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Effects of sudden salinity changes on short-term hematological and biochemical responses in Walton's mudskipper, *Periophthalmus waltoni* Koumans, 1941 (Perciformes: Gobiidae)

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Abstract: The present study investigates and reports the effects of sudden salinity changes on short-term hematological and biochemical responses in Walton's mudskipper, *Periophthalmus waltoni*. For this purpose, mudskippers caught from coastal area of Persian Gulf (Bandar Khamir, Hormozgan Province, Iran) were acclimated and fed with frozen blood worms (*Chironomus*) for one month prior to the start of experiments. After acclimation, groups of 15 individuals were fully submerged in either dechlorinated tap water (fresh water; Group A), 50% seawater (SW) (17‰ salinity; Group B), or 100% SW (35‰ salinity; Group C) during a period of 24 hours. Fish which were free to be in or out of 50% SW served as controls. Statistical analysis showed a significant influence of salinity levels on erythrocyte, haemoglobin, haematocrit, leucocytes, lymphocytes, neutrophils, monocytes and on all biochemical parameters tested during the study. It seems that the changes recorded in haematological and biochemical parameters were the best strategy that the Walton's mudskipper has to adapt itself to an environment with different salinity levels.

Keywords: Oxudercine gobies, Euryhaline, Physiological adaptation, Persian Gulf.

Introduction

Salinity is one of the most important environmental factors influences the osmotic pressure and metabolism (Mustafayev & Mekhtiev 2008; Fazio et al. 2013), causes changes to the activity, structure, and physiological function of fish digestive enzymes; and affects development, habits, and survival of fish (Chen et al. 1998; Wang & Zhu 2002; Ruscoe et al. 2004). There are some species that can tolerate various salinities and a few that can survive extended exposure in water with different salinities (Nordlie 1985; Nordlie et al. 1992; Nordlie & Haney 1998). According to Francis et al. (2007), salinity is one of the most fluctuating water quality parameters in brackish water environment. Growth of euryhaline species is often affected by salinity because the energy used for osmoregulation is not available for growth (Brett 1979; Wootton 1990). Consequently, many of these species have an optimum salinity level at which the growth rate is highest and the cost of osmoregulation lowest, which may affect fish distribution in the wild (Blaber 1997).

The haematological profile represents a good indicator of physiological dysfunctions since there is a close association of circulatory system with the external environment (Elahee & Bhagwant 2007). It provides information not only about the health status of fish and the physical and chemical parameters of water in which they live, but also to assess the relationship between these factors and to know the susceptibility of organism to changes in environmental conditions (Elahee & Bhagwant 2007;

Debala Devi & Usha Anandhi 2010; Maceda-Veigaet al. 2010; Ayoola et al. 2011).

The physiological response to variable salinity levels in an aquatic environment has been investigated in a number of fish species (Woo & Wu 1982; Hutchinson & Hawkins 1990; Proverches et al. 1993; Kelly & Woo 1999; Foss et al. 2001; Luz et al. 2008; Mohammadi Zarejabad et al. 2009; Salati et al. 2010; Farabi et al. 2011; Fazio et al. 2012, 2013; Liu et al. 2013; Peyghan et al. 2014).

Mudskippers (Teleostei: Gobiidae: Oxudercinae: Periophthalmini) are gobies that are "fully terrestrial for some portion of the daily cycle" (Murdy 1989). They are investigated for their biological and eco-toxicological studies, to determine its potential use as a bio-indicator in environmental assessments of coastal waters, tropical or subtropical soft bottom intertidal systems. The mudskippers are euryhaline and can withstand rapid and drastic changes in salinity (Aligaen & Mangao 2011). The Walton's mudskipper, *Periophthalmus waltoni* Koumans, 1941 is one of the three species of oxudercine gobies (Gobiidae) (Agorreta et al. 2013) living in the Persian Gulf and Gulf of Oman (Murdy 1989; Carpenter et al. 1997; Ghanbarifardi et al. 2014a,b,c, 2016; Ghasemian et al. 2015). Iranian mudskippers are differentially distributed from more-aquatic to more terrestrial habitats, respectively, from *Scartelaos tenuis* to *Boleophthalmus dussumieri* to *P. waltoni* (Clayton 1985).

