peak levels at 15 than at 34‰. At 22 8C, the pattern of changes in plasma lactate of EX-fish was similar at both (Erich et al., 2003).

In the experiment 2 was conducted by Fakharzadeh (2011). The value of each parameter regarding the hormonal treatment method and concentration used for hormonal treatment is shown in Table 2. As shown in Table 2, there was no significant difference between daphnia enrichment and bathing methods, but the cortisol concentration was significantly higher ($P < 0.05$) in 3 ppm hormonal treatment (regardless the hormonal treatment method employed) as compared to the other treatments. In other words, significant differences were observed between different concentrations of hormonal treatments (Fakharzadeh (2011). No interaction was observed between the main factors (hormonal treatment method and concentration of hormone) in this regard. Blood cortisol level variations over the experiment are shown. As shown in, there was no significant difference between treatments 1 to 3 and treatments 4 to 6 after hormonal treatment. However, the highest cortisol level was observed in treatment 4. Blood cortisol level 30 min after hormonal treatment (Figure 2) was similar in treatments 1 to 3, however, the cortisol level in treatments 5 and 6 was significantly higher ($P<0.05$) than treatment 4 (Fakharzadeh (2011).

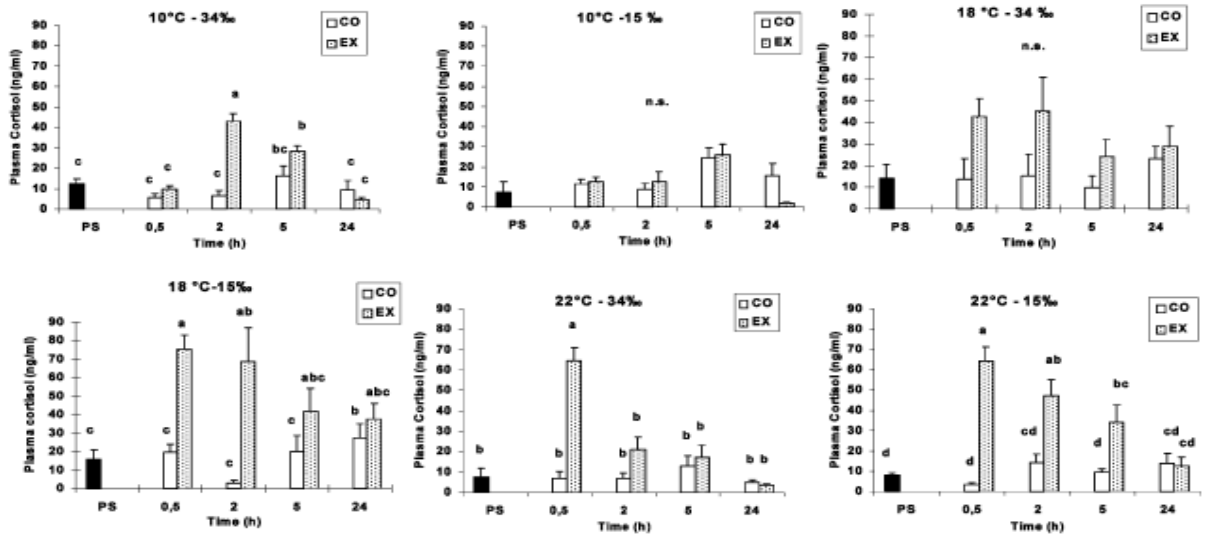

Fig. 1: Plasma cortisol concentrations (ngyml) before (PS) and after stress-treatment (exercise) in turbot reared at 10, 18 and 22 8C and 33.5 and 15‰. Mean values"S.E., *n*s6–8. CO, control fish; EX, exercised fish. Values with different letters are significantly different (P-0.05). n.s., no significant differences (Erich et al., 2003).

