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Effects of gradual salinity increase on osmoregulation in Caspian roach *Rutilus caspicus*

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This study was carried out to determine the effects of gradual salinity increase on osmoregulatory ability of the Caspian roach *Rutilus caspicus*, under conditions which mimic stocking conditions of hatchery-raised fish. Initially, 30 juvenile fish (mean ± s.d. 3·20 ± 0·34 g) were transferred to 20 l circular tanks, in which salinities were changed in a stepwise fashion, from 0 to 5, 10 or 15 at 48 h intervals. The fish at salinity 15 were held for an additional 48 h at this salinity. Forty-eight hours after salinity transfer, survival rate, haematocrit, plasma Cl^-, Na^+ and K^+ concentrations, osmolality and gill Na^+/K^+-ATPase (NKA) activity were measured. The only effect of exposure to 5 was a significant reduction in haematocrit compared to the freshwater control group. Exposure to salinity 10 raised haematocrit, Cl^- and Na^+ concentrations and osmolality. At 48 h exposure to salinity 15, haematocrit, Cl^- and Na^+ concentrations and osmolality were significantly higher than freshwater controls, and gill NKA activity was significantly lower, but the effect on NKA was no longer evident at 96 h exposure. There were no effects on survival. These results indicate that *R. caspicus* juveniles experience an initial non-lethal iono-osmotic perturbation following salinity increase but can adapt to brackish water at salinity 15.

Key words: brackish water; gill; Na^+/K^+-ATPase; NKA; osmolality.

INTRODUCTION

Teleosts, the most advanced group of fishes, need to have highly efficient ion and osmoregulatory mechanisms in order to maintain their body fluid homeostasis, which is necessary for normal operation of all biochemical and physiological processes. To compensate for passive water loss, marine teleosts drink seawater and actively secrete salt *via* the gills as well as kidneys. In contrast, freshwater (FW) fishes do

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not drink (or drink very little) water but produce diluted urine *via* the kidneys for balancing the passive water gain, while actively absorbing salt through the gills from the environment (Evans *et al*., 2005; Marshall & Grosell, 2006; Hwang & Lee, 2007).

Fishes challenged with an altered environmental salinity must maintain their body osmolality and ionic balance by changing activities, such as drinking rate (Marshall & Grosell, 2006) and stress hormone levels, which can disturb hydromineral balance and blood variables such as haematocrit (McCormick, 1993; Brown *et al*., 2001) and functions of the osmoregulatory surfaces (Arai *et al*., 1997; Kelly & Woo, 1999; Fielder *et al*., 2007). Gill Na^+/K^+-ATPase (NKA) is the primary driving force for flux of intra and extra-cellular NaCl and is present in high concentrations on the basolateral side of gill mitochondrion-rich cells (MRC) (McCormick, 1995; Evans *et al*., 2005). Specifically, it is localized to the tubular system membranes, which are extensions of the basolateral membranes (Wilson & Laurent, 2002).

Juvenile osmoregulatory capacity is one of the most important physiological factors in re-stocking success at release as well as during transport (Hoar, 1988; Ataeimehr *et al*., 2005; Portz *et al*., 2006). The time course of changes in gill NKA activities after transfer to different environmental salinities is species dependent. Changes in gill NKA activity are observed 2–3 days after transfer from a hypoosmotic to hyperosmotic environment in the euryhaline teleost killifishes *Fundulus heteroclitus heteroclitus* (L. 1766) (Mancera & McCormick, 2000) and 3–7 days in anadromous and other euryhaline species such as European sea bass *Dicentrarchus labrax* (L. 1758) (Jensen *et al*., 1998), coho salmon *Oncorhynchus kisutch* (Walbaum 1792) (Wilson *et al*., 2002) and gilthead seabream *Sparus aurata* L. 1758 (Laiz-Carrión *et al*., 2005).