Periophthalmus waltoni is a filter and detritus-mud feeder that is in contact with pollutants in the water column and sediments (Sarhadizadeh et al. 2014). This species has been recorded in a wide range of intertidal habitats, including tidal mudflats and mangrove forests (Clayton 1985; Rahimian & Pehpuri 2006; Ghanbarifardi & Malek 2007), and ecosystems characterized by different physicochemical regimes (Yao 2008). Such environments, are particularly harsh during low tide, including rapid and wide fluctuations of temperature (Tytler & Vaughan 1983), salinity (Sasekumar 1994), and oxygen levels (Ishimatsu et al. 2000). The natural habitat of *P. waltoni* lies usually in a dynamic state of fluctuating salinities. Movement between fresh water and brackish or seawater causes physiological changes, including blood composition fluctuations.

Limited information exists with respect to the effects of salinity on physiology of *P. waltoni*. Hence, the present work aims to examine the possible variations of blood profile in *P. waltoni* subjected to sudden acclimation to different salinities.

Material and Methods

Collection and maintenance of mudskippers: Live specimens of Walton's mudskipper , (10.6±2.8cm total length; 7.2±1.9g weight) were collected by hand from the mudflats of the coastal area of the Persian Gulf in Bandar Khamir (Hormuzgan Province, Iran; 26°56'40"N, 55°35'55"E). They were morphologically identified to species level by means of the available morphological keys (Murdy 1989). The animals were brought to the laboratory in plastic troughs containing only sea water to 3cm level. They were maintained under laboratory conditions in 50% SW (17% salinity) in small aquaria (70cm×35cm×35 cm, depth of water approximately 2cm) at 25°C, providing appropriate terrestrial areas to the fish. Different salinities were provided by dilution of coastal Persian Gulf saline water (approximately 35‰) with dechlorinated municipal freshwater. Salinity was determined by using hand held refractometer (Model HRN-2N Atago Product Japan).

The fishes were fed with frozen blood worms (*Chironomus*) once daily in the morning, and the water was changed every other day. They were allowed to adapt to such environment for a month before experiments were performed.

Exposure of mudskippers to waters of various salinities: After acclimation, the fish were randomly divided into four equal groups and transferred into four tanks, each with a different level of salinity. Groups of 15 individuals of *P. waltoni* were fully submerged in either dechlorinated tap water (fresh

water; Group A), 50% SW (17‰ salinity; Group B), or 100% SW (35‰ salinity; Group C) during a period of 24 hours. Fish which were free to be in or out of 50% SW served as controls. Fish were fasted during salinity treatment. Temperature, salinity, dissolved oxygen levels and ammonia concentration were measured based on the methods described by APHA (1985). During the trial, all fish were maintained under natural photoperiod (sunrise at 05:55 h, sunset at 20:00 h). The dissolved oxygen (DO), nitrate and nitrate levels were determine using the methods described by APHA (1985).

Blood and serum collection: The fish were bled immediately after treatment. Six fish were sampled at random from each experimental group and were anaesthetized by 0.1ppm of MS222. After anaesthetizing the fish, the blood samples were collected from caudal vein using a 2ml syringe and a 24-gauge needle. Haematological parameters were assessed within one hour of blood collection. One part of each blood sample was transferred into preheparinised plastic Eppendorf tubes while the other part was transferred into blood collecting tubes or Eppendorf tubes without anticoagulant and was allowed to clot for 2hrs at room temperature in a slanting position. The clot was then cut with a glass rod and care was taken not to haemolyse the clot. The tubes were kept at 4ºC overnight and were then centrifuged at 2500g for 15min and the supernatant serum was collected. The serum was stored at -20ºC in screw cap glass vials until use.

Determination of serum biochemical and hematological parameters: Blood samples were immediately analyzed for the estimation of numbers of erythrocytes (RBC) and leucocytes (WBC), Hemoglobin (Hb) and Hematocrit (Hct). RBC and WBC count were performed as described in Schaperclauset al. (1991). Hemoglobin contents (Hb) were determined using cyanmethemoglobin method with Drabkin's solution (Goldenfarb 1971). Hematocrit was determined by the microhematocrit method (Fox 1997). The mean corpuscular volume (MCV; μm^3), the mean corpuscular hemoglobin (MCH; pg) and the mean corpuscular hemoglobin concentration (MCHC; %) were calculated using the following formulas (Jain 1993):

$$MCV = (\% \text{ of Hct/RBC in millions}) \times 10 \ (\mu m^3)$$

$$MCH = (\text{Hb in g/RBC in millions}) \times 10 \ (pg)$$

$$MCHC = (\text{Hb in g/ } (\% \text{ of Hct}) \times 100 \ (\text{g per } 100 \text{ ml})$$

