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Stress responses of starry flounder, *Platichthys stellatus* (Pallas) following water temperature rise

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Abstract

Stress responses of starry flounder, *Platichthys stellatus* (Pallas) following water temperature rise were investigated to establish the influence of ambient temperature on this species. The physiological indicators of stress were plasma cortisol, glucose, aspartate aminotransferase, alanine aminotransferase, sodium, chloride, osmolality and triiodothyronine (T_3). No significant difference in plasma parameters were observed among the experimental groups of 15ºC, 18ºC and 21ºC. Level of plasma cortisol (49.0-95.0 ng ml^{-1}) and glucose (56.1-58.1 mg dl^{-1}) of starry flounders kept at 24ºC-27ºC were significantly higher than those (cortisol: 20.4-23.6 ng ml^{-1}, glucose: 40.6-47.1 mg dl^{-1}) observed in the 15ºC-21ºC groups. Changes in aspartate aminotransferase and alanine aminotransferase following water temperature rise showed a similar pattern to plasma cortisol and glucose. Starry flounders exposed to 27ºC exhibited higher plasma sodium (164.7 mmol l^{-1}), chloride (147.6 mmol l^{-1}), and osmolality (450.7 mOsm kg^{-1}) than those (sodium: 154.0-158.7 mmol l^{-1}, chloride: 139.1-140.4 mmol l^{-1}, osmolality: 375.1-383.8 mOsm kg^{-1}) fish exposed to 15-21ºC. Though plasma T_3 (29.4 ng ml^{-1}) of starry flounder increased at 24ºC, this hormone was significantly lower (19.3 ng ml^{-1}) in fish kept at 27ºC than those (24.6 ng ml^{-1}) the fish at 15ºC. This phenomenon seems to be directly associated with long-term fasting. Accordingly, the results suggested that starry flounders got stressed with osmoregulatory disturbances above 24ºC.

Key words

Platichthys stellatus, Starry flounder, Stress responses, Water temperature rise

Introduction

In aquaculture, it is well established that fish are stressed by stressors such as rapid water temperature changes, capture, handling, transport, confinement, grading or high stocking density (Iwama *et al*., 2006). Stress induced by stressors can increase the incidence of diseases and mortality, and is therefore an important factor affecting the economics of aquaculture (Tsuzuki *et al*., 2001). Understanding the stress response of fish is essential for optimal performance and production in aquaculture. Commercial benefits of minimising stress and thus optimizing growth and quality at harvesting would be considerable (Nolan *et al*., 1999). It is suggested that stress response of fish is characterized by disturbances in biochemistry and physiology (Barnett and Pankhurst, 1998). The perturbations resulting from stress are often classified as primary, secondary or tertiary. The primary stress response of fish results in activation of hypothalamus-pituitary-interrenal (HPT) axis, prompting secretion of cortisol into the blood (Wendelaar Bonga, 1997; Mommsen *et al*., 1999). Secondary responses include imbalance concerns blood ion levels, increase in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) by destruction of hepatocytes, kidney and heart, increase in cardiac impulse, increase in oxygen consumption and energy stimulus, *i.e.* increased blood glucose (McDonald and Milligan, 1997; Chang *et al*., 2007; Choi *et al*., 2007).

Thyroid hormones such as L-thyroxine (T_4) and triiodo-L-thyronine (T_3), are known to stimulate metabolism and growth in most vertebrates (Szischa *et al*., 2005) and plasma thyroid hormones increase during metamorphosis in flatfish (Inui *et al*., 1995) and smoltification of salmon (McCormick, 2001). In addition, T_3 and T_4 are secondary indices of stress and are influenced by cortisol level (Peter, 2011).

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The starry flounder, *Platichthys stellatus* (Pallas) of Pleuronectidae family is common flatfish distributed from East Asia, Okhotsk Sea to North Pacific Ocean, is also found in water with a wide range of salinities (coastal areas, bay, estuaries, blackish and occasionally freshwater lagoons). This suggests that starry flounder has strong tolerance against cold water and low salinity as marine euryhaline teleost (Lim *et al*., 2013). The distinctive features of starry flounder include combination of black and white-to-orange bar on dorsal and anal fins as well as skin covered with scales modified into tiny star-shaped plates or tubercles (thus both the common name and species epithet), resulting in a rough feel. The eye side is black to dark brown while the lower side is white or cream-coloured. Although classed as "right eye flounders" individuals may have their eyes on either right or left.