The Caspian roach *Rutilus caspicus* (Yakovlev 1870) is a cyprinid species that is prized in both sport and commercial fisheries (Keyvanshokooh *et al*., 2007). It is moderately euryhaline and omnivorous, feeding on small crustacean and insect larvae. It is migratory with a spring migration from the Caspian Sea into rivers to spawn and an autumn migration back to sea to overwinter. This species is a significant prey item for beluga sturgeon *Huso huso* (L. 1758) in the Caspian Sea. Recently, because of over fishing and deterioration of spawning grounds, the species has been considered for inclusion in the list of threatened species in the region (Kiabi *et al*., 1999).

The most economically important teleosts in the southern Caspian Sea are all anadromous and deterioration of their spawning grounds has lead to problems with sustainability of their stocks. In Iran, fisheries organizations have released millions of larvae and juveniles, derived through artificial propagation, into the rivers that discharge into the southern Caspian Sea in an effort to rebuild these resources. *Rutilus caspicus* is among the species that are reared for re-stocking as juveniles.

The main goal of the present study was to determine the effects of a gradual increase of salinity, to Caspian Sea levels, on osmoregulation by juvenile *R. caspicus*, reared in aquaculture, using blood haematocrit, serum chemistry and gill NKA activity as indicators. The salinity treatments were selected to represent the salinity range that *R. caspicus* may encounter in Bandare Torkaman coastal region, Gorgan Bay, south-eastern Caspian Sea, Iran.

MATERIALS AND METHODS

ANIMALS

Approximately 800 juvenile *R. caspicus* aged between 3 and 4 months were obtained from Sijual Teleost Fish Propagation and Rearing Center, close to Bandare Torkaman, Iran. The fish were transferred to the Aquaculture Research Center of the Faculty of Fisheries and Environmental Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran.

All fish were acclimatized to laboratory conditions for at least 2 weeks prior to experiments in six 400 l fibreglass tanks provided with a flow of dechlorinated tap water, with *c*. 150 juveniles in each tank, to avoid any confounding effects of handling stress on osmoregulation (Biswas *et al*., 2006). Fish were fed twice daily with a commercial 0·8 mm pellet (INICIO Plus, Biomar Co.; www.biomar.com) during holding. Fish were not fed during experiments. Fish were exposed to an ambient photoperiod of *c*. 14L:10D.

Caspian seawater (SW) with a maximum salinity 15 (obtained from Bandare Torkaman sea shore, Gorgan Bay, Iran) was added to dechlorinated tap water to achieve the experimental salinities. Salinity, temperature (range 15·0–16·5° C), pH (range 8·2–8·6) and dissolved O_2 (range 7·6–13·3 mg l^{-1}) were measured daily (Table I) using a water quality metre (U-10, Horiba Ltd; www.horiba.com).

SALINITY ACCLIMATION

Three salinity levels were investigated, 5, 10 and 15, for comparison with a freshwater control. Initially, 30 juveniles (mean ± S.D. mass = 3·20 ± 0·34 g) were transferred to 20 l circular tanks, in which salinities were changed in a stepwise fashion, from 0 to 5, to 10 and then 15 at 48 h intervals. A group of fish was then maintained a further 48 h at salinity 15. Salinities were raised by removing water from the circular tanks and adding an appropriate amount of Caspian seawater at salinity 15.

Six individuals were sampled four times, just before raising the salinity (at 5, 10 and 15 at 48 h, then 15 at 96 h), *c*. 24 individuals in total. Forty-eight hours after salinity transfer, survival rate, haematocrit, plasma Cl^-, Na^+ and K^+ concentrations, osmolality and gill NKA activity were measured.

SAMPLING

The six fish from each treatment were anasthetized with clove powder (100 mg l^{-1}) and samples of blood were taken immediately into 75 mm heparinized capillary tubes following caudal transection. Tubes were centrifuged at 5000 *g* (D_78532, Hettich Co.; www.hettichlab.com) for 15 min, for the measurement of haematocrit (Hct) and plasma aliquots, then sampled and stored at −80° C.

TABLE I. Water quality variables of *Rutilus caspicus* in various salinities (values are means ± S.D.).

