Effects of Cortisol, Vasotocin and Salinity on the Expression of Aquaporin-1 in Silver Sea Bream *Sparus sarba*

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Abstract of thesis entitled:

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The present experiments investigated the effects of salinity and hormones on the relative expression of hypothalamic pro-vasotocin, and aquaporin-1 (AQP-1) in intestine, gills and kidney of the silver sea bream *Sparus sarba*. With the use of immunohistochemical techniques, immunoreactivity of AQP-1 was detected at the basal side of enterocytes and gill chloride cells, and at the apical brush border of kidney tubules whereas AQP-3 was only localized in similar positions in the gills and intestines. AQP-1 was relatively more ubiquitous than AQP-3 and was localized with same cell types as the electrogenic Na^+-K^+-ATPase in gills and kidney.

In the second part of our study, cDNA of AQP-1 and pro-vasotocin were cloned from the silver sea bream. An AQP-1 full clone was isolated from kidney and intestine and it consists of 904 bp with an open reading frame of 774 bp. The deduced amino

sequence of sea bream AQP-1 shares highest identity with AQP-1a of gilthead sea bream (97.7%) and AQP-1a of other fish species (83.6% to 95.8%), however, considerably low identity was found between the silver sea bream AQP-1 and AQP-1b of gilthead sea bream (56%). The silver sea bream AQP-1 possesses basic features of a functional aquaporin and AQP-1, which includes two channel-forming asparagine-proline-alanine (NPA) signature motifs, six transmembrane domains, residues of the pore-forming region and a potential mercurial inhibiting site (Cys-178). The water channel was ubiquitously expressed in gills, liver, intestine, rectum, kidney, heart, urinary bladder and blood cells. A partial fragment of pro-vasotocin was isolated from hypothalamus of silver sea bream and consists of 184 bp, including encoding regions for the processing and amidation signal, vasotocin hormone and part of the neurophysin.

Semi-quantitative RT-PCR analysis was used for studying the effect of salinity and hormones on expression of AQP-1 and pro-vasotocin. In the long-term salinity acclimation experiment, the sea bream were acclimated to six different salinity regimes (0‰, 6‰, 12‰, 33‰, 50‰, 70‰) for four weeks. The abundance of AQP-1 transcript was the highest in intestine of 70‰-acclimated fish among different salinity groups and there was also a statistically significant increase in 12‰-acclimated fish.

Branchial AQP-1 expression was significantly upregulated in sea bream acclimated to freshwater. In contrast, the hypothalamic pro-vasotocin expression was significantly downregulated during freshwater acclimation. In addition, the sea bream were also subjected to an abrupt 6‰ transfer at different time intervals (2, 6, 12, 72 and 168 hours). RT-PCR analysis revealed there was a transient decrease in branchial AQP-1 expression two hours after abrupt hypo-osmotic exposure and the expression levels subsequently returned to the seawater control levels. The expression levels of hypothalamic pro-vasotocin were not significantly altered by the abrupt exposure treatment.

Lastly, single doses of cortisol (50 μg/g tissue) or vasotocin (1 μg/g tissue) were administered to seawater-acclimated sea bream with further three-day stabilizing period in seawater followed by an abrupt 6‰ exposure or administered to seawater transfer controls for three days. Cortisol markedly stimulated intestinal expression of AQP-1 in both the seawater transfer control and abrupt 6‰ transfer groups. Vasotocin treatment did not significantly modify AQP-1 expression in all tested organs. Hypothalamic pro-vasotocin expression levels were similar among different treatment groups.

The present study had demonstrated the responsiveness of intestinal and branchial AQP-1 expressions of the silver sea bream to environmental salinity perturbations. Further to this, cortisol was observed to upregulate the transcription of AQP-1 in the intestine. Pro-vasotocin expression was altered by long-term salinity adaptation, however, the linkage of this alteration to AQP-1 functioning in different osmoregulatory organs is yet to be elucidated.

摘要

本研究探討在體外的鹽度和激素對鯛魚(*Sparus sarba*)下丘腦的前管催產素(pro-vasotocin)和腸、鰓及腎的水通道蛋白-1 (Aquaporin-1)的傳信核糖核酸相對表達的影響。在實驗的第一部份，我們利用免疫組織化學方法，分別在腸的腸胃道細胞基部、鰓的泌氯細胞及部份腎小管的刷狀邊緣發現了對水通道蛋白-1抗體所產生的免疫染色反應。在相同的測試方法下，只有腸的腸胃道細胞基部及鰓的泌氯細胞對水通道蛋白-3產生免疫染色反應。以上結果顯示水通道蛋白-1較水通道蛋白-3更廣泛地分佈於各主要滲透調節器官。另一方面，在鯛魚鰓及腎中，水通道蛋白-1與鉀鈉腺苷三磷酸酶共處於相同種類的細胞之中。

在本研究的第二部份，我們利用基因克隆方法將水通道蛋白-1及前管催產素的cDNA從鯛魚分離。水通道蛋白-1 的全長cDNA從腎臟及腸分離出來，全段cDNA及開放閱讀框架的長度分別是904 bp及774 bp。本種鯛魚的水通道蛋白-1的推導胺基酸序列與另一種鯛魚(*Sparus aurata*)的水通道蛋白-1a最為相似(97.7%)，與其他魚類的水通道蛋白-1a亦有很高的相似度(83.6%至95.8%)，但與鯛魚(*S. aurata*)的水通道蛋白-1b的序列的相似度較低(56%)。本種鯛魚的水通道蛋白-1具備很多代表水通道蛋白的功能性之主要特徵，包括兩個天門冬酰胺-脯胺酸-丙氨酸序列(NPA motif)、六個跨膜結構域、形成水通道區域的主要殘基及

一個潛在的汞抑制部位(半胱氨酸-178)。另一方面，從鯛魚的下丘腦分離出來的前管催產素的cDNA片段共有184 bp，其編碼包括了負責處理及酰胺化的部份、管催產素及部份神經垂體激素運載蛋白。

本研究的第三部份採用半定量逆轉錄聚合酵素連鎖反應，探討體外的鹽度和激素對前管催產素及水通道蛋白-1的傳信核糖核酸相對表達的影響。。在長期鹽度馴化實驗中，鯛魚適應於六個不同鹽度的環境(0‰、6‰、12‰、33‰、50‰及 70‰)，適應期為四星期。在超高鹽度(70‰)的情況下，腸水通道蛋白-1的傳信核糖核酸相對表達達至最高水平，而適應於鹽度12‰的鯛魚亦錄得顯著的向上調節。另一方面，鰓水通道蛋白-1的相對表達在淡水(0‰)的環境之下錄得明顯的增加，而對前管催產素的相對表達卻明顯地向下調節。在突然低滲透壓轉變的實驗中，鯛魚突然被轉移至鹽度6‰的環境。鰓水通道蛋白-1的相對表達在低滲透壓轉移的兩小時後明顯地向下調節，但逐漸地回復至海水對照組的水平。腦下丘的前管催產素的傳信核糖核酸相對表達並不受突然低滲透壓轉變影響。

最後，鯛魚接受單劑量的皮質醇(50 微克/ 克組織)或管催產素(1 微克/ 克組織)注射，經過三天在海水(‰)的穩定期後，再被突然低滲透壓轉移(6‰)或海水對照轉移。不論在突然低滲透壓轉移或海水對照轉移情況下，皮質醇能夠刺激

腸水通道蛋白-1的相對表達。管催產素治療沒有明顯地改變各已檢驗器官內水通道蛋白-1的相對表達。此外，前管催產素的相對表達不受激素治療影響。

本研究驗證了腸及鰓水通道蛋白-1對體外鹽度變化的反應性。除此之外，皮質醇令腸水通道蛋白-1的傳信核糖核酸表達向上調節。另一方面，雖然前管催產素的相對表達隨鹽度適應而改變，但這轉變與各主要滲透壓調節器官的水通道蛋白-1功能之間的關係仍有待進一步探討。

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Chapter 1

Introduction

Like all aquatic animals, teleost fishes face a basic problem for regulation of body fluid composition and osmolality, particularly when faced with a variable external environment. Osmoregulation in teleosts is accomplished by integrated effort of ion and water transport activities of gills, kidney and intestine (Evans, 1993; Bentley, 2002). In hypo-osmotic freshwater environment, teleosts need to overcome excessive water gain and ion losses by excreting water through kidney and absorb salts through intestine and gill. Seawater teleosts need to compensate their salt loading and water loss by water absorption in intestine after drinking large amounts of seawater and secrete salts through gills and kidneys. Compared with extensive research on ionic homeostasis, relatively scarce studies and information are known about the cellular mechanisms regulating water transport in these animals.

