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Full Length Research Paper

Calmodulin Gene Expression in Sarotherodon melanotheron: A Salinity-Dependent Study in Drainage Basins

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Calmodulin (CaM) is an ubiquitous Ca^{2+} binding protein that plays an important role in signalling events mediating the hormone secretion such as pituitary hormones. Previous laboratory experiments have demonstrated that this gene is relatively over expressed in gill tissues of *Sarotherodon melanotheron* acclimated to freshwater. In this study, the relationship between mRNA levels of the CaM in the gills and environmental salinity was investigated in wild populations of this species sampled during both the rainy and the dry season from six coastal, estuarine and freshwater sites in Senegal and Gambia. In both seasons the highest CaM mRNA levels were recorded in freshwater where the highest prolactin (PRL) and lowest growth hormone (GH) expression levels were previously reported. In the dry season CaM expression was highest at the lowest salinities (Guiers lake and Balingho) than in the hypersaline water sites of the Saloum estuary (Missirah, Foundiougne and Kaolack) and did not differ between these three sites. The amounts of CaM mRNA were not different between Balingho and Hann bay locations. The expression of CaM showed a similar pattern in rainy season, being highest in freshwater locations of Guiers lake and lowest in the most saline sites (Hann bay, Missirah, Foundiougne and Kaolack).The CaM mRNA levels were significantly negatively correlated with environmental salinity in both the rainy and the dry season. All together these results may indicate a role of CaM in the acclimation to hypo-osmotic stress possibly through the regulation of cell volume, calcium uptake and the expression of genes involved in osmoregulation such as PRL and GH. In the rainy season, overall mean expression of the CaM was higher than in the dry season, which may have reflected more variable particularly sudden fluctuations in salinity and poorer overall water quality.

Keywords: Acclimation, Calmodulin, Estuary, Gene expression, Salinity, Fish, *Sarotherodon melanotheron.*

INTRODUCTION

Important fluctuations (diurnal and seasonal) in physicochemical factors such as water temperature, dissolved oxygen and salinity (McKinsey and Chapman, 1998, Whitfield et al., 2006, Brinda and Bragadeeswaran, 2005, Jaureguizar et al., 2004) occur in estuarine environments. Estuarine fishes and those that spend time in these areas to feed or reproduce must be able to quickly respond to these environmental changes. These species have different and specific tolerance ranges for each of these abiotic factors (Chung, 2001, Rajaguru and Ramachandran, 2001, Yamanaka et al., 2007), and respond to their variations by physiological, biochemical and molecular adjustments, which in turn may affect normal biological functions such as growth and reproduction (Cossins et al., 2006, Jackson et al., 1998, Nikinma and Waser, 2007). An example for this is the black-chinned tilapia *Sarotherodon melanotheron,* which is euryhaline estuarine teleost particularly notable for its ability to tolerate a wide range of environmental salinities. The species is widely distributed in West-African coastal, estuarine and lagoon ecosystems such the Saloum and the Gambia rivers, which exhibit extreme variations in salinity (from freshwater to up to 130 psu) and where seasonal variations in salinity can be considerable (Panfili et al., 2004, Panfili et al., 2006). While *S. melanotheron* can colonise a broad range of salinities, fish inhabiting extremely hypersaline waters of the Sine Saloum estuary

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exhibited impaired growth performance and precocious reproduction. These phenotypic differences have been interpreted as indicative of hypersaline stress, but relatively little is known about mechanisms of salinity adaptation in this species, in particular the molecular responses which may underlie its exceptional euryhalinity.

The acclimation to particular environmental stress involves the transcriptional activation of gene complexes involved in regulatory pathways. These genes encode for a variety of proteins involved in signalling events mediating the expression, synthesis and secretion of a variety of proteins such as metabolite transport proteins involved in the maintenance of homeostatic balance, enzymes that allow alternative metabolisms, regulatory hormones or protective proteins including chaperones and antioxidants specialised in avoiding protein disruption and cellular damage. Amongst these gene complexes involved in regulatory pathways, there is the Calmodulins (CaM), an ubiquitous Ca^{2+} binding protein that acts through Ca^{2+}dependent signaling pathways to regulate various biological processes. When associated with calcium ions, the CaM forms a CaM/Ca^{2+} complex that interacts with its target proteins to activate cellular processes such as transcriptional and translational mechanisms or hormone secretion. CaM is well known for its regulatory role on the synthesis and/or secretion of hypothalamic factor (growth hormone (GH), somatostatin (SRIF) and pituitary hormones including prolactin (PRL), luteinizing hormone (LH), and growth hormone (GH), hormones that are involved in fish osmoregulation. It has been also demonstrated that CaM is involved in the restoration of cellular volume toward its normal state further to hypoosmotic stress (Edmonds and Koenig, 1990). The CaM may, therefore, play an important role in the acclimation of *S. melanotheron* to the salinity variations that prevail in its natural environment.