In recent years, increasing demand for starry flounder as food makes, it an important species for commercial aquaculture and for stock enhancement in South Korea. For this reason there has been continuous increase in aquaculture production of the flounder (from 17 ton in 2007 to 552 ton in 2010). With resistance to low temperatures, starry flounder is commonly cultured in land-based farming tanks (flow-through) in the East coast of Korea with low water temperature. However, in summer high water temperature can reduce its resistance to pathogens, increasing disease susceptibility and thus affecting its growth and survival.

The physiological responses of starry flounder to hypoosmotic media have been studied recently (Kim *et al*., 2009; Min *et al*., 2009; Lim *et al*., 2013), but such responses to water temperature have not been investigated. Therefore, the present study was carried out to examine the effect of water temperature rise on circulating cortisol as primary stress response as well as on levels of glucose, AST, ALT, ions and osmolality as secondary stress responses with the aim to obtain fundamental data for starry flounder aquaculture. It also examined how the stressor affected plasma T_3 concentrations.

Materials and Methods

Starry flounders (average weight: 267.6±20.8 g) were produced at East Sea Mariculture Research Center of National Fisheries Research and Development Institute (Uljin, Korea) and were reared for about a year. Prior to experiment, the fish were randomly distributed into 6 circle 500 l tanks (10 fish $tank^{-1}$) which were supplied with full seawater (water flow rate: 6 l min^{-1}) at stable water temperature (WT) of 15ºC, using thermostat (Aquatron®, Youwon Electric, Inc., Korea) and were maintained for 2 weeks before WT rise. The fish were fed with commercial diet twice a day during acclimation but were kept without feeding for 1 day before and during the experiment.

WT rise and sampling : 5 tanks were kept at same WT (15ºC) at the beginning of experiment. Experimental WT of each tank was increased at a rate of 1ºC per day using the thermostat. When WT of tanks reached the desired levels of 15, 18, 21, 24 and 27 ºC, all fish from one of the experimental tanks were sampled. In other words, when WT of 5 tanks reached 15ºC, fish from one of the 5 tanks were sampled. Again, when WT of the remaining 4 tanks reached 18ºC, fish from one of the 4 tanks were sampled. This process was repeated until WT reached 27ºC. The remaining fish in other tank were held at 15ºC and sampled at the same time as fish from the last experimental tank with 27ºC. WT were sampled (Day 12) to investigate stress responses to fasting during the experimental period. Fish was anesthetized with 200 mg l^{-1} solution of tricaine methan sulphonate (MS-222, Sigma, USA) and blood was taken from the caudal vasculature using a 3 ml syringe coated with heparin as anticoagulant. Plasma samples were separated by centrifugation at 6,500 rpm at 4ºC for 15 min and stored at -80ºC till analysis.

Plasma parameters analysis : Plasma cortisol was analyzed by enzyme immunoassay (EIA) with cortisol EIA kit (Oxford Biomedical, USA) according to the manufacturer's protocol. Briefly, plasma of 100 µl was mixed with 1 ml of ethyl ether for extraction of cortisol, then organic phase was evaporated with nitrogen. The wells coated with a rabbit anti-cortisol antibody were treated with 50 µl of solution of standard and plasma samples in duplicate. After adding 50 µl of diluted Cortisol-HRP conjugate, the wells were incubated at room temperature for 1 hr. After washing, 150 µl of TMB substrate was added to the wells, incubated at room temperature for 30 min and then OD was read at 650 nm with microplate reader. Intra- and inter-assay coefficients of variation for cortisol were 8.6% and 9.2% respectively.

Plasma T_3 concentration was measured by EIA with Kit (Syntron Bioresearch, USA). Briefly, anti-T_3 (goat anti-mouse IgG) coated wells were treated with 50 µl of standard and samples in duplicate. After adding 100 µl of T_3-HRP conjugate, the wells were incubated at 37ºC for 1 h. After washing, 50 µl of 0.05 M acetate buffer and TMB were added and incubated at 20ºC for 15 min. After adding stop solution (1 N HCl), OD was read at 450 nm. Intra- and inter-assay coefficients of variation for T_3 were 7.6% and 8.8% respectively.

To determine the levels of plasma glucose, AST, ALT, sodium and chloride, 10 µl of plasma were put on Dri-CHEM slides (Fuji, Japan) and measured using Dry-chem 3500 (Fuji, Japan). Plasma osmolality was measured with Vapor Pressure Osmometer (Vapro 5520, WESCOR Co., USA) and expressed as mmol kg^{-1}.