1.1 Movement of water molecule across cell membrane

Over a century ago, Overton (1896) has demonstrated permeability of water and some nonelectrolytes across cell membranes is directly proportional to their lipid solubility. Diffusion of water molecules across biological membrane is very slow and requires a high activation energy (E_a>10 kcal/mol). The osmotic permeability of some types of epithelial cells, however, was much too large that could be explained by

simple diffusion through the plasma membrane. Overton suggested small hydrophilic solutes could also penetrate the membrane regardless of their molecular sizes. He proposed a 'mosaic' membrane which is largely lipid but with aqueous patches which allow those small hydrophilic molecules to pass through.

Later studies revealed high water permeability in human erythrocytes and this high water permeability appeared to be sensitive to mercurial sulfhydryl reagents. By measuring red blood cell osmotic water permeability and diffusional water permeability, the osmotic permeability coefficient (P_f) is demonstrated to be 2.5 times higher than the diffusional permeability coefficient (P_d) (Sidel & Solomon, 1957; Paganelli & Solomon, 1957). In addition, diffusion of water through pure lipid bilayers occurs with high activation energy (E_a > 10 kcal/mol) while water flow through human red blood cell is much faster with a relatively low activation energy (E_a < 5 kcal/ mol). This indicated the presence of water-filled channels or pores in the membrane. Since then, restless work has been carried out for decades and this situation continued until the unexpected discovery of aquaporin by Preston & Agre (1991).

1.2 Discovery of aquaporin water channels

In 1993, CHIP28 and related proteins were renamed as "aquaporins" with regarded to its role on water transport by Agre et al. (1993). And a formal name 'aquaporin-1 (AQP-1)' was officially adopted by the Human Genome Organization (Agre, 1997). The discovery of the water channel earned Professor Agre the 'Nobel Prize in Chemistry' in 2003 (The Royal Swedish Academy of Sciences, 2003).

AQP-1 exists as a tetramer with each monomer containing its own pore. Topological studies (Figure 1.1) revealed that AQP-1 consists of six transmembrane-spanning domains joined by two highly-conserved and hydrophobic connecting loops (B and E), which form a pore by two signature motifs NPA (N, asparagine; P, proline; A, alanine). The connection of loop B and E eventually forms an "hour-glass-like" protein, which was firstly proposed by Jung et al. (1994, Figure 1.2).

AQP-1 is a membrane channel with properties of selective passage for water and non-electrolytes. Molecular dynamic studies demonstrated the presence of residues in the pore-forming regions of AQP-1 responsible for selectivity against

passage of ions and protons (De Groot et al., 2001). The residues Phe-56, His-180 and Arg-195 in the human AQP-1 form an aromatic/arginine region, which acts as a proton filter. Arg-195 in loop E provides a functionally positive charge at the narrowest segment of the channel. His-180 in TM5 is uncharged at neutral pH, but becomes protonated at lower pH, providing a second positive charge. The hydrophobic Phe-56 side chain orients the water molecules such as to enforce strong hydrogen bonds to Arg-195 and His-180. Together with short α-helices with partial positive charges in the terminal part of loops B and E, these residues create strong repelling positive charges to resist passage of protons and ultimately provide selectivity against ions and protons (Agre et al., 2002; de Groot et al., 2001; de Groot & Grubmuller, 2001).

Diversity and classification of aquaporins in living organisms has been critically reviewed by Campbell et al. (2008); Heymann & Engel (1999); Ishibashi (2009) and Zardoya (2005). Phylogenetic analysis of the aquaporins (AQP) suggests a classification into three subfamilies. Subfamily AQP includes AQPs0-2, 4-6 and 8, which allow water permeation across the membrane. In addition to the passage of water molecule, the second subfamily glycerol facilitator-like protein (GLP) permits passage of glycerol and some other molecules. Enhanced glycerol permeation by AQP-3 has been documented by Zeuthen & Klaerke (1999), but the physiological

significance of glycerol permeation is still not fully understood. Apart from AQP-3, AQPs-7, 9 and 10 also belong to the glyceroaquaporins. The third subfamily is subcellular aquaporins which includes AQP-11 and AQP-12.

1.3 Osmoregulatory role of aquaporins

Terrestrial mammals face tough challenges of water loss due to evaporation. In order to maintain a steady internal osmotic environment, they save water and produce concentrated urine by kidney and the osmoregulatory role of aquaporin water channels in renal reabsorption had been well documented. Four types of aquaporins: AQP-1, AQP-2, AQP-3 and AQP-4, with their specific roles in different parts of the renal tubule, work together to achieve renal water reabsorption in human (Nielsen et al., 1995; Nielsen, 2002; Nielsen et al., 2002). Their functions are known to be under modulation of different hormones such as adrenocorticoids and vasopressin.

In addition to kidney, gills and intestine of teleost fishes play a dual role in ion and water transport (Evans, 1993; Bentley, 2002). In the past decade, the mechanism of water transport in teleosts has received growing attention and studies have provided information on the properties of the AQP-1 group (black porgy, *Acanthopagrus*

schlegeli, An et al. 2008; Gilthead sea bream *Sparus aurata*, Raldua et al., 2008; European eel, *Anguilla anguilla*, Martinez et al., 2005a, 2005b, 2005c; Japanese eel, *Anguilla japonica*, Aoki et al. 2003; Sea bass, *Dicentrarchus labrax*, Giffard-Mena et al., 2007; Rainbow wrasse, *Coris julis*, Brunelli et al., 2010) and AQP-3 group (*A. Anguilla*, Cutler & Cramb 2002; Osorezan dace, *Tribolodon hakonensis*, Hirata et al. 2003; Mozambique tilapia, *Oreochromis mossambicus*, Watanabe et al. 2005; *A. japonica*, Tse et al. 2006; Silver sea bream, *Sparus sarba*, Deane & Woo, 2006; *C. julis*, Brunelli et al., 2010) in these organs. Some basic information of aquaporin studies in teleost species has been summarized in Table 1.1. Their findings suggested that these aquaporins are responsive to external osmotic changes or other environmental distress (e.g. low pH, Osorezan dace, *T. hakonensis*, Hirata et al. 2003). Recently, responsiveness of newly isolated homologues of mammalian AQP-8 was reported in intestine of European eels (Cutler et al., 2009). Other than osmoregulatory tissues, there were evidences to demonstrate osmosensitivity of prolactin cells were enhanced by AQP-3 in euryhaline Mozambique tilapia (Watanabe et al., 2009).

Research focus has recently been extended to regulatory processes of cortisol and vasotocin regulatory axis on these water channels (Martinez et al., 2005a, 2005b & 2005c; An et al., 2007; Cutler et al., 2007; Tingaud-Sequeira et al.. 2008). Substantial

evidences demonstrated the correlations of AQP-1 with the regulatory systems of these hormones, but the underlying mechanism, from cellular regulatory pathway to aquaporin functioning, still left open a large void to be filled in.

Aquaporin research in teleosts began with euryhaline and catadromous species, but lately some research groups have published their findings about the function of aquaporins in marine teleosts like gilthead sea bream (*Sparus aurata*, Raldua et al., 2008) and black porgy (*Acanthopagrus schlegeli*, An et al., 2008). Throughout their lives, marine teleosts rarely experience a vast and sudden change of environmental salinity (ecologically stenohaline) though they may be capable of tolerating wide salinity fluctuations (physiologically euryhaline) (Woo & Chung, 1995). Therefore, the applicability of a model that was based on euryhaline or diadromous fishes to marine teleosts remains doubtful. The present study aims to provide insights in aquaporin biology in fish, using silver sea bream (*Sparus sarba*) as a model of marine teleosts.

In the first part of the experiment, antisera of mammalian homologue AQP-1, 2, 3 and 4 were adopted for immunolocalisation study for localization of these AQPs in the major osmoregulatory organs (gill, kidney and intestine) of silver sea bream.

Secondly, in order to further our study on the influence of salinity perturbations and hormone modulation on aquaporin functions, cDNAs of silver sea bream AQP-1 and pro-vasotocin were characterized by cloning techniques and the sequence results were used in the primer design for semi-quantitative mRNA expression study.

Chronic and abrupt salinity acclimation experiments were performed to investigate the influence of osmotic perturbations on AQP-1 and pro-vasotocin transcription levels in intestine, gills and kidney of the silver sea bream. In the chronic salinity acclimation experiment, the fish were gradually adapted to environments of broad spectrum of salinity (0‰, 6‰, 12‰, 33‰, 50‰, 70‰) for four weeks. For abrupt salinity experiment, the fish were directly transferred from 33‰ to 6‰ and adapted for a maximum period of up to 168 hours before sampling.