We have previously isolated (Tine et al., 2008) a copy of a gene encoding CaM1 [ES881188] in a freshwater SSH library created from gills of *S. melanotheron* acclimatised either to freshwater water or hypersaline. Accordingly, we (Tine et al., 2008) proposed that the gene must be involved in the acclimation of this species to salinity variations. The aim of this study was, therefore, to investigate the hypothesis that the relative expression of CaM1 would be correlated with environmental salinity in wild population of *S. melanotheron* from various coastal marine, estuarine and freshwater drainage basins. Gene expression analyses were conducted on fish sampled during both the rainy and the dry season, because the estuarine environments are known to vary significantly in their salinity regimes between these two seasons. The same sampling sites have previously been studied for the effects of salinity on life history traits and induction of osmoregulatory genes (growth hormone and prolactin) in *S. melanotheron* (Panfili et al., 2004, Tine et al., 2007). The relative expression of CaM1 mRNA was quantified by real time PCR.

MATERIAL AND METHODS

Sampling design of natural populations

Six natural populations (Figure 1) of the black-chinned tilapia *S. melanotheron* were sampled in 2006, at the end of the dry season (May). Two sampling sites, Guiers Lake and Hann Bay have the particularity to not undergo salinity variations throughout the year. Fish were also collected at three locations of the Saloum estuary (Missirah, Foundiougne, and Kaolack) and in one location of the Gambia estuary (Balingho). These sites are known to exhibit large spatial and seasonal variations in salinity. For each location, the salinity and temperature were measured *in situ* with a refractometer (ATAGO) and a thermometer, respectively. Fish sampling was carried out by local fishermen using castnets with small mesh size. Only five fish were sampled from each castnet thrown in order to limit fish stress and prevent variability due to manipulation. Since gene expression could conceivably be influenced by differences in developmental or sexual stage, only size classes between 120 and 160 mm fork length with sexual stage 1 or 2, corresponding to immature individuals were selected for the analyses. Gills were extracted from these individuals and stored in *RNA later* (Ambion) at 4 °C for 24 h and then at -20 °C until processing.

Total RNA extraction, reverse transcription and real-time PCR analysis

Total RNA was extracted from the gills conserved at -80°C for experimental samples and in RNAlater of natural samples with TRIZOL® reagent (Gibco-BRL, USA), according to the manufacturer's instructions. RNA concentrations were determined spectrophotometrically and RNA integrity was verified by 1% TAE 1X agarose gel electrophoresis (Tris 40mM, acetate, EDTA 1mM). The relative expression of CaM was analyzed in both the rainy and the dry season. Primers (CaM-F: TTTTGACGGATTCTTTGTCG ; CaM-R : GATCAGGGAAGCAGACATCG) used to amplify these genes were designed using Primer 3 software. Gene expression was quantified using quantitative real-time PCR (qRT-PCR) on a Light Cycler (Roche Molecular Biomedicals) using the QuantiTect SYBR Green PCR Master Mix (Qiagen) kit. Quantification of each sample was performed in total volume of 10 µl containing 1 µl of cDNA, 0.5 µl of each primer and 1X of SYBR Green master mix (Qiagen). Each qRT-PCR reaction was conducted in duplicate with initial denaturation step of 15 min at 95°C, followed by an amplification of the target cDNA for 40 cycles, each cycle consisting of a denaturation at 95°C for 15 seconds, annealing between 54°C and 55°C for 15 seconds and elongation at 72°C for 15 seconds. To determine qRT-PCR efficiency of each used primer pair, standard curves were generated using five serial dilutions (1, 1/10, 1/50, 1/ 100, 1/500) of a unique cDNA sample constituted of a pool of 6 cDNA from each population to be analysed. Efficiencies qRT-PCR (*E*) were calculated from the given slope of the standard curve according to the equation $E=10^{(-1/slope)}$. The products of amplification were validated by analysing the amplicon size on agarose gel electrophoresis. Results are shown as changes in relative expression normalised to the reference gene, β-actin using the $2^{-(\Delta\Delta Ct)}$ method described by Ptaffl (2001). β-actin was previously analysed and shown no change with salinity acclimation.