Statistical analysis : Results were presented as means±S.E.M. and data were analyzed using SPSS (version 17.0) software. Significant differences among the groups were tested by one-way ANOVA, followed by the Tukey-HSD mean comparison test (significance level $P<0.05$). Differences between the 15ºC group

(fasting for 1 day) and the 15ºC-con. group (fasting for 13 days) were also analyzed by Student's t-test. Differences were considered significant at $P<0.05$.

Results and Discussion

Plasma cortisol levels at different WT as primary stress response were analyzed. The hormone concentrations increased with temperature rise of the ambient water. Plasma cortisol concentration was 20.6±3.3 ng ml^{-1} in the 15ºC group. In fish exposed to 18ºC and 21ºC, the concentrations were 23.6 ng ml^{-1}, 20.4 ng ml^{-1} respectively, which were not significantly different from the 15ºC group. In the 24ºC and 27ºC groups, the concentrations were 49.2 ng ml^{-1} and 95.0 ng ml^{-1}, respectively, which were higher as compared to other temperature groups (Fig. 1). Basic levels of plasma cortisol in fish may differ depending on species, size, sex and rearing conditions; however, given that it was 5-50 ng ml^{-1} for tilapia, *Oreochromis niloticus* (Auperin *et al*., 1997) and 10-35 ng ml^{-1} for black porgy, *Acanthopagrus schlegeli* (Chang *et al*., 2002; Min *et al*., 2003). According to Min *et al.* (2009) and Kim *et al*. (2009), stable levels of cortisol in circulation of starry flounder were found to be less than 30 ng ml^{-1} and 10 ng ml^{-1} respectively. Therefore, this study suggest that the values (approximately 20-24 ng ml^{-1}) observed at temperatures up to 21ºC are basic levels which did not cause stress in fish.

Plasma glucose concentrations showed similar changing patterns to those of cortisol. Fish exposed to 15, 18 and 21ºC showed few variations of glucose concentrations ranging from 40.6 to 47.1 mg dl^{-1}. However, the concentrations were significantly increased to 56.1 and 58.1 mg dl^{-1} at 24ºC and 27ºC respectively (Fig. 2). Glucose is generally increased by stress. Increase of plasma glucose is caused by cortisol which enhances the gluconeogenic capacity of phosphoenolpyruvate carboxykinase, a gluconeogenic enzyme in the liver (Hanson and Reshef, 1997). In this study, plasma glucose showed a pattern of increase similar to that of cortisol, suggesting that cortisol mediated the gluconeogenesis. Hyperglycemia is known to satisfy the increased energy requirements caused by stress (Vijayan *et al*., 1997). When Atlantic salmon, *Salmo sala* (Olsen *et al*., 1995) was subject to acute stress, their plasma cortisol and glucose levels increased in tandem. In addition, hyperglycemia has been reported in sunshine bass, *Morone chrysops* × *Morone saxatilis* (Davis, 2004), rainbow trout, *Oncorhynchus mykiss* (Meka and McCormick, 2005) and bullhead, *Ictalurus melas* (Ottolenghi *et al*., 1995) as a stress response to temperature rise. This study has also shown that WT rise increased plasma glucose levels in starry flounder.

No differences were observed in plasma sodium and chloride among the 15, 18, 21 and 24ºC groups. In fish exposed to 27ºC, however, the electrolyte levels were significantly increased (Table 1). Plasma osmolality of the 24ºC and 27ºC group (435.7, 450.7 mmol kg^{-1}, respectively) were significantly higher than those of the 15, 18 and 21ºC groups (375-390.5 mmol kg^{-1}) (Table 1). In seawater fish, the osmotic loss of water and diffusional influx of ions over, integument and in particular over the branchial epithelium, are offset by uptake of seawater via intestine, the extrusion of large amount of Na^+ and Cl^- via chloride cells (Wendelaar Bonga, 1997). In general, handling, rapid changes in salinity, heavy metals and pollutants, ammonia, water acidification, or sudden water temperature changes cause increase passive ion influxes and water loss in seawater fish (Choi *et al*, 2007). These effects are associated with disturbed plasma ion homeostasis and acid-base balance. In this study, we have observed a significant increase in plasma sodium, chloride and osmolality values in both the 24ºC and 27ºC groups. The results of the present study demonstrate that higher WT (more than 24ºC) causes ionic/osmotic imbalances and induces mortality of starry flounder, maybe as a result of theses osmoregulatory problems.