Physical variables Salinity	Temperature (° C)	pH	O_2 (mg l^{-1})
Freshwater control	15·2 ± 2·1	8·5 ± 0·1	12·6 ± 1·8
5	16·5 ± 1·1	8·4 ± 1·0	7·6 ± 1·4
10	16·3 ± 0·9	8·2 ± 0·3	8·0 ± 0·3
15	15·0 ± 0·4	8·6 ± 1·0	13·3 ± 0·6
15*	16·2 ± 0·4	8·5 ± 0·6	8·5 ± 0·7

*15 after 96 h.

ANALYTICAL TECHNIQUES

Plasma Na^+, K^+ and Cl^- concentrations were measured using ion-selective electrodes (Electrolyte analyzer XI-921E, Caretium Medical Instruments Co.; www.caretium.com) and results were reported in mmol l^{-1}.

Plasma osmolality was determined in fresh samples using freezing-point depression (Melting Point Osmometer, N 961003, Roebling Co.; www.melting-point.buchi.com) and reported as mOsm kg^{-1}.

Gill NKA activity was measured according to the microassay protocol of McCormick (1993) with some modifications. Gill filament samples from the second arch on left side were severed from the anasthetized fish by fine point scissors and immersed in 100 µl of ice-cold SEI buffer [sucrose (150 mM), EDTA (10 mM), imidazole (50 mM), pH 7·3] and frozen at −80° C.

The filaments were thawed, homogenized with pestle in SEI buffer containing 0·1% deoxycholic acid and centrifuged at 8000 *g* for 60 s to remove large debris. For the assay, 25 µl of the supernatant was added to 500 µl of assay mixture [imidazole buffer (50 mM), phosphoenolpyruvate (PEP) (2·8 mM), nicotinamide adenine dinucleotide (NADH) (0·22 mM), ATP (0·7 mM), lactate dehydrogenase (LDH) (4·0 U) and pyruvate kinase (PK) (5·0 U)]. Assays were run in two sets of duplicates, one set containing the assay mixture and the other assay mixture plus ouabain (1·0 mM, Sigma–Aldrich Chemical Co.; www.sigmaaldrich.com) to specifically inhibit NKA activity. ATPase activity was detected by enzymatic coupling of ATP dephosphorylation to NADH oxidation measured at 340 nm with a spectrophotometer (Photometer clinic Π, Tajhizat Sanjesh Co.; www.tajhizatsanjesh.com) for 10 min at 30° C. Total protein concentrations were determined by modification of the Bradford (1976) dye binding assay with a bovine serum albumin (BSA) standard at 630 nm and the results expressed as µmoles ADP mg^{-1} protein h^{-1}.

STATISTICAL ANALYSIS

All the data are expressed as means ± S.D. Analysis of data was carried out using SPSS (version, 17.0.; www.ibm.com/uk/SPSS). One-way ANOVA was used to examine differences among the experimental groups and for comparing means, Duncan's *post hoc* test was used. Statistically significant differences were expressed as $P < 0{\cdot}05$.

RESULTS

No mortality occurred in any of the treatments.

Plasma ion levels are presented in Fig. 1. Plasma Cl^- levels remained similar to those of control fish after 48 h exposure at salinities 5 or 10. Transfer to salinity 15, however, resulted in a significant incremental increase with time compared with the freshwater control [Fig. 1(a)]. Similar results were observed for plasma Na^+, whereby only exposure to salinity 15 for 48 and 96 h caused significant increase compared to controls [Fig. 1(b)]. Plasma K^+ levels were not altered by 48 h exposure to salinities 5 or 10 but, in contrast to Na^+ and Cl^-, plasma K^+ levels were significantly lower following exposure to salinity 15 [Fig. 1(c)].

Plasma osmolality increased after 48 h exposure to salinities 10 and 15 [Fig. 2(a)], but there was no effect of exposure to salinity 5.

Blood haematocrit was significantly lower after 48 h exposure to salinity 5 but increased significantly at salinities 10 and 15, peaking after the first 48 h in salinity 15 [Fig. 2(b)].