Statistical analysis

CaM expression data at each site were expressed as mean ± SD. Bartlett and Kolmogorov-Smirnov tests were first conducted to respectively evaluate the variance homogeneity and the normality of the data. Since the gene expression data were not normally distributed and did not have uniform variance, a Kruskal-Wallis

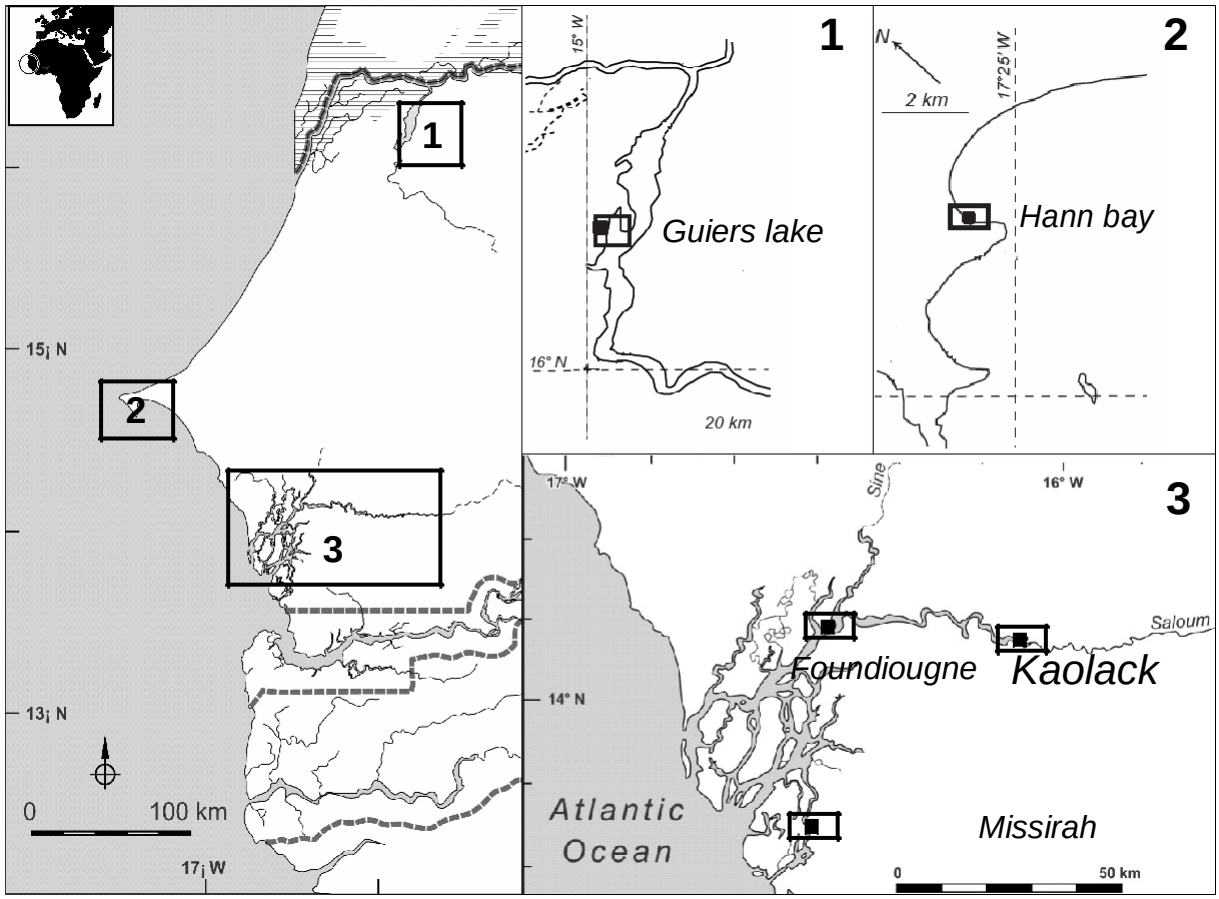

Figure 1. Sampling locations of the black-chinned tilapia *Sarotherodon melanotheron* in Saloum and Gambia estuaries. Fish were collected in 2006, in rainy season when salinity varied only between 0 and 37 psu amongst sites, and in the dry season when extremely hypersaline conditions were observed (up to 100 psu) in some estuarine sites.