In the last part of this study, seawater-adapted sea bream were administered with either cortisol or vasotocin and the fish were then transferred to 6‰ regimes for three days. Responses of AQP-1 and pro-vasotocin transcription in those osmoregulatory organs were measured. The results obtained in all expression studies were discussed and hopefully, it can contribute to the extension of understanding on the role of aquaporin water channels in osmoregulation of fishes.

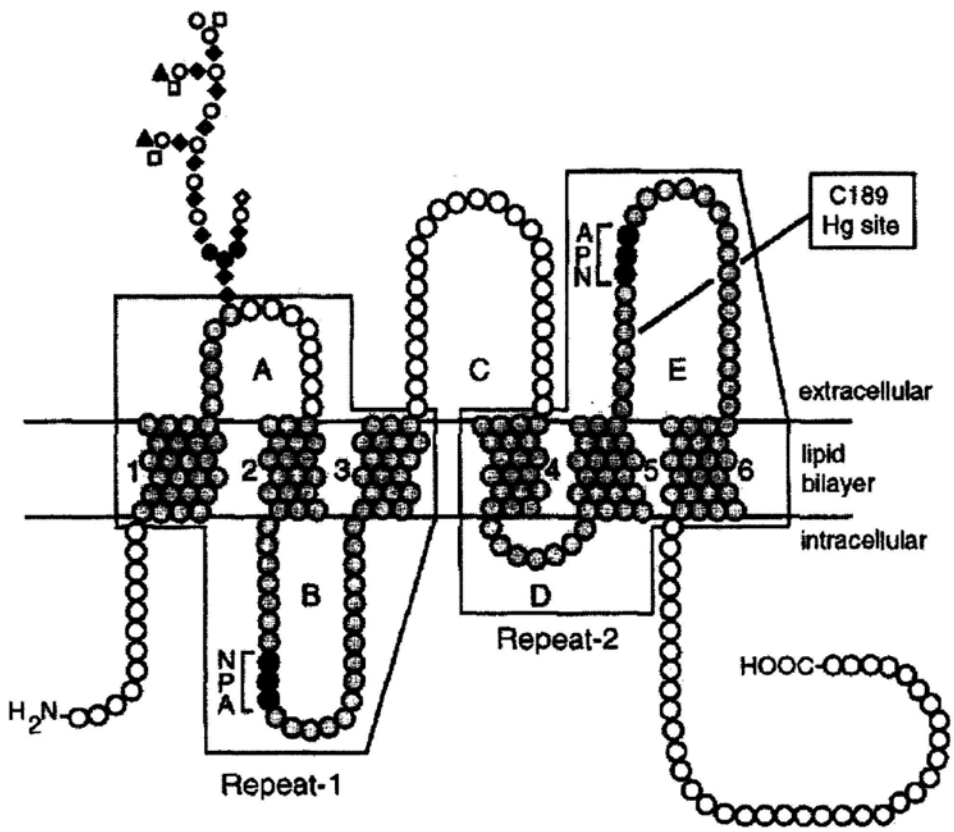

Figure. 1.1 Membrane topology of AQP-1 subunit (Bognia et al., 1999).

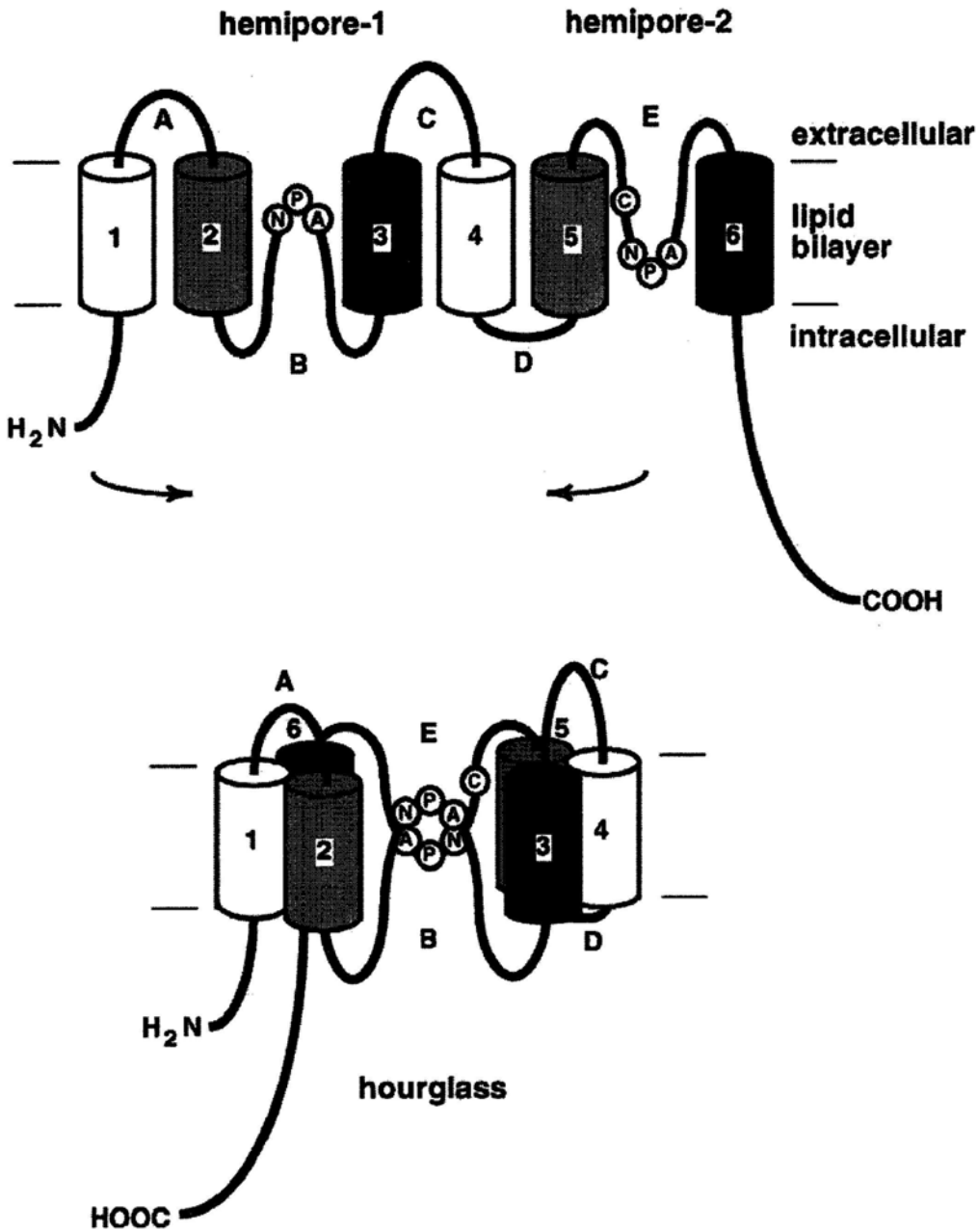

Figure 1.2. An hourglass model of AQP-1 (Jung et al, 1994).

Table 1.1 Summary of aquaporins discovered in teleosts

Teleost Species	AQP family	Target organ studied	References
Acanthopagrus schlegeli	AQP-1	Gill, intestine and kidney	An et. al. 2008
Anguilla anguilla	AQP-1	Kidney	Martinez et. al. 2005a
		Intestine	Martinez et. al. 2005b
		esophageal epithelium	Martinez et. al. 2005c
	AQP-1dup	Kidney	Martinez et. al. 2005a
		Oesophagus	Martinez et. al. 2005c
	AQP-1a	Ovary	Tingaud-Sequeira et al. 2008
	AQP-1b		
	AQP-3	Gill and gastrointestinal	Lignot et. al. 2002
		tract	Cutler & Cramb 2002
	AQP-8	Intestine	Cutler et al. 2009
	AQPe	Kidney	Martinez et. al. 2005a
		Intestine	Martinez et. al. 2005b
Anguilla japonica	AQP-1	Intestine	Aoki et. al. 2003
Danio rerio	AQP-1a	Ovary	Tingaud-Sequeira et al. 2008
	AQP-1b		
Fundulus heteroclitus	AQP-0	Lens	Virkki et al. 2001
Oreochromis mossambicus	AQP-3	Gill	Watanabe et. al. 2005
Solea senegalensis	saAQP-1b	Ovary	Tingaud-Sequeira et al. 2008
Sparus aurata	saAQP-1a	Intestine	Raldua et. al.2008
	saAQP-1b		
	saAQP-1b	Ovary and oocyte	Fabra et al. 2005
			Fabra et al. 2006
			Tingaud-Sequeira et al. 2008
	sbAQP	Gill, intestine and kidney	Santos et al. 2004
Sparus sarba	AQP-3	Various organs, larval	Deane & Woo 2006
		developmemt	
Tribolodon hakonensis	AQP-3	Gill	Hirata et al. 2003

Chapter 2

Literature review

In the past decade, osmoregulatory role and regulation of the aquaporin water channels had been well studied in mammals and human. Kidney is the specialized organ for osmoregulation in mammals and water transport is best-defined along different regions of renal nephrons (Knepper et al., 1996). High water permeability was observed in renal proximal tubules and the descending thin limbs of Henle's loop. In contrast, water is generally impermeable in the ascending thin limb and thick limbs of Henle's loop while water permeability in the collecting duct is vasopressin-mediated. Recent findings have thus firmly established the roles of AQP-1, 2, 3 and 4 in these renal water transport processes.