Gill NKA activity was similar to the freshwater control group after 48 h exposure to salinity 5 but, at 48 h exposure at salinities 10 and 15, activity levels were significantly lower by comparison with the control group. Following an additional 48 h

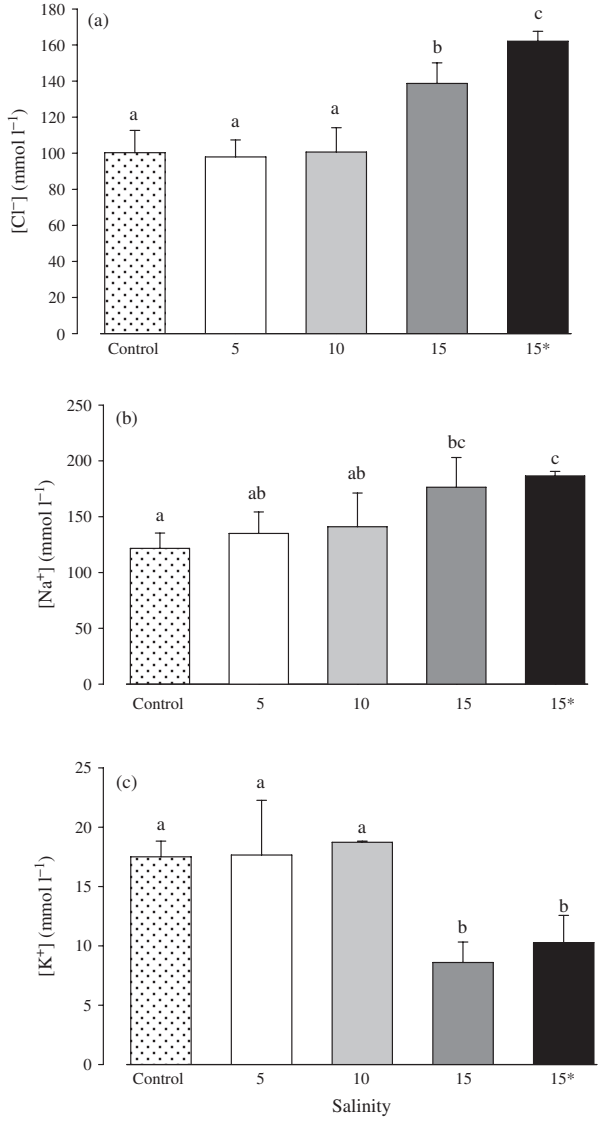

FIG. 1. (a) Chloride, (b) sodium and (c) potassium of *Rutilus caspicus* transferred stepwise from fresh water to salinities of 5, 10 and 15 and acclimated for 48 h at each salinity. Fish at salinity 15* were sampled after an additional 48 h. Values are means + S.D. ($n = 6$). Bars with the same lower case letters are not significantly different from each other ($P < 0{\cdot}05$).

at salinity 15, however, gill NKA activity was no longer significantly different from the control group [Fig. 2(c)].

DISCUSSION

Rutilus caspicus is capable of surviving gradual transfer to water having salinity 15, which is the maximum natural salinity of the south-eastern Caspian Sea, although