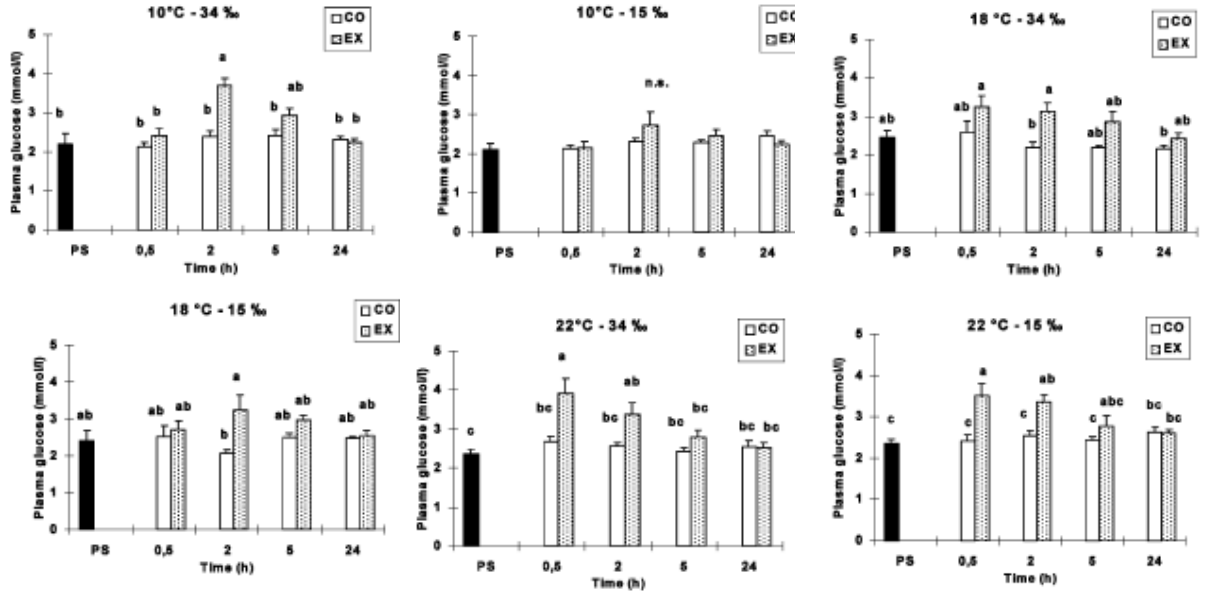

Fig. 2: Plasma glucose concentrations (mmolyl) before (PS) and after stress-treatment (exercise) in turbot reared at 10, 18 and 22 8C and 33.5 and 15‰. Mean values"S.E., ns6–8. CO, control fish; EX, exercised fish. Values with different letters are significantly different (P-0.05). n.s., no significant differences (Erich et al., 2003).

Table 1: Different treatments employed in the study (Fakharzadeh (2011)

Treatment	Treatment method	Hormonal concentration
1	Daphnia enrichment	3
2	Daphnia enrichment	5
3	Daphnia enrichment	7
4	Hormonal bathing	3
5	Hormonal bathing	5
6	Hormonal bathing	7

In overall, the cortisol level in enrichment method was significantly higher than bathing method in the similar concentration of hormone used. 60 min after the stress, cortisol level in treatments 1 to 3 was similar, while the cortisl level in treatment 4 was significantly higher (P<0.05) than treatments 5 and 6. In overall, the highest cortisol value among all treatments was observed in treatment 4. 24 h after the stress, cortisol level in treatments 1 to 3 was similar, however the cortisol value in treatments 4 to 6 was different and treatment 5 was significantly higher (P<0.05) than treatment 4 regarding this parameter (Fakharzadeh (2011). As shown in Table 2, there was no significant difference between daphnia enrichment and bathing methods, but the glucose level was significantly higher (P<0.05) in 3 ppm treatment compared to other treatments (regardless the hormonal treatment method used). Meanwhile, there was an interaction between the two main factors (treatment method and concentration of hormone). Results showed

Table 2: Comparison (mean±SD) of the parameters investigated over the experiment

Parameter	Daphnia enrichment method	Cortisol bathing method	3 ppm concentration	5 ppm concentration	7 ppm concentration	Interaction
Cortisol (ng/ml)	18.50±1623a	17.55±12.23a	20.01±11.8a	16.58±9.80b	17.57±12b.	P>0.05
Glucose (mmol/l)	3.21±0.24a	3.07±0.53a	3.30±0.3a	3.07±0.43b	3.05±0.30b	P<0.05
Hematocrit (%)	0.65±0.02a	0.53±0.01b	0.52±0.01a	0.53±0.02b	0.54±0.02b	P<0.05
Mortality (%)	0.17±0.28a	0.33±0.32a	0.37±0.2a	0.02±0.02b	0.37±0.32a	P<0.05

* Values in the same row with different superscript letters were significantly different (P < 0.05).

that the glucose level in treatment 5 was significantly higher (P<0.05) than treatment 4 at the end of the experiment. There was no sample available due to the high mortality rate observed in treatment 6 at the end of the experiment. The hematocrit values in different treatments are shown in Table 2. There was a significant difference between hormonal treatment methods and the level of hematocrit was significantly higher (P<0.05) in fish treated with daphnia enrichment method Fakharzadeh (2011).

The hematocrit value was significantly lower (P<0.05) in 3 ppm treatment compared to other treatments (regardless the hormonal treatment method used). Meanwhile, there was an interaction between the main factors (treatment method and concentration of hormone). As shown in Figure 6 the hematocrit value in treatment 3 was significantly higher (P<0.05) than other treatments at the end of the experiment. No hematocrit value could be reported due to the high mortality observed in treatment 6 at the end of the experiment.

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