Fish exposed to 15, 18 and 21ºC showed no significant differences (35.3±3.2-37.8±2.9 IU l^{-1}) in AST. At 24ºC and 27ºC,

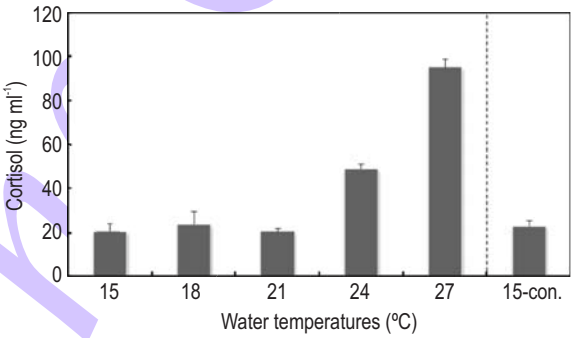

Fig. 1 : Level of plasma cortisol in starry flounder, *Platichthys stellatus* with water temperature rise (15ºC→27ºC). Each value represents mean±S.E. (n=10). Different letters indicate statistical differences among the water temperature groups ($P<0.05$). ND: no difference between 15ºC group (fasting for 1 day) and 15ºC -con. group (fasting for 13 days).

Table 1 : Level of plasma sodium, chloride and osmolality in starry flounder, *Platichthys stellatus* with water temperature rise (15ºC→27ºC).

WT (ºC)	Na^+ (mmol l^{-1})	Cl^- (mmol l^{-1})	Osmolality (mmol kg^{-1})
15	158.7±1.3^a	141.9±1.7^a	383.8±4.6^a
18	154.0±0.8^a	139.1±1.6^a	375.1±8.8^a
21	156.2±1.1^a	140.4±2.2^a	390.5±9.2^a
24	157.3±1.4^a	142.0±1.4^a	435.7±18.6^b
27	164.7±1.1^b	147.6±1.4^b	450.7±15.5^b
15-con.	157.6±2.9ND	141.9±3.6ND	373.3±9.6ND

Each value represents mean±S.E. (n=10). Different letters indicate statistical differences among the water temperature groups ($P<0.05$). ND: no difference between 15ºC group (fasting for 1 day) and 15ºC-con. group (fasting for 13 days)

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the levels were 105.9±9.1 IU l^{-1} and 127.7±12.6 IU l^{-1} respectively, which were significantly higher than those of the other temperature groups (Fig. 3 a). ALT also showed a similar tendency like AST following temperature rise. ALT levels were not significantly different among the 15º, 18º and 21ºC groups (15.5-22.3 IU l^{-1}). Fish exposed to 24ºC and 27ºC, however, the concentrations were 32.0±3.0 IU l^{-1} and 78.6±8.4 IU l^{-1} respectively, which were significantly higher than those of the 15ºC and 18ºC group (Fig. 3 b). In normal circumstances, AST and AST mainly exist in hepatocytes, playing important roles in protein metabolism. When liver is damaged, AST and AST are released into the blood (Ming *et al*., 2012). Therefore, the activities of amino transfer enzymes can be used to monitor health status of fish and also to evaluate the stress response caused by rapid changes in salinity and temperature, low oxygen, pH, ammonia, or heavy metals (Pan *et al*., 2003). Choi *et al*. (2007) reported an increase in enzymes in black porgy kept at high temperature. Similar phenomenon occurred in this study with AST and ALT levels of fish at 24ºC and 27ºC being significantly higher than those of the fish kept at 15ºC-21ºC. This study suggests that hepatocytes in starry flounder are damaged due to high WT, which will result in poorer liver function.

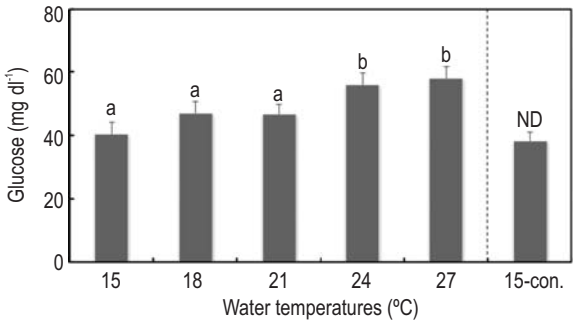

Fig. 2 : Level of plasma glucose in starry flounder, *Platichthys stellatus* with water temperature rise (15ºC→27ºC). Each value represents mean±S.E. (*n*=10). Different letters indicate statistical differences among the water temperature groups (*P*<0.05). ND: no difference between 15ºC group (fasting for 1 day) and 15ºC-con. group (fasting for 13 days)

