Comparative Biochemistry and Physiology, Part A 185 (2015) 107–114

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Osmoregulation by juvenile brown-banded bamboo sharks, *Chiloscyllium punctatum*, in hypo- and hyper-saline waters

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ARTICLE INFO

Article history:
Received 19 December 2014
Received in revised form 30 March 2015
Accepted 2 April 2015
Available online 11 April 2015

Keywords:
Elasmobranch
Euryhaline
Gills
Ion transporters
Osmoregulation
Rectal gland
Sodium–potassium ATPase
Stenohaline
Vacuolar H^+-ATPase

ABSTRACT

While there is a considerable body of work describing osmoregulation by elasmobranchs in brackish and saltwater, far fewer studies have investigated osmoregulation in hypersaline waters. We examined osmo- and ionoregulatory function and plasticity in juvenile brown-banded bamboo sharks, *Chiloscyllium punctatum*, exposed to three experimental salinities (25, 34 and 40‰) for two weeks. *C. punctatum* inhabits sheltered coastal areas and bays which can naturally become hypersaline as a consequence of evaporation of water but can also become hyposaline during flood events. We hypothesised that *C. punctatum* would demonstrate a phenotypically plastic osmoregulatory physiology. Plasma osmolality, urea, Na^+ and Cl^- levels increased significantly with increasing environmental salinity. Rectal gland and branchial sodium–potassium ATPase (NKA) activities were unaffected by salinity. Using immunohistochemistry and Western Blotting we found evidence for the presence of the key ion-regulatory proteins vacuolar H^+-ATPase (VHA), pendrin (Cl^-/HCO_3^- co-transporter) and the Na^+–H^+ exchanger isoform 3 (NHE3) in discrete cells within the branchial epithelia. These results indicate that *C. punctatum* is a partially euryhaline elasmobranch able to maintain osmo- and ionoregulatory function between environmental salinities of 25‰ and 40‰. As suggested for other elasmobranchs, the gills of *C. punctatum* likely play a limited role in maintaining Na^+ homeostasis over the salinity range studied, but may play an important role in acid–base balance.

1. Introduction

Although the majority of elasmobranchs are largely stenohaline, at least 170 species of the ~1100 recognised elasmobranch species are able to tolerate brackish or fully freshwater (FW) environments for extended periods (Martin, 2005) suggesting that some degree of euryhalinity is more common than previously thought. While much is known about elasmobranch osmoregulation in normal seawater (~34‰ salinity, SW), considerably less is known about their responses to changes in environmental salinity, and particularly those relating to hypersalinity (where environmental salinities may exceed that of the open ocean). Hypersaline seawaters can develop in coastal environments where exchange with open waters is restricted, and with high evaporation rates and/or low freshwater inputs such as in coastal lagoons, inverted and/or tidal estuaries, and embayments (Potter et al., 2010). In rare cases these environments can be as much as 8.5 times more saline than the open ocean (Brauner et al., 2012), though more typically they range in salinity from 40–160‰ (Potter et al., 2010; Brauner et al., 2012). Elasmobranchs appear to be well represented in many coastal hypersaline environments; for example, 28 species of sharks and rays alone are known to inhabit the Shark Bay area on the West Australian coastline (where waters may range in salinity from 45–70‰ (Vaudo and Heithaus, 2009)). For elasmobranchs, hyposaline waters are more commonly encountered than hypersaline. Hyposaline waters can develop in coastal regions where freshwater runoff from the rivers and streams enters the sea. Only a small number of elasmobranchs are fully euryhaline, allowing them to occupy riverine niches which are uninhabitable to most elasmobranchs.

Living outside the usual salinity of the open ocean poses considerable ion regulatory problems for elasmobranchs. Typically, the fluids and tissues of sharks and rays living in SW are hypertonic with respect to the surrounding seawater which facilitates a slight osmotic influx of water and the net diffusional gain of Na^+ and Cl^- ions (Smith, 1931; Piermarini and Evans, 1998; Wood et al., 2002; Pillans et al., 2008). This gradient means that sharks in SW generally do not need to drink, and any net gain of ions is handled largely by a salt-secreting rectal gland. The accumulation of high concentrations of urea in cells and extracellular fluids accounts for a large proportion of the high internal osmotic pressure of elasmobranchs and, while high levels of urea can affect protein function in other organisms, some unique elasmobranch proteins can only function in the presence of

Abbreviations: FW, freshwater; H_{CT}, haematocrit; MRC, mitochondrial rich cell; NKA, sodium–potassium adenosine triphosphatase; NKCC, sodium–potassium-2-chloride cotransporter; NHE3, sodium–proton exchanger type 3; Pi, inorganic phosphate; SW, seawater/saltwater; VHA, vacuolar-type proton adenosine triphosphatase.