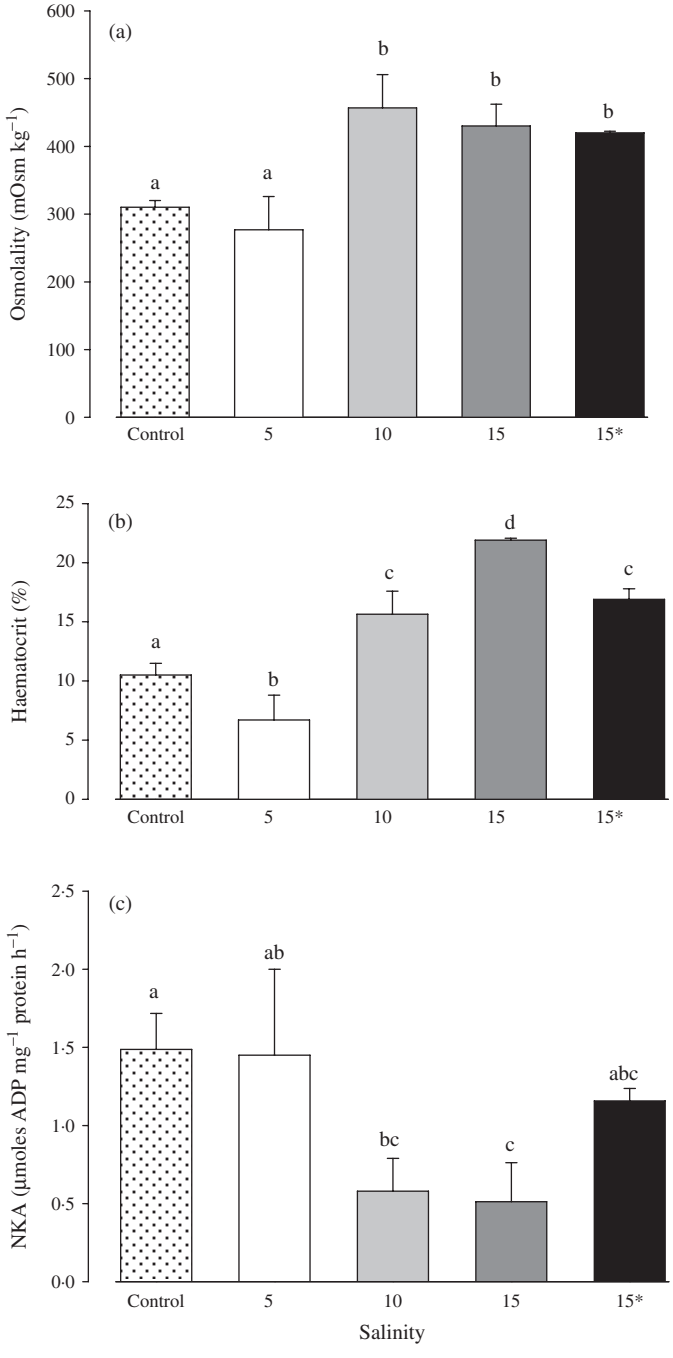

FIG. 2. (a) Osmolality concentrations of blood plasma and (b) haematocrit and (c) gill Na^+/K^+-ATPase activity of *Rutilus caspicus* transferred stepwise from fresh water to salinities of 5, 10 and 15 and acclimated for 48 h at each salinity. Fish at salinity 15* were sampled after an additional 48 h. Values are means + S.D. ($n = 6$). Bars with the same lower case letters are not significantly different from each other ($P < 0{\cdot}05$).

clear osmoregulatory perturbations were observed. No mortality occurred in any of the salinity treatments, which indicates that these fish have the ability to tolerate gradual stepwise salinity changes. In contrast, abrupt transfer to different coastal salinities (12–15) resulted in some mortality (S. Malakpour Kolbadinezhad, unpubl. data). Schofield *et al*. (2006) reported high survival of another cyprinid, the goldfish *Carassius auratus* (L. 1758) under chronic exposure to salinities of 5 and 10, but significant mortality at salinities of 15 and 20.

Changes in haematology, in general, can be explained by changes in ionoregulatory status (Mojazi Amiri *et al*., 2009). Blood haematocrit was reduced after 48 h exposure in 5 compared to the initial levels in the freshwater control group. This can be explained by the osmotic water movement over the course of the 48 h exposure from the red cell, resulting in cell shrinkage. Cleary *et al*. (2002) reported that haematocrit levels of silver seabream *Pagrus auratus* (Forster 1801) decreased following exposure to handling and confinement stress. Following this initial decrease, haematocrit increased in parallel with salinity increase in *R. caspicus*, as has been reported for other species (Gallaugher *et al*., 2001; Baker *et al*., 2005; Mojazi Amiri *et al*., 2009).