2.1 AQP functions in mammalian kidney

2.1.1 AQP-1 in proximal tubules and descending limbs of Henle's loop

Distribution of AQP-1 in kidney is well studied by Nielsen and his co-workers. AQP-1 is primarily located in kidney and as well as tissues of other organs. By using immunohistochemical methods, AQP-1 was found to be located in the apical membrane of brush border and basolateral membrane of proximal tubules and descending loop of Henle (Nielsen *et al.* 1993a). In addition, the protein was identified in descending vasa recta (Pallone *et al.* 1997), defining the pathway for the transfer of

large amounts of water from tubular lumen to the interstitium and then into the vascular space. This was confirmed in AQP-1 gene knockout mice, which have a severe urinary concentrating defect (Schnermann et al., 1998).

2.1.2 AQP-2, AQP-3 and AQP-4 in collecting duct

AQP-2 is primarily located in the apical plasma membrane and cytoplasmic vesicle of principal cells of collecting duct of kidney. This water channel exhibits a different mechanism of water reabsorption from that of aforementioned AQP-1. Upon activation of arginine vasopressin (AVP), vesicles containing the ready-to-use AQP-2 are trafficked to the apical membrane of the principal cells and the water channel is then inserted into the membrane for its function (Nielsen, 2002). The main role of AQP-2 is for entry of water molecules into the cell whereas the exit of water is facilitated by basolaterally located AQP-3 and AQP-4 (Nielsen et al., 2002).

2.1.3 Hormonal regulation of renal aquaporins

In mammals, renal multi-AQPs water transport is governed by the anti-diuretic hormone, vasopressin (Nielsen et al., 2002) and the underlying mechanism of this

hormone on AQP-2 function was well established after a series of experiments carried out by Nielsen and his co-workers. Perfusion of inner medullary collecting ducts with vasopressin resulted in translocation of vesicular-resident AQP-2 to the apical membrane (Nielsen et al., 1995). The mechanism of vasopressin on AQP-2 trafficking is now clear: Vasopressin firstly binds to its receptor, activating adenyl cyclase and promoting cAMP production. cAMP stimulates protein kinase A (PKA), which phosphorylates AQP-2 (Christensen et al., 2000; Katsura et al., 1997). AQP-2 has a phosphorylation site (serine at position 256) for PKA (van Balkom et al., 2002). This phosphorylation is essential to promote trafficking of AQP-2 (Nielsen, 2002).

Adrenocortical hormones, glucocorticoid and mineralocorticoid, are also importantly involved in the normal modulation of renal water excretion and regulation of extracellular fluid volume. Kwon et al. (2002) demonstrated that aldosterone-deficient rat was associated with a dramatic downregulation of renal AQP-3 abundance and a consistent decrease in AQP-3 labeling in the basolateral plasma membranes of collecting duct principal cells by using immunocytochemistry and immunoelectron microscopy. Besides, aldosterone stimulates urine production and decrease AQP-2 expression in rats with diabetes insipidus (Nielsen et al., 2006).

2.2 Aquaporins in teleosts

Fish are aquatic vertebrates and regulation of water movements is a preamble to survival. However, little is known regarding aquaporins in fish and to date, there are only few published reports on piscine aquaporins. Information pertaining to studies on specific piscine aquaporins has been summarized in Table 2.1, 2.2, and 2.3. Among the aquaporins characterized in teleost species, experiments indicated homologues of mammalian AQP-1 and AQP-3 are most likely related to osmoregulation in the light of their high abundance in teleostean osmoregulatory tissues.

AQP-1 is believed as the archetypal member of the aquaporin family (Nielsen et al., 2002) and is apparently present in all groups of vertebrates and cDNAs of AQP-1 isoforms has been isolated from a number of teleostean species. cDNAs of AQP-1 encode 258-262 amino acids. Figure 2.1 shows a comparison of AQP-1 amino acid sequence among mammals, amphibians and teleosts. The piscine AQP-1, now commonly known as AQP-1a, shares approximately 58-59% and 75-76% similarity with mammalian and amphibian AQP-1 respectively. The amino acid sequence of AQP-1a was highly conserved among *Anguilla anguilla* and two species from the Family Sparidae, with 89% similarity between the two groups. The sequences

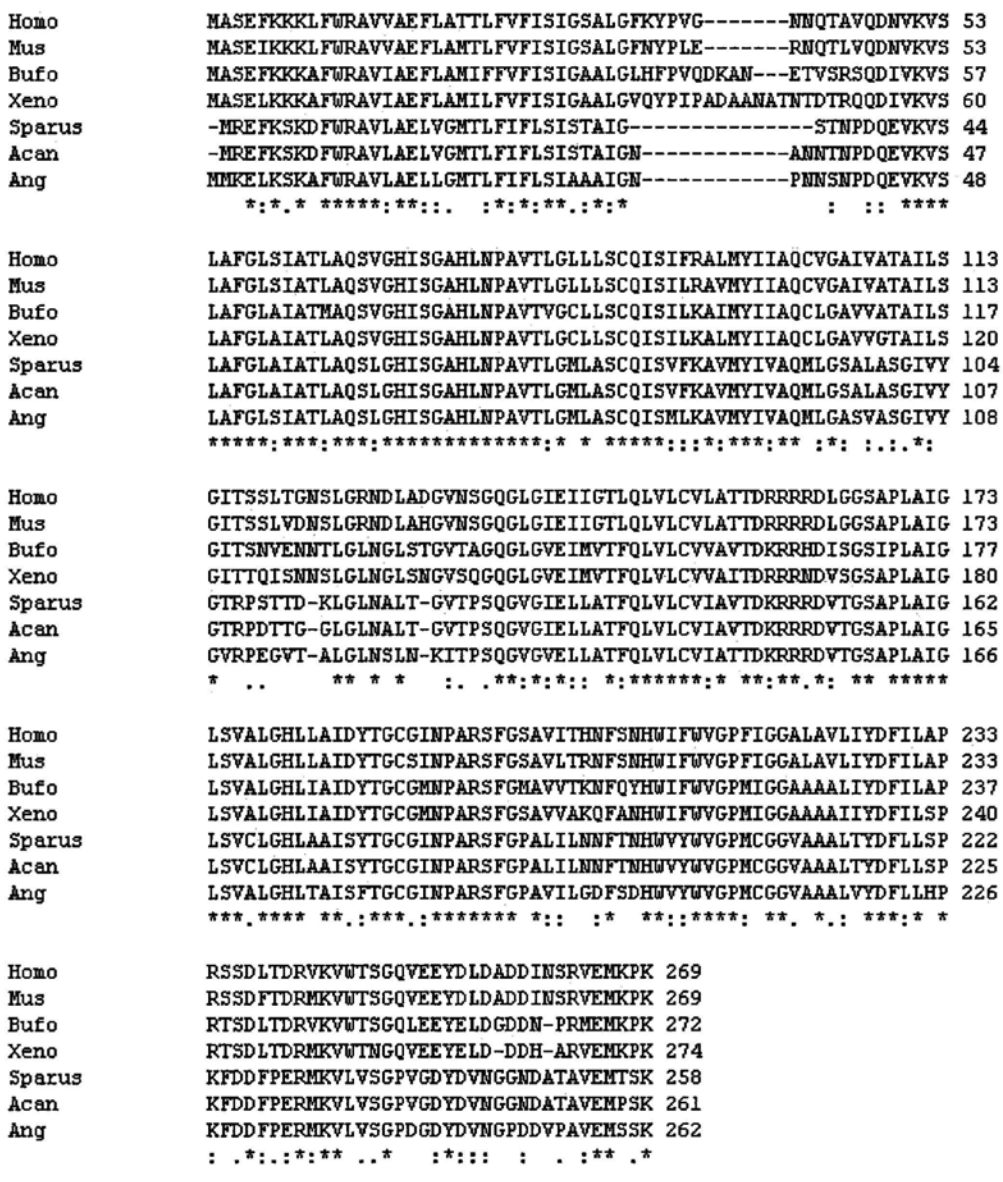

Figure 2.1. Comparison of deduced amino acid sequences of AQP-1 homologues of mammals, amphibian and fish. Homo: *Homo sapiens*; Mus: *Mus musculus*; Bufo: *Bufo marinus*; Xeno: *Xenopus tropicalis*; Sparus: *Sparus aurata*; Acan: *Acanthopagrus schlegeli*; Ang: *Anguilla anguilla*. Asterisk denotes similar sequence among species; full stop denotes two different sequences present and; colon indicates more than two different sequences exist.

upstream and downstream from the two NPA motifs are virtually identical and this forms a crucial piece of information for development of molecular studies on aquaporins of other teleosts of interest. Similar to mammalian AQP-1, AQP-1a is ubiquitously present in most fish tissues including brain, gills, kidney and intestine (An et al., 2008; Martinez et al., 2005a).