non-parametric analysis of variance (ANOVA) followed by Mann-Whitney post-hoc test was performed to reveal differences in means between populations. These tests were performed with Statistica software (http://www.statsoft.com/). For all tests, a probability of less than 5% ($P < 0.05$) and a confidence of 95% are considered as fiducial level of significance.

RESULTS

Sampling characteristics

The dry season was associated with a much wider range of salinities, specifically because salinity was markedly higher at all of the estuarine sites, up to an extreme of 100 psu at Kaolack (Table 1). In the rainy season, the salinity ranged between 0 and 37 psu amongst the sites. Thus, it was 0 psu at the freshwater reference site (Guiers lake) and 37 psu at the reference seawater site (Hann bay), with a range of similar or intermediate salinities at the other estuarine sites (Table 1). The water temperature was overall higher in rainy season than in dry season. In the dry season, the temperature varied slightly, being ranged between 26 and 29°C amongst the sites. In rainy season, temperatures were higher at Guiers lake and Hann bay locations and lower at Kaolack location. The seasonal variations in water temperature were relatively lower. The highest amplitudes (4.5 and 4°C) were respectively recorded at Hann bay and Guiers lake sites

Gill gene expression in the tilapia populations

In the dry season, there were significant differences between sites for the CaM relative expression (Figure 2). CaM expression was highest at the lowest salinities (Guiers lake and Balingho) than in the hypersaline water sites of the Saloum estuary (Missirah, Foundiougne and Kaolack) and did not differ between these three sites. The amounts of CaM mRNA were not different between Balingho and Hann bay. The expression of CaM showed a similar pattern in rainy season, being highest in freshwater locations of Guiers lake and lowest in the most saline sites (Hann bay, Missirah, Foundiougne and

Table 1. Sampling characteristics

Drainage basin	Station	Salinity (psu)		WT(°C)		N	FL ranges (mm)	
		DS	RS	DS	RS		DS	RS
Guiers lake	Guiers lake	0	0	28	32	10	120-155	120-134
Gambia river	Balingho	22	0	28	30	10	124-156	121-132
Hann bay	Hann bay	37	37	28	32.5	10	122-150	128-156
Saloum	Missirah	40	32	28	31	10	120-160	123-160
Saloum	Foundiougne	60	37	28	31	10	120-141	122-144
Saloum	Kaolack	100	28	26	28	10	121-145	120-138

DS: dry season; RS: rainy season; WT: water temperature; N: number of individuals; FL: fork length