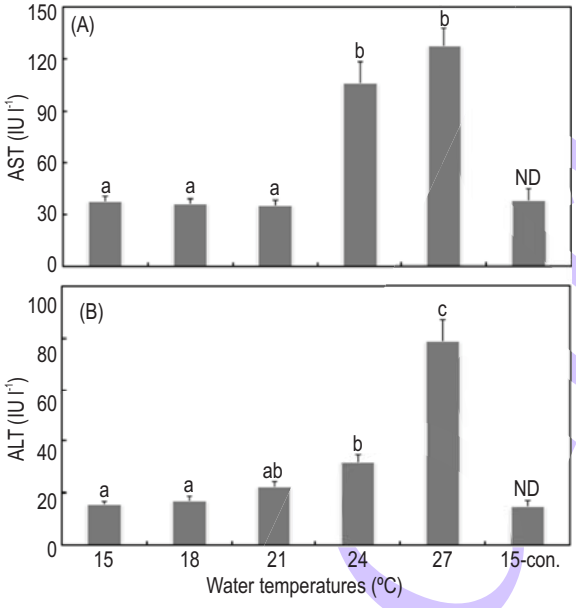

Fig. 3 : Level of AST and ALT in starry flounder, *Platichthys stellatus* with water temperature rise (15ºC→27ºC). Each value represents mean ±S.E. (*n*=10). Different letters indicate statistical differences among the water temperature groups (*P*<0.05). ND: no difference between 15ºC group (fasting for 1 day) and 15ºC-con. group (fasting for 13 days)

Plasma T_3 concentrations were 24.7±1.9 ng ml^{-1} in 15ºC group, but these were significantly increased to 29.4±0.8 ng ml^{-1} at 24ºC. Fish exposed to 27°C had significantly lower T_3 concentrations than those exposed to the other temperatures. Plasma T_3 was also shown to be significantly influenced by fasting. Fish exposed to 15ºC were significantly lower in T_3 concentration while fasting for 13 days than fasting for 1 day (Fig. 4). In fish, thyroid hormones secreted from the hypothalamic-pituitary-thyroid (HPT) axis are essential central regulators that link many biological tasks, including embryonic and post-natal growth, reproductive function, and the behavioural and physiological responses to stress (Crockford, 2003). It is well known that HPT axis responds to many stressors in fish (Peter, 2011). An increase in plasma T_3 due to high WT (more than 25ºC) as stressor was observed in seawater adapted black porgy (Choi *et al*., 2007). In this study, T_3 was expected to increase due to temperature stress. This hormone increased in starry flounder kept at 24ºC. However, T_3 of the fish kept at 27ºC was significantly lower than that of fish kept at 15ºC. One of the possible explanations is that decrease in T_3 level at 27ºC is caused by

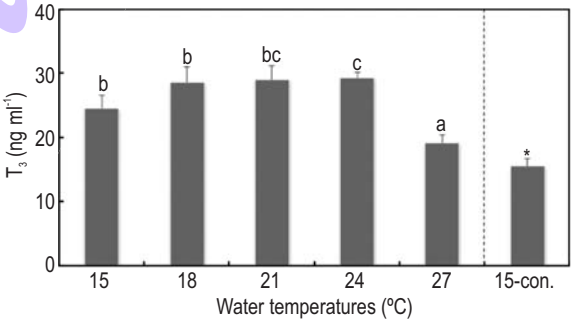

Fig. 4 : Level of plasma T_3 in starry flounder, *Platichthys stellatus* with water temperature rise (15ºC→27ºC). Each value represents mean ±S.E. (*n*=10). Different letters indicate statistical differences among the water temperature groups (*P*<0.05). ND: no difference between 15ºC group (fasting for 1 day) and 15ºC-con. group (fasting for 13 days)

fasting during long-term period (13 days). During feed restriction, it has been well documented that thyroid hormone levels decrease (Navarro and Gutierrez, 1995; MacKenzie *et al*., 1998). Lowered level of T_3 in response to feed restriction observed in this study follows a similar trend reported in other studies (Gaylord *et al*., 2001).

In conclusion, results of the present study showed that the water temperature of over 24ºC significantly affected physiological stress responses in starry flounder. Accordingly, weakened physiological functions, delayed growth, even death can be seen if the species is reared for a long term over 24ºC in summer.

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