For differential count of leukocytes whole blood on glass microscope slides, dried in air, and stained with May-Grunwald/Giemsa. Leucogram was assessed for each fish under an oil immersion lens. One hundred white blood cells from each smear were assessed and the percentage of different types of leucocytes was calculated (Schaperclaus et al. 1991). The quantitative determination of glucose was carried out using commercially available diagnostic experimental protocols kits (Pars Azmun, Iran, 1 500 0178) (Hosseini et al. 2011), at 546nm and 37°C according to the glucose oxidase method suggested by Trinder (1969). Lactate was measured with an enzymatic method by a lactate kit (Pars Azmoon, Tehran, Iran). Total serum protein was measured by the Biuret method (Kwapinski 1965).

Statistical analysis: Data are presented as Mean±SD of the number of fish per group. Hematological and biochemical parameters were analyzed by one way analysis of variance (ANOVA) and Tukey's multiple comparison range. All statistical analyses were tested at the 0.05 level of probability, using the software SPSS 16.0 for Windows.

Results

No mortality was observed during the experiments. All fish were considered healthy on the basis of an external examination for any signs of abnormalities. Mean values ± SD of haemato-biochemical parameters recorded in experimental and control groups are tabulated in Table 1 and 2. ANOVA analysis showed significant changes in all parameters among the groups except MCV, MCH and MCHC. In particular, RBC was lower in groups A and B but higher in group C with respect to the control group (P<0.05) (Table 1). Moreover, Hgb and Hct levels

Table 1. Mean values (±SD) of haematological profile of *P. waltoni* acclimated at different salinities.

Parameters	Salinity			
	Control (17‰)	Group A (0‰)	Group B (17‰)	Group C (35‰)
RBC	1.44±0.12^{c}	0.97±0.08^{a}	1.22±0.06^{b}	1.80±0.11^{d}
Hct	18.66±2.16^{b}	12.66±1.63^{a}	15.66±2.16^{ab}	22.50±1.87^{c}
Hgb	5.3±0.75^{b}	4.3±0.52^{a}	5.2±0.55^{ab}	6.3±0.57^{c}
WBC	8.28±0.66^{b}	11.25±1.10^{d}	8.95±0.70^{b}	5.90±0.49^{a}
Lymphocyte	68.16±4.02^{b}	75.66±3.56^{c}	65.5±3.39^{b}	50.66±3.14^{a}
Neutrophil	5.33±1.2^{b}	7.5±1.05^{c}	5±1.26^{b}	3.66±1.03^{a}
Monocyte	10.5±1.38^{b}	13.5±1.52^{c}	9.83±1.83^{b}	6.5±1.05^{a}
MCV	131.1±24.6^{a}	150±21.6^{a}	128.1±14.8^{a}	125±10.5^{a}
MCH	36.81±4.43^{ab}	44.35±6.61^{b}	42.57±5.50^{ab}	35.19±4.8^{a}
MCHC	28.98±6.7^{a}	34.26±4.9^{a}	33.52±5.2^{a}	28.11±3.1^{a}

Values in the same row showing the same superscript letter are not significantly different (pTukey> 0.05).

Table 2. Mean values (±SEM) of biochemical parameters of *P. waltoni* acclimated at different salinities.

Parameters	Salinity			
	Control (17‰)	Group A (0‰)	Group B (17‰)	Group C (35‰)
Glucose	51.83±6.49^{a}	68.33±7.58^{b}	58.16±5.88^{ab}	64.5±5.72^{b}
Lactate	0.59±0.16^{a}	2.95±0.19^{b}	0.89±0.23^{a}	3.05±0.25^{b}
Total protein	2.95±0.16^{a}	4.21±0.24^{b}	3.20±0.18^{a}	4.06±0.21^{b}

Values in the same row showing the same superscript letter are not significantly different (pTukey> 0.05).

were lower in groups A and C compared with the control group. The number of WBC was higher in group A but lower in group C in comparison to the control group. The percentage of lymphocytes, neutrophils and monocytes was significantly higher in group A but lower in group C with respect to the control group. Glucose, lactate and total protein values increased in groups A and C compared with the control group (P<0.05) (Table 2).