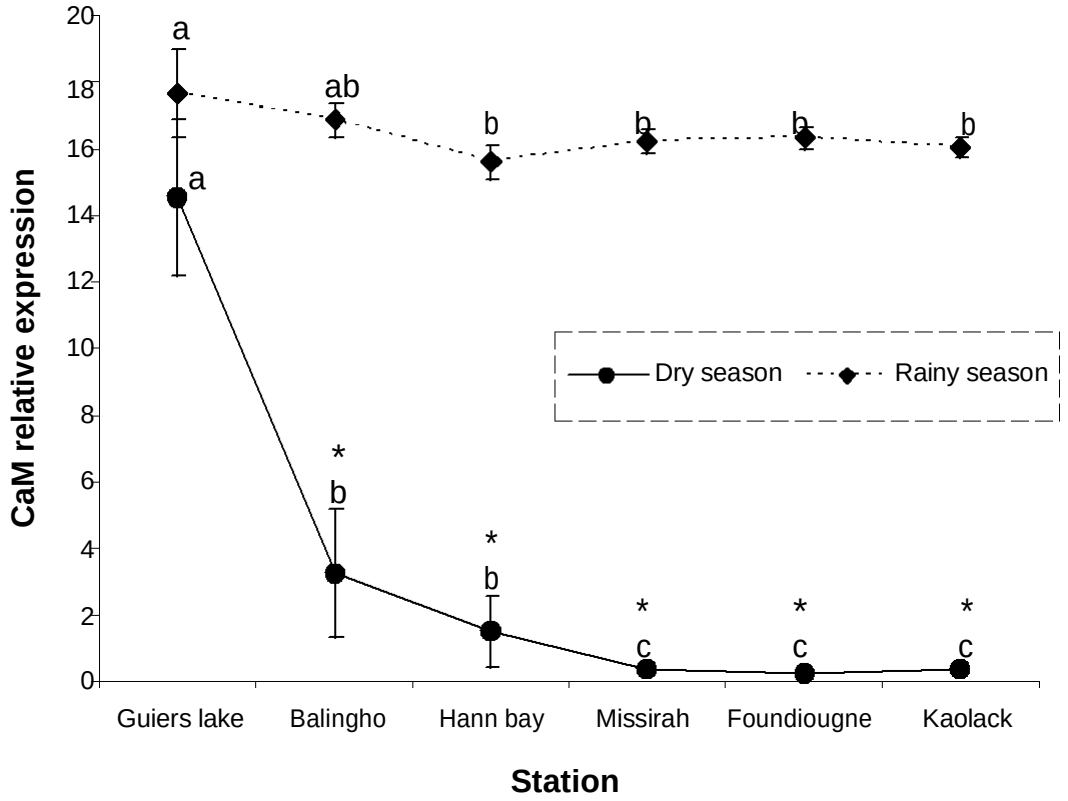

Figure 2. Mean ± (SD) of CaM mRNA levels of the black-chinned tilapia *S. melanotheron* from six wild populations sampled in both the rainy and the dry season. The mRNA expression levels represent the relative expression normalized to β-ACTIN RE: relative expression; RS: rainy season; DR: dry season. The same letters above the bar indicate no significant differences between the mean values (P>0.05) and the asterisk above the bars indicates significantly different mean values (P<0.05).

Kaolack). The CaM mRNA levels did not significantly differ between Balingho, Hann bay and the locations of the Saloum estuary.

The comparison between the dry and the rainy season showed that the wet season was associated with an overall increase in CaM mRNA. These changes in expression were not, however, obviously related to any seasonal change in salinity, and varied between sites. For example, there were significant increases (P<0.05) in the expression of CaM in Hann bay, where the salinity was constant at 37 psu. (Figure 2).

Correlations between Salinity and mRNA levels

A significant impact of environmental salinity on CaM relative expression was observed in wild populations of *S. melanotheron*. There was a significant negative Spearman rank correlation between salinity and relative expression of CaM (R^2 = 47.74%; P < 0.001) in dry