Plasma osmotic pressure, or osmolality, is determined by the total concentration of solutes, mostly inorganic electrolytes present in the body fluid. Since Na^+ and Cl^- are the major electrolytes in the body fluid, regulation of both Na^+ and Cl^- is critical for osmoregulation (Kaneko *et al*., 2008). Euryhaline teleosts acclimated to hyperosmotic environments experienced two periods: (1) a crisis period in which there is a rapid increase in gill-ion fluxes accompanied by elevated plasma ions and osmolality, followed by (2) a regulatory period in which an increase in gill NKA activity, together with a proliferation and development of functional MRC, net sodium and chloride efflux increases and plasma ion balance are restored (Evans *et al*., 2005). The increases in plasma osmolality, Cl^-, and Na^+ in *R. caspicus* following exposure to salinities 10 and 15 might indicate that ion uptake mechanisms were not yet down-regulated, resulting in greater net uptake under conditions of greater NaCl availability. Blood osmolality in teleosts ranges from *c*. 280–360 mmol kg^{-1}, and is tightly regulated within a species-dependent range of salinities (Varsamos *et al*., 2005). A comparison of *R. caspicus* to published values in other euryhaline fishes indicates that the levels of Na^+, Cl^- and osmolality are relatively high suggesting that the *R. caspicus* has a relatively poor salinity tolerance, or that it is at least in a temporary state of ion imbalance, as electrolyte concentrations did not recover as has been reported in other species (Wilson *et al*., 2002; Laiz-Carrión *et al*., 2005). Conversely, a reduction of plasma K^+ was accompanied by increasing salinity. It has been shown that the gills of fishes in seawater are permeable to K^+ and that efflux is greater than influx (Sanders & Kirschner, 1983). This would indicate that reduced uptake, rather than increased loss of K^+, is the more important factor (Partridge & Lymbery, 2008).

Lasserre (1971) first described a U-shaped relationship between NKA and water salinity, although this is found largely in euryhaline species that routinely experience rapid salinity changes (Jensen *et al*., 1998). Gaumet *et al*. (1995) suggested that NKA activity is generally lowest in fishes living in a medium whose salinity is equivalent to that of their blood. Reports in the literature, however, are variable. In *R. caspicus*, gill NKA activity decreased with salinity in the short term with activity being the lowest in fish after 48 h at salinity 15, which return towards control levels

at 96 h. In another experiment, reduced NKA activity was observed after abrupt salinity transfers (S. Malakpour Kolbadinezhad, unpubl. data). Responsiveness of gill NKA to environmental salinity is dependent on species, life-history stage and, in some cases, experimental conditions. There are reports of no effect of salinity on NKA activity in longjaw mudsucker *Gillichthys mirabilis* Cooper 1864 (Yoshikawa *et al*., 1993) or of a strong effect of medium salinity on gill NKA activity [*e.g.* turbot *Scophthalmus maximus* (L. 1758): Imsland *et al*., 2003; Mozambique tilapia *Oreochromis mossambicus* (Peters 1852): Kültz *et al*., 1992; *Oncorhynchus keta* (Walbaum 1792): Uchida *et al*., 1997], while others report a negative correlation between water salinity and NKA activity (*F. h. heteroclitus*: Marshall *et al*., 1999; *O. mossambicus*: Lin *et al*., 2004).

Ecological theory would predict that fishes should be adapted to spend the least amount of osmoregulatory energy in environmental salinities they have evolved to live in (Morgan & Iwama, 1991). Also, physiologically, the energy consuming NKA activity would be expected to be minimal at environmental salinities isosmotic to blood (Saoud *et al*., 2007). In general, the effect of gradual salinity change on physiological–osmoregulatory functions requires more study. It would be of interest to see if the *R. caspicus* can survive long-term exposure ($\geq$2 weeks) to salinities $\geq$15 and to measure the effects on plasma ions and gill NKA activity.

The results indicate that *R. caspicus* juveniles need a period of gradual acclimation for its ion-osmoregulatory system to adapt to brackish water. The absence of mortality in *R. caspicus* gradually transferred to a salinity similar to the south Caspian Sea indicates that this transfer method may be valuable in release of juveniles for re-stocking programme, rather than the common practice of abrupt transfer. Additional studies encompassing different salinities, sampling times and other environmental tolerances such as temperature, culture density or diet are, however, needed to further improve stocking success.

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