Duplicate forms or isoforms of AQP-1a gene, now known as AQP-1b, have been reported in osmoregulatory important tissues of European eel (*Anguilla anguilla*; Martinez et al., 2005a, 2005b, 2005c) and gilthead sea bream (*Sparus aurata*; Raldua et al., 2008). The intraspecific homology between AQP-1a and AQP1b is relatively low (61-64%) and the most divergent region between the amino acid sequence of the isoforms was the C-terminus. Tingaud-Seuqeira et al. (2008) had demonstrated the specific C-terminus residues are essential to the control of AQP-1b intracellular trafficking through phosphorylation-independent and –dependent mechanisms.

Fish homologues of mammalian AQP-3 was firstly isolated from the gills of European eels (Cutler & Cramb, 2002) and the derived amino acid sequence shares 67-70% homology with other vertebrate AQP-3 homologues. More fish AQP-3 homologues were also cloned from Mozambique tilapia (Watanabe et al., 2005) and

Japanese dace (Hirata et al., 2003) and our group had recently isolated a partial clone of AQP-3 in the silver sea bream (Deane & Woo, 2006). Tissue distribution analysis revealed AQP-3 was predominantly expressed in the gill among the major osmoregulatory organs (Cutler & Cramb, 2002; Deane & Woo, 2006; Watanabe et al., 2005) and this suggested a possible role of AQP-3 in branchial water transport as well as osmoregulatory function.

2.3 Water transport and aquaporin functions in osmoregulatory organs of teleosts

Teleost fishes maintain their internal ion and water balance by various osmoregulatory organs. Among these organs, gills, gastrointestinal tract and kidney are the major sites for osmoregulation and their roles in maintaining water balance had been readily studied by fish physiologist. Several homologues of mammalian aquaporins were recently isolated from these tissues and studies on these water channels give us some clues on water transport and osmoregulaotry mechanism in teleost species.

2.3.1 Gills

In fish, gill is one of the organs, which is in direct contact with surrounding waters. The gill epithelium is a major site for gaseous exchange, as well as for ions and water exchanges with the external environment. In freshwater teleosts, gill contributes 90% of total body water influx (Motais et al., 1969; Haywood et al., 1977). Osmotic permeability of the gills in euryhaline teleosts is generally higher in freshwater than in seawater adapted fish, with notably six times higher permeability in European eels (Isaia, 1984). Contrary to the results of Isaia (1984), several studies also reported rapid decline in osmotic water permeability after seawater or freshwater transfer (Ogasawara & Hirano, 1984; Gallis et al., 1979). Isolated gill preparation of Japanese eels demonstrated retarded water permeability was measured at three hours after transfer from seawater to freshwater (Ogasawara & Hirano, 1984). These results may reflect substantial 'flexibility' of branchial water permeability as immediate and long-term strategies in response to low salinity acclimation.

The pathway for water fluxes across gill is believed to take place in chloride cells (mitochrondria-rich cells). This flux of water is associated with ion transporting mechanism through the basalateral tubular network in gill chloride cells (Isaia, 1984).

Chloride cell population is generally higher in seawater adapted fish (Potts, 1984), this may help to explain part of the water flux in hyperosmotic environment. Freshwater fish have relatively lower abundance of chloride cells and more extensive tight junctions, which apparently does not match with its high osmotic water permeability in gill epithelium.

Morphometric studies on gill chloride cells of sea bream revealed that the apical area of chloride cells remained unchanged or even higher after hypo-osmotic exposure in 6‰ or freshwater regimes (Kelly et al., 1999; Kelly & Woo, 1999). Reduction of chloride cell population is compensated by increase in individual chloride cell surface area to external environment. Hypo-osmotic exposure also results in morphological alteration to chloride cell, the apical surface of these cells are either at same level or protruding above the adjacent pavement cells instead of being invaginated as observed in most seawater acclimated sea bream. Despite lower gill chloride cell population in freshwater acclimated fish, apical functional area apparently is not reduced.

In addition, Rankin and Bolis (1984) suggested the bulk of osmotic water fluxes, particular in freshwater fish must take place through or between other surface epithelial cells such as second lamellar respiratory cells other than chloride cells. The

functional changes in chloride cells and other gill cells may account for higher permeability happened in freshwater acclimated fish.

Recent research has extended to the largely unknown regulatory mechanism of water transport across gill epithelium. The cDNAs of AQP-1, AQP-3 and a novel aquaglycerporin have recently isolated from few teleost species (An et al., 2008; Brunelli et al., 2010; Giffard-Mena et al., 2007). The effect of freshwater acclimation on branchial AQP-1 expression was reported in black porgy *Acanthopagrus schlegeli* (An et al., 2008) and sea bass *Dicentrachus labrax* (Giffard-Mena et al., 2007). Branchial mRNA expression of freshwater-acclimated black porgy was significantly higher than those of seawater-acclimated fish, but there was no significant change found between sea bass adapted to freshwater and seawater for nine months. The results from these two studies gave totally different conclusions for the role of AQP-1 during salinity acclimation. In addition, immunohistochemical studies on gills of rainbow wrasse *Coris julis* revealed the distribution of AQP-1 water channels coincided with Na^+-K^+-ATPase in the interlamellar spaces where chloride cells are located in gills (Brunelli et al., 2010).

Compared with AQP-1, extensive investigation had been done for branchial

AQP-3 functions in European eel (*Anguilla anguilla*) and Japanese eel (*Anguilla japonica*). After seawater transfer, branchial AQP-3 was downregulated in these fish (Cutler & Cramb, 2002; Lignot et al., 2002; Tse et al., 2006). In addition to these observations in eels, our group had isolated a partial cDNA of AQP-3 homologue from the gills of silver sea bream (Deane & Woo, 2006). The abundance of AQP-3 transcript in gills of silver sea bream adapted to 6‰ and 12‰ was significantly higher than those of seawater- and 50‰-adapted fish (Deane & Woo, 2006).

Immunohistochemical studies revealed that this water channel primarily co-localized with Na^+-K^+-ATPase, an enzyme known to be found intensely in gill chloride cells (Brunelli et al., 2010; Hirata et al., 2003; Lignot et al., 2002). Immuno-positive staining of AQP-3 was localized throughout the cell with more intensive staining occurring at the apical pit of these chloride cells (Lignot et al., 2002). Unlike the situation in mammals, AQP-3 is usually localized on or near the basolateral membrane of renal tubules and epithelial cells of intestine (Frigeri et al., 1995a; Ramirez-Lorca et al., 1999; Koyama et al., 1999). The presence of AQP-3 in tubule-vesicular system surrounding the apical pit suggests possible existence of another regulated water pathway within the chloride cells of both seawater and freshwater adapted fish (Lignot et al., 2002).

There were no observable differences in expression between the chloride cells of freshwater and seawater acclimated European eels, however, higher intensities of AQP-3 immunoreactivity were found near the periphery of the non-chloride cells in the gills of freshwater acclimated fish (Cutler & Cramb, 2002; Lignot et al., 2002). In freshwater eels, cells near the central cavity of the primary filament epithelium and basal layer cells within the gill arch epithelium exhibited strong immunoreactivity near the plasma membrane. These signals are less intense in seawater eels. *In situ* hybridization study also localized the mRNA of a unique aquaglycerporin (sbAQP) at gill chloride cells in a marine teleost, gilthead sea bream (*Sparus aurata*; Santos et al., 2004).