Discussion

Haematological parameters of fishes are closely related to the response of fish to the environmental and biological factors such as age, weight, sex, food, bacteria, parasite and water quality parameters, including water temperature, salinity, oxygen availability and pH (Haider 1973; Steinhagen et al. 1990; Fernandes & Mazon 2003). Our results for *P. waltoni* showed a significant influence of different levels of salinity on most of the parameters studied.

In accordance with the previous studies (Gabriel et al. 2007; Akinrotimi et al. 2010, 2012), no changes occurred in MCV, MCH and MCHC values among the treatments, however, significant reduction was observed in the level of RBC, Hct and Hgb in group A compared with the control group. These reductions may be attributed to salinity-induced osmoregulatory dysfunction which leads to erythrocyte fragility

(Montero et al. 1999; Girling et al. 2003). In fact, osmotically obliged water movement due to an increase in blood osmolality reduced blood hematocrit and hemoglobin concentrations at lower salinities. Furthermore, the observed haemodilution may be a consequent of impaired erythropoiesis (production of erythrocytes) caused by disrupted osmoregulation (Zhiteneva et al. 1989; Gabriel et al. 2007).

The RBC, Hct and Hgb significantly increased with increasing salinity levels up to 35‰ salinity in group C. It was revealed that in a hyperosmotic environment, first the fish would lose water passively and concentrations of blood-cell elements increase. Furthermore, the elevation of these parameters might be in part due to introduction of red blood cells by splenic contraction (Mojazi Amiri et al. 2009). Similar findings were obtained in previous investigations (Plaut 1998; Martínez-Álvarez et al. 2002).

Hyperglycemia is an expected result of stress or exhaustive exercise in fishes (Barton & Iwama 1991; Hrubec et al. 1997). It is known that the degree of hyperglycemia may change depending on the type of stress and the sampling times (Rotlland et al. 1997). The increase in blood glucose levels found in groups exposed to extreme salinities (group A and C) could be attributed to liver glycogenolysis initiated by catecholamines to meet metabolic demands for osmoregulatory tissues like the gills and kidney (Lea Master et al. 1990; Vijayan et al. 1996; Laiz-Carrión et al. 2003; Sangiao-Alvarellos et al. 2003, 2005).

Lactate is produced by anaerobic metabolism in the muscle under stressful conditions of hypoxia or strenuous exercise and released to the plasma (Milligan & Girard, 1993; Mommen et al. 1999; reviewed in Begg & Pankhurst 2004). In addition, lactate can provide energy for brain, gills and kidney (Mommsen 1984). In our study, increase in the lactate levels observed in fish exposed to both extreme salinities (group A and C), suggests that this metabolite is presumably used as an energy source by osmoregulatory organs. Similar results were obtained in common carp and Senegalese sole (*Solea senegalensis*) when fish were exposed to a sudden change of salinity (Salati et al. 2009; Herrera et al. 2012).

Total plasma protein concentration relative to a reference interval is used as a broad clinical indicator of health, stress, and well-being of aquatic organisms (Riche 2007). In the current study, protein levels increased in fish treated with both acute salinity levels (group A and C). This contrasts with previous findings reporting either no changes or decreases in protein levels following increased salinity. In fact, our data are in agreement with the findings of Fazio et al. (2013), who addressed the possible importance of increased serum protein as a fuel for tissues during osmotic acclimation once carbohydrate stores have been mobilized. Amino acids seem to play an important role in allowing fish to adjust to the different environmental salinities, either as energy sources or as important to smolytes for cell volume regulation (Aragão et al. 2010). Elevated serum or plasma protein levels had previously been reported in starved red sea bream (*Chryrosphrys major*) and black sea bream (*Mylio macrocephalus*) and red grouper (*Epinephelus akaara*) exposed to low salinity environments (Woo & Murat 1981; Woo & Wu 1982). Furthermore, in accordance with our study, serum protein concentrations were significantly elevated in silver Seabream (*Spams sarba*) as the ambient salinity diminished (Luk Chun-yin 2001).