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http://dx.doi.org/10.1016/j.cbpa.2015.04.001
1095-6433/

high urea levels (Yancey and Somero, 1978). Indeed, retaining sufficiently high levels of urea is thought to be one of the reasons why so few elasmobranch species live in FW (Ballantyne and Fraser, 2012).

In low salinity environments, euryhaline elasmobranchs must lessen the osmotic gradient acting on the animal, and do so by lowering plasma urea and Na^+ and Cl^- levels (Hazon et al., 2003; Pillans and Franklin, 2004; Pillans et al., 2006). And although lower than in their SW counterparts, many FW elasmobranchs still maintain relatively high levels of urea in their blood and tissues meaning that animals must contend with a substantial diluting gradient. In FW, the rectal gland is often non-functional and in any case, is unable to reverse its function, so the gills and kidneys take on a larger role in ion and water balance and urea retention. Branchial Na^+ and Cl^- uptake in euryhaline elasmobranchs is achieved via at least two distinct subclasses of mitochondria rich cells (MRC). One MRC subclass possess an apical Na^+/H^+ exchanger (NHE) and a basolateral Na^+K^+ ATPase (NKA), while another subclass demonstrates and apical HCO_3^-/Cl^- exchanger (pendrin) and a basolateral vacuolar H^+-ATPase (VHA) (Piermarini and Evans, 2000, 2001; Piermarini et al., 2002; Reilly et al., 2011). While these major transporters are also present on the gills of SW elasmobranchs, it is thought that their function is most likely acid–base balance (Choe et al., 2007). An increase in the number of MRCs and in the expression patterns of several of these transporters is consistent with the idea that the ion-regulatory role of the gill is greater in FW than in SW (Piermarini and Evans, 2000; Choe et al., 2005; Reilly et al., 2011).

In contrast with the hyposaline environment, relatively less is known about the osmoregulatory responses of marine elasmobranchs in hypersaline environments. In normal SW, the slight hyperosmotic gradient between the fish and the environment means that animals do not need to drink to maintain water balance (Ballantyne and Fraser, 2012). In hypersaline environments, the challenge remains to ensure that the directionality of the osmotic gradient is maintained; however this may mean contending with significant osmolyte loads and the energetic costs of maintaining them. Urea production and retention is a significant energetic cost for elasmobranchs (Ballantyne and Fraser, 2012) and acute transfer of elasmobranchs to a higher salinity environment can stimulate drinking in some species (Anderson et al., 2002b). As a consequence, plasma Na^+ and urea levels have been shown to increase in a few elasmobranchs acclimated to hypersaline (120–140%) SW (Mandrup-Poulsen, 1981; Anderson et al., 2002a; Good et al., 2008; Yamaguchi et al., 2009). However, little else is known about the effect of acclimation to hypersaline environments on the osmoregulatory physiology of elasmobranchs.

We examined the effects of environmental salinity on the osmoregulatory physiology of juvenile brownbanded bamboo sharks, *Chiloscyllium punctatum*. *C. punctatum* is an oviparous species that inhabits the continental shelf and coastal waters of the Indo-West Pacific including Australia (Compagno, 1984; Michael, 2001; White et al., 2006; Last and Stevens, 2009). They have been found not only in the hypersaline waters of Shark Bay, Western Australia (at 41.6‰; White and Potter, 2004) and Hervey Bay, Queensland (36–38‰; Gutteridge et al., 2011) but also in dynamic estuarine areas such as Mooloolaba (Sunshine Coast, Queensland) where the salinity can drop as low as 25‰ during a flood event (pers. obs.). Due to its relatively broad tolerance of high and low environmental salinities, *C. punctatum* is an interesting model system for investigating the effects of environmental salinity on osmo- and ionoregulation in elasmobranchs. We acclimated juvenile *C. punctatum* to three environmental salinities (25‰, 34‰ and 40‰) and compared haematocrit, plasma osmolyte levels and branchial and rectal gland NKA activities. We also qualitatively compared the abundance and distribution of four key ion transporters in the branchial epithelium: vacuolar H^+-ATPase (VHA), pendrin (Cl^-/HCO_3^- co-transporter) and the Na^+–H^+ exchanger isoform 3 (NHE3), but found no evidence of the $Na^+K^+2Cl^-$ (NKCC) transporter. We hypothesised that animals would demonstrate acclimatory responses in haematological and ion transport systems to changing environmental salinity that would allow them to contend with the osmoreglatory challenges that each environment poses.