Curiously, by using rabbit antibody against AQP-3, high AQP-3 immunoreactivity was localized all over the gill epithelium in freshwater- acclimated Japanese eels (Tse et al., 2006). The mRNA of AQP-3 was highly expressed in the isolated pavement cells. This result contrasts to those of seawater- acclimated eels where there is intense immunoreactivity in isolated chloride cells. The immunolocalisation of AQP-3 in chloride cell suggests a possible association with the basolateral tubular network of chloride cells, and this could be related to the osmotic water flux pathway that has been hypothesized to be operating in the system (Isaia,

1984). High expression of AQP-3 in non-chloride gill epithelial cells suggests existence of alternative pathway of water fluxes, which may explain prominent branchial water permeability in freshwater fish.

2.3.2 Intestine

In marine teleosts, the gut is vital to water uptake in order to compensate for the diffusive water loss to the external hyperoosmotic environment. Seawater adapted teleosts drink large amounts of seawater (Marshall & Grosell, 2005; Wong, 2001) and salts are partially removed by oesophagus. Since oesophagus has an extremely low permeability to water, the oesophageal fluid can be diluted up to 33 to 50% of the initial osmolality after desalinization (Hirano & Mayer-Gostan, 1976; Parmelee & Renfro, 1983; Nagashima & Ando, 1993). This salt removal step in oesophagus is a crucial step for subsequent water uptake processes that occurs in the intestine (Skadhauge, 1969).

As very limited fluxes of ion and water occur in stomach, the osmolality of the luminal fluid that reaches the intestine is more or less similar to that of the oesophageal fluid (Hirano & Mayer-Gostan, 1976). Eventually most of the remaining water is absorbed in intestine (Skadhauge, 1969; Hirano & Mayer-Gostan, 1976) through

secondary-active transport and passive water fluxes (Skadhauge, 1969).

A secondary-active transport is thought to take up water against osmotic gradient through a trans-/paracellular shunt pathway, and a number of studies demonstrated the transport is tightly coupled with transport of chloride and sodium and/or potassium ions (Skadhauge, 1969; Skadhauge, 1974; Ando, 1975; Ando, 1980; Ando, 1981; Ando, 1983; Ando, 1985). In intestine, salts are actively absorbed and as the osmolality of the luminal fluid is continuously reduced, it reaches a level where no net water flux occurs (known as turning point osmolality, Skadhauge, 1969). This turning point osmolality is higher than the plasma osmolality. As ions are further absorbed, net water is absorbed against the opposing osmotic gradient. In these studies, the osmolality at which turning point osmolality occurs was used as a measure of this solute-coupled secondary-active salt/water transport in eels. This osmolality is directly proportional to the salinity of the external environment, suggesting the capacity of the solute-coupled water fluxes increases as the external salinity increases (Skadhauge, 1969; Skadhauge, 1974). After freshwater-to-seawater acclimation, this active water absorption increased for 3-3.4 fold in intestine of eels (Utida et al., 1972; Ando, 1975). Intestinal passive osmotic water permeability also increased by 2-6 fold following seawater acclimation (Skadhauge, 1969; Ando, 1975). Comparison of water fluxes in

various parts of the intestine had been carefully examined by Ando and Kobayashi (1978) and Ando (1980). Highest level of water transport was detected in midgut and followed by posterior, anterior intestine and rectum.

Cutler & Cramb (2000) suggested that a regulated cellular pathway took up the role of water transport in intestine, which appears to be more important for hypoosmoregulation in seawater teleosts. Although there is no direct evidence showing the involvement of aquaporin water channels in intestinal water transport, a number of studies on these proteins confirmed their responsiveness to external salinity changes (An et al., 2008; Aoki et al., 2003; Cutler & Cramb, 2002; Cutler et al., 2009; Giffard-Mena et al., 2007; Lignot et al., 2002; Martinez et al., 2005b; Raldua et al., 2008).

AQP-1 and its isoform had been well studied in gastrointestinal tract. In study of European eel (*Anguilla anguilla*), intestinal AQP-1 mRNA expression of sea-water acclimated yellow and silver eels is higher than that of freshwater acclimated eels (Martinez et al., 2005b). Similar observations were also found in AQP-1 of Japanese eels (Aoki et al., 2003), sea bass (Giffard-Mena et al., 2007) and two AQP-1 isoforms (AQP-1a and AQP-1b) of gilthead sea bream (Raldua et al., 2008). Meanwhile,

intestinal sac preparations from seawater- and freshwater-adapted Japanese eels revealed higher water absorption in seawater sacs than in freshwater sacs, along with elevated drinking rates observed in seawater adapted eels (Aoki et al., 2003). However, reverse trend was reported in black porgy with stimulated AQP-1 expression after freshwater exposure (An et al., 2008). These contradictory observations are yet to be solved by more future researches on teleost fishes.

Localization studies of AQP-1 in intestine had been carried out simultaneously in studies on European eels and gilthead sea bream. Using a specific eel AQP-1 antibody; Martinez et al. (2005b) detected immunoreactivity on the endothelium layer of blood vessels in gut of freshwater and seawater-adapted European eels, but AQP-1 immunoreactivity was detected only at the apical brush border in both posterior intestine and rectum of seawater-adapted eels.

In gilthead sea bream, differential localization of two AQP-1 isoforms (AQP-1a and AQP-1b) was observed in intestine (Raldua et al. 2008). Sea bream AQP-1a was mainly immunolocalized in both the apical and basolateral membranes of enterocytes and higher immunoreactivity was found in the duodenum and hindgut. AQP-1a also immunoreacted with and was localized in the cytoplasm of some rectal enterocytes. In

contrast to its isoform, AQP-1b appears to be more prominent in rectum and its immunolocalisation was restricted to apical brush border. Immunohistochemical studies on fish AQP-1s conforms some results obtained from their expression studies on intestine, suggesting an upregulation after hyperosmotic exposure. It also suggests a possible site-specific localization for AQP-1 in the gut, which may be related to the properties of water transport in each organ.

Aquaporins from aquaglycerporin family were also characterized in telesot intestinal tissues. In European eels, homologue of mammalian AQP-3 may be involved with immunofunction or mucus secretion as they are primarily localized in macrophage-like cells of intra-epithelial layers in intestine and mucous cells in rectal gut respectively (Lignot et al, 2002). Another aquaglyceroporin, AQPe, which has been isolated from European eels, was upregulated after seawater acclimation (Martinez et al., 2005b). The importance of these aquaglycerporin may be undermined by their insufficient studies in intestine, with respect to their versatility of transporting many types of solutes. In addition to aquaglyerporins, characterization of the homologue of mammalian AQP-8 and its role in intestine were reported in European eels (Cutler et al., 2009). The expression of eel AQP-8 was restricted to the intestine and the levels of expression increased after salinity acclimation. There is strong

evidence for a role of AQP-1 in intestinal fluid absorption, and the intestine-specific AQP-8 can be an additional channel for water transport in the organ (Cutler et al., 2009).

2.3.3 Kidney

Lacking the advanced water reabsorption processes that take place in mammalian kidney, volume of urine production is mainly regulated by glomerular filtration rate and limited water transport in teleosts. Kidney in freshwater teleosts serves to excrete excess osmotic loads by producing very dilute urine, with higher glomerular filtration rates and very low net reabsorption rate for water. Even in seawater teleosts, only minimal water absorption occurs in renal tubular regions. Tubular water secretion may occur in kidneys of marine aglomerular fish, as a way to excrete excessive salts out of the body. The role of kidney on water reabsorption is considerably less important in teleost than in mammals (Cutler & Cramb, 2000).

The homologue of mammalian AQP-1 was detected at limited levels in teleost kidney, in contrast to the high abundance in the same organ of mammalian species. Cutler & Cramb (2000) suggested a possibility of other aquaporins picking up a more

active role in renal water transport. The same research group had successfully isolated the eel AQP-1 isoform (AQP-1dup) and a homologue of mammalian aquaglyceroporin, AQPe from kidney tissues of European eels, with considerably high abundance in the kidney (Martinez et al. 2005a).

Tissue distribution study revealed that the relative expression level of AQP-1dup and AQPe are higher than AQP-1 in renal tissues of eels (Martinez et al. 2005a). By using Northern-blot analysis, expressions of AQP-1, AQP-1dup, and AQPe were shown to be significantly reduced in renal tissue of yellow eels after seawater adaptation from freshwater. Conversely, renal expression levels of these aquaporins in silver eels were independent of salinity changes.