Leukocytes are good fish physiological stress indicators (Heath 1995; Tillmann & Biron 2000; Svobodová et al. 2001). The present study demonstrated significant increase in the number of white blood cell (WBC) as well as the differential counts namely lymphocytes, neutrophils and monocytes in the lowest salinity treatment (Group A). This is in line with the results of Tavares – Dais et al. (2001) in tambaqui *Colossoma macroponum*. Comparable finding was observed in green back flounder *Rhombosulea tapirina* (Girling et al. 2003) and in *Tilapia guineensis* (Akinrotimi et al. 2010) exposed to fresh water. This increment is due to a

non- specific immune response to stress as a result of interaction of prolactin and cortisol hormone to restore ion balance in hyposmotic environment. This supports the finding of Eckert et al. (2001) who observed similar changes in channel catfish (*Ichtalurus punctatus*) exposed to different salinities. An increase in the count of WBC may also be caused by a release of cells accumulated in the spleen, to combat the stressor (Houston et al. 1996; Ajani et al. 2007).

In the present study, the number of WBC and the differential counts was significantly reduced in fish subjected to highest level of salinity (Group C). This corroborates the finding of Fazio et al. (2013) on cultured mullet, *Mugil cephalus*. The decrease in the value of leukocytes and differential counts suggests a suppression of production of these elements from haemopoietic organs (Anyanwu et al. 2007). In contrast with our finding, significant elevation of number of WBC was found in silver barb (*Barbonymus gonionotus*) transferred directly from freshwater to brackish water (16ppt) (Binte Aamin 2014). Likewise, rainbow trout (*Oncorhynchus mykiss*) cultured in brackish water exhibited higher value of WBC than those cultured in freshwater (Hosseinzadeh Sahafi et al. 2013). Previous studies revealed that salinity change can modulate the immune response in fish species (Cuestaa et al. 2005; Ky et al. 2007; Birrer et al. 2012). However, these changes in blood parameters can be different among species based on genetic make-up, life history, nutritional status, and the fish's environment (Mohammadi Zarejabad et al. 2009).

Collectively, it seems that the changes recorded in haematological and biochemical profiles in experimental groups were the best strategy the mudskipper has to adapt itself to an environment with different salinity levels.

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اثر تغییرات ناگهانی شوری بر پاسخ‌های کوتاه‌مدت بیوشیمیایی و خون‌شناختی ماهی گل‌خورک والتونی *Periophthalmus waltoni* Koumans, 1941 (سوف ماهی‌شکلان، گاوماهیان)

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چکیده: در این مطالعه اثر تغییرات ناگهانی شوری بر پاسخ‌های کوتاه مدت بیوشیمیایی و خون‌شناختی ماهی گل‌خورک والتونی ارزیابی و گزارش می‌گردد. برای این منظور نمونه‌های ماهی گل‌خورک از نواحی ساحلی خلیج‌فارس (بندر خمیر در استان هرمزگان) صید گردید و قبل از شروع آزمایش به مدت یک‌ماه جهت سازگاری با شرایط آزمایشگاه با شیرونویده تغذیه شدند. بعد از سازگاری، ماهیان در چهار گروه متشکل از ۱۵ قطعه ماهی تقسیم شدند. در تیمارهای ۱: آب شهری فاقد کلر (آب‌شیرین)، ۲: آب با شوری نصف آب دریا (۱۷ گرم در لیتر) و ۳: آب دریا (شوری ۳۵ گرم در لیتر) ماهیان به شکل کاملاً غوطه‌ور به مدت ۲۴ ساعت نگهداری شدند. در تیمار ۴ ماهیانی که به‌طور آزادانه در داخل یا خارج از آب با شوری ۱۷ گرم در لیتر نگهداری شدند به‌عنوان گروه شاهد در نظر گرفته شدند. واکاوی آماری حاکی از تاثیر معنادار سطوح شوری بر گلبول‌های قرمز، هموگلوبین، هماتوکریت، گلبول‌های سفید، لنفوسیت‌ها، نوتروفیل‌ها، مونوسیت‌ها و همه فراسنجه‌های بیوشیمیایی مورد آزمایش بود. به‌نظر می‌رسد تغییرات ثبت شده در فراسنجه‌های خون‌شناختی و بیوشیمیایی از مهمترین راهبردهای ماهی گل‌خورک والتونی برای سازگاری با زیستگاه با تغییرات دائمی شوری می‌باشد.

کلمات‌کلیدی: گاوماهیان، یوری هالین، سازگاری فیزیولوژیک، خلیج فارس.