2. Materials and methods

2.1. Animal husbandry and acclimation

Juvenile *C. punctatum* (3–6 months old, mass = 36.37 ± 29.1 g; n = 15) were collected from a captive bred population at UnderWater World (Mooloolaba, QLD, Australia) and transported by car to The University of Queensland, Brisbane. Sharks were divided across nine 40 L aquaria (25 °C, 34‰) and allowed to acclimatise for a week. Salinity was monitored daily using a portable optical refractometer. Saltwater was made using reverse osmosis water and commercial sea salt (Ocean Nature, Aquasonic). Sharks were fed daily with raw prawns and fish. Each of the sharks were then assigned to one of three experimental salinities: hyposaline (25‰; n = 5), hypersaline (40‰; n = 4) or control (34‰; n = 6) SW. Tanks were adjusted to these salinities slowly over a period of 10 days through the addition of reverse osmosis water (hyposaline) or extra salt (hypersaline). When appropriate salinities were attained for all treatments, the sharks were allowed to acclimate for a period of two weeks. Sharks were fasted for 72 h before being anaesthetised in clove oil (Sigma Aldrich, Australia; 175 mg/L) and then pithed.

2.2. Tissue sampling

A blood sample was taken from each euthanized animal via caudal puncture. The first hemibranch of the gills were then excised and snap frozen in liquid nitrogen before being stored at −80 °C until required for NKA activity analyses. The second hemibranch on each side were also excised and fixed in neutral buffered formalin (NBF) overnight at 4 °C. Fixed samples were placed into 70% ethanol and stored until processing at −20 °C. The rectal gland was also removed from each shark, snap frozen and stored at −80 °C.

2.3. Haematocrit and plasma solute concentrations

Blood samples were immediately centrifuged at 5000 *g* for 3 min and the plasma was removed and stored at −20 °C prior to analysis. A small capillary tube of whole blood was centrifuged at 10,000 *g* for 1 min to determine haematocrit (H_{CT}). The osmolality of the plasma was determined in triplicate using a Vapro 5520 vapour pressure osmometer (Wescor, Logan, UT, USA). Osmolality of the aquarium water was also measured. Plasma samples were diluted by 55% in ultrapure water (Millipore) and ion concentrations (Na^+, Cl^- and K^+) were determined using an iStat Analyser (Abaxis, USA) and EC8+ cartridges. Plasma urea concentration was determined in triplicate using a Quantichrom™ Urea Assay Kit (BioAssay Systems, USA). Briefly, plasma samples were diluted 1:60 in ultrapure water (Millipore) with 5 μL then added to 200 μL of working reagent. This reaction mixture was allowed to incubate at room temperature for 30 min and absorbance read at 485 nm.

2.4. NKA activity

The activity of NKA in samples of rectal gland and gill tissue was measured as per Else et al. (1996) as described in Cramp et al. (2009). Briefly, samples of rectal gland and gill tissue were taken from storage at −80 °C, weighed and suspended in ice-cold homogenisation buffer (in mmol L^{-1}: 250 sucrose, 5 EDTA, 20 imidazole and 2.4 sodium deoxycholate, pH 7.4) at a concentration of 2.5% w/v and 5% w/v, respectively. Homogenates (50 μL) were incubated for 10 min at room temperature in assay buffer (in mmol L^{-1}: 80 Tris (hydroxymethyl) aminomethane HCl, 5 $MgCl{\cdot}6H_2O$, 120 NaCl, 20 KCl and 5 NaN_3, pH 7.5) with or without ouabain (1 mmol L^{-1}). The difference in