Differential distribution of AQP-1 was found between renal tissues of yellow and silver eels (Martinez et al. 2005a). In yellow eels, immunoreactivity of AQP-1 was predominately localized in vascular endothelium. In contrast, the silver eels have their AQP-1 strongly expressed in the apical brush border of some kidney tubules. The type of immunolocalized tubular cells was not identified in the study, however, their disparity of AQP-1 localization suggests an adaptive function for the seawater-migrant silver eels.

Apical-residence of AQP-1 in tubular cells may support early observations of tubular water secretion as a result of salt excretion in proximal tubule II of seawater teleosts. Beyenach (1982, 1986) utilized an isolated renal tubule preparation to investigate salts and fluid mechanism in glomerular winter flounder, *Pseudopleuronectes americanus*. The oil-filled tubule was surrounded by a peritubular bath in which fluid was secreted to the lumen and displaced the oil out of the tubule over time. The collected fluid is of similar Na^+ and Cl^- concentrations of the peritubular bath.

The fluid secretion process is driven by Na^+ and Cl^- secretion and this net secretion of NaCl and water is essential for maintenance of renal excretion under very low glomerular intermittency during seawater adaptation as well as in aglomerular marine teleosts. Along with the parallel extrusion of divalent ions, marine teleosts are capable of producing a resultant urine with an osmolality slightly to a few times higher than that of plasma (Stanley & Fleming, 1964; Fleming & Stanley, 1965; McDonald & Grosell, 2006), representing a measure to hypoosmoregulate their body. The role of water secretion was well evident and more emphasized in previous studies, however, the direct linkage between the aquaporins and water movements in renal tubules is still undetermined.

2.4 Hormonal regulation of osmoregulation

Fish osmoregulation is under two classes of endocrine control: (1) fast, short-acting hormones for immediate responses, such as arginine vasotocin (AVT) and angiotensin; (2) slow, long-acting hormones for long-term strategies for persistent changes in osmotic conditions, including cortisol, growth hormone and prolactin.

There have been fruitful evidences for activation of on aquaporin-2 (AQP-2) by administration of AVT and its mammalian analogue, arginine vasopressin (AVP) in amphibian and mammals. AQP-2 has not been characterized in piscine species, however, the possibility that AVT may have an effect on other piscine aquaporins and epithelial water transport shall not be underestimated.

Cortisol is regarded as seawater adapting hormone in teleosts. Plasma cortisol level rises following the transfer of teleosts to hyperosmotic regimes (Mommsen et al., 1999). It also has pronounced effects on ion-transporting proteins and recently a few research groups have revealed its role on aquaporin expression in a number of teleost species (Martinez et al., 2005a, 2005b & 2005c).

2.4.1 Arginine vasotocin (AVT)

AVT belongs to a group of neuropeptide hormones that are defined as peptides synthesized in neurons of the nervous system. The first step of AVT biosynthesis is transcription and translation of the pro-vasotocin gene (precursor of AVT) in the hypothalamic magnocellular neurons of the nucleus preopticus. The pro-vasotocin is partially proteolysed and is transported to the secretory cells of neurohypophysis for storage and release.

The amino acid sequence of pro-vasotocin consists of a signal peptide, AVT hormone, neurophysin and an elongated carboxyl-terminal segment with copeptin-like sequence. The AVT is stored as a complex of AVT-neurohypophysin in secretory cells and the hormone complex dissociates spontaneously as AVT and neurophysin after being released from the neurohypophysis through exocytosis. AVT is composed of nine amino acids and the AVT sequence is highly similar to those of other neurohypophysial peptides in vertebrates (Fig. 2.3).

AVT has dual role on osmoregulatory functions in gills, gastrointestinal tract and kidney. The nonapeptide facilitates ion outflux in gills of flounder transferred from

freshwater to seawater (Motais & Maetz, 1967). It acts mainly on V1-type receptors (Guibbolini & Lahlou, 1990; Warne, 2001) and is closely associated with the respiratory cells (Guibbolini and Avella, 2003) and chloride cells (Marshall, 2003) of gill. The neuropeptide hormone was found to have greater impact on branchial resistance than systemic resistance (Conklin et al, 1997). Changes in AVT levels can affect the redistribution of blood flow into the branchial arterial system and thus influence ions and water transport via epithelium (Maetz & Lahlou, 1974).

To date, there is no evidence that suggests a direct osmoregulatory role of AVT on the gastrointestinal tract. More information is known about the fish kidney, but the picture is much less clear than the well-established model of its analogue, AVP, in mammals. In mammalian kidney, AVP possesses two components of action, vascular and tubular. Action of AVT on vascular regulation in fish kidney is obvious. Unlike mammals, the glomerulotubular balance seems to be poorly developed and the glomerular filtration rate (GFR) is readily affected by renal perfusion pressure (Nishimura & Bailey, 1982). Early studies revealed that AVT induces a diuresis in teleosts at pharmacological AVT doses (100 and more times AVT physiological levels), this possibly being a consequence of increase in systemic blood pressure (Henderson & Wales, 1974).

By adopting a perfused trout kidney preparation supplied with a constant perfusing pressure, administration of different doses of AVT (10^{-9} and 10^{-11} M) was observed to reduce the filtering population of glomerulus which resulted in an antidiuresis (Amer & Brown, 1995). Similar observations have also been found in freshwater eel (Henderson & Wales, 1974) and these models suggest that the renal response to AVT would be largely a result of glomerular action of the hormone rather than tubular one (Warne et al., 2002).

Pro-vasotocin synthesis, and circulatory and pituitary AVT concentrations are related to alterations of external osmotic conditions (Maetz & Lahlou, 1974; Haruta et al., 1991; Hyodo & Urano, 1991; Perrott et al., 1991). Initial study on pro-vasotocin synthesis demonstrated a significant decrease in AVT precursor mRNA level in rainbow trout transferred from freshwater to 80% seawater. After reintroducing these fish from 80% seawater to freshwater, provasotocin mRNA level returned to the initial freshwater level (Hyodo & Urano, 1991).

A completely different story was told by studies on the euryhaline flounder *Platichthys flesus* after direct exposure from seawater to freshwater (Warne et al., 2000; Warne et al., 2005). After exposing the flounder *Platichthys flesus* to

hyposmotic salinity for 3 days, hypothalamic pro-vasotocin mRNA level apparently dropped, which was then followed by an elevated level of pituitary AVT and lowered plasma AVT concentration (Warne et al., 2000).

In order to measure the responsiveness of AVT system in the flounder, more precise sampling time interval was employed in an abrupt hyperosmotic exposure experiment performed by Warne et al. (2005). Pituitary AVT concentrations fell while plasma AVT level rose significantly eight and 24 hours following transfer from freshwater to seawater. Pro-vasotocin level in hypothalamus was elevated significantly at four and eight hours after transfer to seawater. Similar fluctuations in pituitary and plasma AVT levels have been reported in rainbow trout (Kulczykowska, 1997).

The response of vasotocin to environmental osmotic changes is quick and occurs at the early phase of hyperosmotic challenge. The rapid synthesis of provasotocin and AVT release from pituitary are consistent with the rapid actions of AVT on osmoregulation, including antidiuretic response that fish require to accommodate the dehydrating water losses and salt loading on exposure to the new hyperosmotic environment.

Like other neuropeptides, AVT carries out its specific action by binding with their receptors located in different target organs. Homologues of mammalian AVP receptor type 1 (AVPR1) have been isolated from a few teleostean species (*Catostomus commersoni*, Mahlmann et al, 1994; *Platichthys flesus*, Warne, 2001; *Acanthopagrus schlegeli*, An et al., 2008; *Sparus sarba*, Luk & Woo, unpublished data).

Both V1 type (AVPR1s) and V2 type (AVPR2) are G-protein coupled receptors. The V1 type is further divided into alpha (AVPR1A) and beta (AVPR1B) subtypes and these AVP1 receptors are activated through the phospholipase C pathway (Morel et al., 1992). Alternatively, activation of AVPR2 stimulates adenylate cyclase (Lolait et al., 1992).

In *P. flesus*, the V1-type receptor is primarily located at the afferent and efferent arterioles of glomerulus and on the capillary bed that extends from the efferent arteriole to the smooth muscle surrounding the collecting duct (Warne et al., 2005). AVT acts on the receptors of these arterioles and thus adjusts the volume of blood passing through the glomerulus. This may be essential for glomerular intermittency as a means of adjusting glomerular filtration rate and urine production rate. Localization and expression levels of the receptor are comparable in freshwater- and

seawater-adapted flounders (Warne et al., 2005).