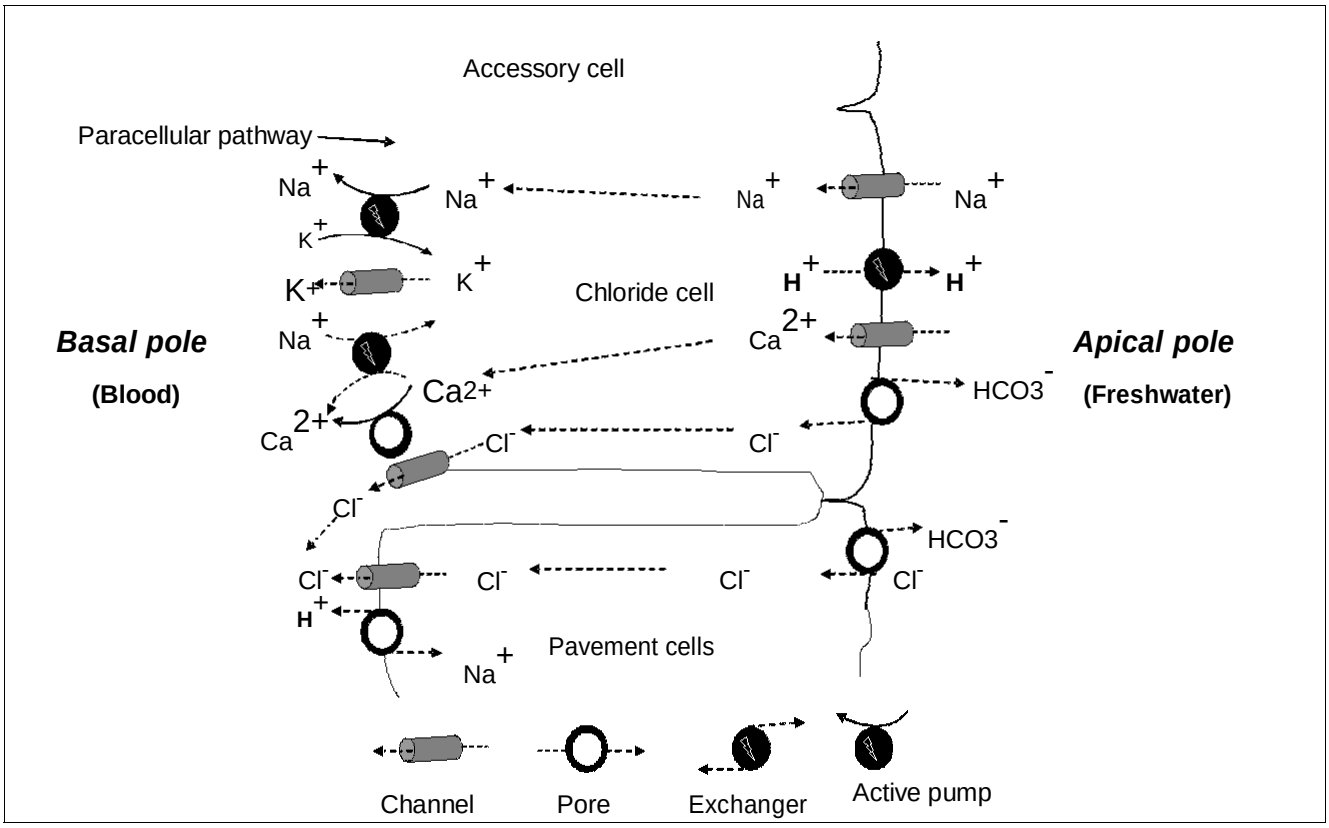

Figure 3. Model of ion uptake in the teleost fish gill in freshwater (adapted from Marshall (2002))

season. Likewise, the relative expression of CaM was negatively correlated to the salinity in dry season (R^2 = 40.18%; $P < 0.001$), although the correlation was lower than in the dry season.

Discussion

The results demonstrate that CaM which we have identified from laboratory studies of salinity acclimation in *S. melanotheron* (Tine et al., 2008) show differential expression as a function of environmental salinity in wild populations. The CaM mRNA levels were significantly negatively correlated with environmental salinity in both the rainy and the dry season. Overall mean expression of the CaM was higher the rainy season than in the dry season, which may have reflected more variable particularly instability in salinity and poorer overall water quality.

Cellular volume regulation

Our results showed that CaM mRNA levels in both the rainy and the dry season were higher in freshwater acclimatized fish by comparison to seawater and hypersaline water acclimatized fish. Thus, the CaM expression pattern in wild population fully matched results obtained during experimental settings (Tine et al., 2008), where its mRNA levels were highest in fish acclimated to freshwater by comparison to those living in hypersaline water. It has been demonstrated that hypoosmotic stress cause osmotic swilling that cells compensate by increasing the membrane permeability to specific intracellular inorganic ions (most commonly K^+ and Cl^-) and organic compounds with small molecular weight (Graf et al., 1995 , Wehner et al., 1992, Haddad et al., 1991). The efflux of K^+ and water occurs essentially through a channel calcium/calmodulin dependent the membrane which is activated by the CaM/Ca^{2+} complex (Wheatly et al., 1999). Therefore, the highest expression levels in freshwater might reflect a direct role of CaM in the regulation of cellular volume.

Figure 3 illustrates the nature of the ions transport across the membrane when the cells are exposed to hypo-osmotic conditions. According to this model, the ions H^+ are extruded from the cell via the H^+-ATPase localized on the apical surface of the cell. This generates a negative potential inside the cell leading a passive transport of Na^+ via the Na^+ channels. This phase is followed by absorption of Na^+ at the basolateral membrane via the Na^+, K^+-ATPase pump. The K^+ ions

absorbed in the cell by the Na^+, K^+-ATPase are recycled via the channels of the basolateral membrane. The Ca^{2+} are then absorbed by the Ca^{2+}-dependent ATPase and by the Na^+/Ca^{2+} exchangers located on basolateral membrane of the cell. The CaM has been thought to play a regulatory role in the membrane Ca^+-ATPase which is the mean source of Ca^{2+} uptake from the external medium. Therefore, in addition to its evolvement in the restoration of cellular volume, the highest expression levels of CaM in freshwater may be attributed to a role of this protein in Ca^{2+} homeostasis. This conclusion is supported by observations on the pearl oyster, *Pinctada fucata* that has the highest CaM expression levels in the gill, the mean organ for Ca^{2+} uptake in this species (Li et al., 2004). This interpretation is also in agreement with the fact that the Ca^{2+} uptake in crayfish via Ca^{2+}-ATPase is calmodulin dependent (Wheatly et al., 1999).

Regulation of gene expression

CaM/Ca^{2+} complex is known as an important factor in regulating gene expression particularly, prolactin and growth hormone, two genes whose the involvement in the acclimation to salinity changes has been demonstrated in many teleost fishes. It has been demonstrated that CaM activates the expression of prolactin by acting on its promoter through a proximal enhancer element (Davis et al., 1991) whereas an overexpression of CaM suppressed grass carp GH promoter activity (Huo et al., 2005). Many GH biological functions such as somatic growth, body metabolism and cell differentiation are mediated by IGF, a polypeptide produced under the stimulatory influence of GH. IGF can also exert a feedback action to inhibit GH synthesis and secretion and it has been demonstrated that IGF inhibits GH synthesis in carp pituitary cells through up-regulation of CaM gene expression (Huo et al., 2005). In this study we do not have measures of PRL and GH expression, but a previous study conducted on same populations has shown that the PRL expression exhibited a similar pattern, with higher expression levels in freshwater by comparison to seawater and hypersaline water. GH showed a different pattern, being high in seawater in comparison to freshwater and hypersaline water. Although these results do not bear on the same samples, the higher CaM expression levels in freshwater probably reflects its involvement in acclimation of hypo-osmotic condition though regulation of the expression genes related to osmoregulatory processes.

Seasonal variations in CaM expression

It is not clear why overall relative expression for these genes was higher in the rainy season than in the dry season. This could not be ascribed to salinity per se, because large changes in expression were observed in sites such as Guiers lake and Hann bay, which did not vary in salinity between the seasons. The water temperature cannot explain overexpression during the rainy season, because it did not vary greatly between seasons (4.5°C maximum), as previously reported by Albaret et al. (2004) and Simier et al. (2004). One likely explanation is that it reflected poorer overall water quality at most of the sites. The torrential downpours which characterise the rainy season in this area cause large and sudden variations in water quality, due to runoff of soil and other, potentially toxic, materials. The water turbidity in these estuarine environments is significantly increased in the rainy season (Albaret et al., 2004) which, at extreme levels, is known to cause gill damage in tilapia (Ardjosoediro and Ramnarine, 2002). Another possible explanation is that during the rainy season, salinity in the estuaries is very unstable and can decrease considerably due to inputs of freshwater from precipitation. Thus, unstable salinity may represent a major stress factor in this season and it may affect the populations of *S. melanotheron*. Therefore, the highest expression levels of CaM1 may have reflected a need to respond to rapid changes in salinity of unknown amplitude.

CONCLUSIONS

The expression levels of CaM differed significantly among six natural populations of the black-chinned tilapia *S. melanotheron* seasonally acclimatised to salinities ranging from 0 to 100 psu. The significant correlations between CaM transcription levels and salinity strongly suggest that the gene is involved in the adaptation of *S. melanotheron* to the salinity variations it encounters in its natural environment. The CaM expression pattern during the dry season, when the salinity range was very wide and relatively stable, provided some evidence that the gene is activated in freshwater to participate to the restoration of hydromineral balance. Overall, a higher CaM expression in the rainy season may have reflected a need to respond to sudden and unpredictable variations in salinity and/or poorer overall water quality by comparison with the dry season. The results of this study are the first step towards the characterization of the adaptive mechanisms of *S. melanotheron* to quite different environmental conditions. Understanding these mechanisms will be valuable tools for marker development for aquaculture related questions such as growth, reproduction or survival.

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