Fish analogue of the mammalian V2 type receptors has not yet been isolated although increase in cAMP production was reported after administration of AVT in trout isolated nephron preparation (Perrott et al., 1993). In mammalian kidney, V2 type receptor is responsible for stimulation of aquaporin-2 trafficking from cytoplasmic vesicles to apical membrane of tubular cells (Christensen et al., 2000; Katsura et al., 1997).

An et al. (2008) attempted to investigate the responsiveness of AQP-1 and AVT receptor expression to salinity changes in black porgy. Expression levels of AQP-1 and AVT receptors are both higher in gills and kidney of black porgy acclimated to 10‰ SW. While a direct linkage of the water channel and nonapeptide receptor is still in doubt, further investigation is necessary to examine the hypothesis.

2.4.2 Cortisol

Cortisol is a steroid hormone which is synthesized by the interrenal tissue of teleost fish. The corticosteroid stimulates hyperosmoregulatory actions in many ways.

The hormone increases number of gill chloride cells, where aquaporin-3 is highly abundant (Perry et al., 1992). Cortisol acts on the intestinal epithelium by enhancing water absorption and Na^+-K^+-ATPase activity (Cornell et al., 1994; Veillette et al., 1995) and exerts pronounced effect on aquaporin expression in osmoregulatory organs. Cortisol administration in freshwater-adapted eels increased the net absorption of monovalent ions and water through the intestine in freshwater eels (Epstein et al., 1971; Gaitskell & Chester Jones, 1970; Hirano & Utida, 1968, 1971; Hirano et al., 1976). Early experiments have demonstrated the stimulation of intestine fluid uptake by cortisol in salmonid species (Cornell et al., 1994; Veillette et al., 1995). Besides, administration of cortisol led to increased drinking rates in *Oncorhynchus mykiss* and *S. salar* after transfer to seawater (Fuentes et al, 1996). More recent evidences revealed that administration of cortisol increased intestinal AQP-1 expression levels in freshwater yellow European eels while seawater eels reamined unaffected (Martinez et al., 2005b). A reduction of renal AQP-1 and AQP-1dup expression levels exclusively occurred only in freshwater yellow eels (Martinez et al, 2005a).

The aquaglyceroporin, AQPe, is apparently not responding to cortisol treatment in kidney and intestine of European eels (Martinez et al., 2005a; Martinez et al., 2005b). However, another aquaglyceroporin, AQP-3, was found to be significantly

downregulated in branchial tissues after infusion of cortisol (Cutler et al, 2007) with the expression in intestine unaffected. These findings suggested actions of cortisol on AQP-1 is tissue specific, in accord with the specific role in each osmoregulatory organ to achieve ultimate goal for hyper- or hypo-osmoregulation under particular environmental osmotic conditions.

Table 2.1 Aquaporins in gills of teleosts

AQPs	Species	Effects on expression and distribution	References
AQP-1	*Acanthopagrus schlegeli*	↑ after FW acclimation	An et. al. (2008)
	Coris julis	Immunolocalized in chloride cells	Brunelli et al. (2010)
	Dicentrarchus labrax	No significant difference between SW- and FW-acclimated fish	Giffard-Mena et al. (2007)
AQP-3	*Anguilla anguilla*	↓ after SW acclimation	Cutler & Cramb (2002) &
		Immunolocalized in chloride cells	Lignot et al. (2002)
	Anguilla japonica	↓ after SW acclimation	Tse et al. (2006)
		FW eels: Immunolocalized mainly in pavement cells	
		SW eels: Immunolocalized mainly in chloride cells	
	Coris julis	Immunolocalized in chloride cells	Brunelli et al. (2010)
	Dicentrarchus labrax	↑ in FW-acclimated fish	Giffard-Mena et al. (2007)
	Sparus sarba	↑ in sea bream acclimated to 6‰ and 12‰	Deane & Woo (2006)
	Oreochromis mossambicus	Immunolocalized at basolateral membrane of chloride cells	Watanabe et. al. (2005)
	Tribolodon hakonensis	↑ after transfer to acidic regime	Hirata et al. 2003
		Immunolocalized at basolateral membrane of gill chloride cells	
sbAQP	*Sparus aurata*	Immunolocalized in chloride cells	Santos et al. (2004)

Table 2.2 Aquaporins in intestine of teleosts

AQPs	Species	Effects on expression and distribution	References
AQP-1	*Anguilla japonica*	↑after SW acclimation. Intense immunoreactions in apical surface of epithelial cells in SW eels whereas weak in FW eels; Posterior >> Anterior intestine;	Aoki et al. (2003)
	Anguilla anguilla	↑ in yellow and silver eels after SW acclimation Posterior and rectal intestine >> anterior intestine Immunofluorescence within the vascular endothelium in both FW and SW eels; and in the epithelial apical brush border in the posterior/rectal gut regions of SW eels	Martinez et al. (2005b)
	Acanthopagrus schlegeli	↑ after FW acclimation	An et. al. (2008)
	Dicentrarchus labrax	↑ anterior intestine, posterior intestine and rectum of SW sea bass	Giffard-Mena et al. (2007)
AQP-1a	*Sparus aurata*	↓ after FW acclimation Higher abundance in duodenum and hindgut, with immunoreactivity in brush border and basolateral membrane of enterocytes. Immunostained with cytoplasm of some rectal enterocytes.	Raldua et. al.2008
AQP-1b	*Sparus aurata*	↓ in rectum after FW acclimation Higher abundance in rectum, with immunolocalized in brush border of rectal enterocytes.	Raldua et. al.2008
AQP-3	*Anguilla anguilla*	Immunoreactivity in intra-epithelial macrophage-like cells within the intestine of FW and SW-acclimated eels and in the mucous cells of the rectal epithelium of SW eel.	Lignot et al. (2002)
AQPe	*Anguilla anguilla*	↑ in midgut of silver eels after SW acclimation	Martinez et al. (2005b)
sbAQP	*Sparus aurata*	Localized in *lamina propria* and interface between muscle layer of hindgut	Santos et al. (2004)
AQP-8	*Anguilla anguilla*	↑ during salinity acclimation	Cutler et al. (2009)

Table 2.3 Aquaporins in kidneys of teleosts

AQPs	Species	Effects on expression and distribution	References
AQP-1	*Anguilla anguilla*	⇩ in yellow eels after SW acclimation	Martinez et al. (2005a)
	Acanthopagrus schlegeli	Highest at 10% seawater	An et al. (2008)
	Dicentrarchus labrax	⇩ after SW acclimation	Giffard-Mena et al. (2007)
AQP-1dup	*Anguilla anguilla*	⇩ in yellow eels after SW acclimation	Martinez et al. (2005a)
AQP-3	*Sparus sarba*	No significant difference among different salinity groups	Deane & Woo (2006)
AQPe	*Anguilla anguilla*	⇩ after SW acclimation	Martinez et. al. (2005a)
sbAQP	*Sparus aurata*	Localized in epithelial cells of some kidney tubules	Santos et al. (2004)

Chapter 3

Immunological characterization of aquaporins in major osmoregulatory organs of silver sea bream

3.1 Introduction

In the past decade, AQP-1 and AQP-3 had been characterized in major osmoregulatory organs of teleost species with the aid of immunohistochemical techniques. AQP-1 was ubiquitously immunolocalized in gill chloride cells (Brunelli et al., 2010), enterocytes (Aoki et al., 2003; Raldua et al., 2008; Martinez et al., 2005b), and apical membrane of renal tubules (Martinez et al., 2005a). Immunoreactivity of AQP-3 was mainly found in chloride cells and pavement cells of gills (Brunelli et al., 2010; Lignot et al., 2002) and macrophage-like body and goblet cells of intestine (Lignot et al., 2002). In addition, immunoreactivity of these aquaporins were colocalized with that of Na^+-K^+-ATPase in the branchial chloride cells suggesting a potential linkage between water transport and ionoregulatory process. With reference to their respective tissue location, it is generally accepted that these AQPs are related to water transport or cell volume control although the role of AQP-3 is more likely to have a secretory role in the goblet cells of intestine.

In most of these immunohistochemical studies, specific fish antibodies to respective AQPs were adopted as probes, however, Tse et al. (2006) and Brunelli et al. (2010) had also demonstrated high efficacy of mammalian AQP anti-sera in probing

corresponding AQPs in eels and rainbow wrasses. The present study adopted mammlian AQPs anti-sera to characterize AQP-1, AQP-2, AQP-3 and AQP-4 in the major osmoregualtory organs (gills, intestine and kidney) of silver sea bream (*Sparus sarba*) using immunohistochemical methods. The four aquaporins are chosen in the experiment because they are highly abundant in various tissues in human and other mammalian species and are better understood among the animal-type aquaporins.

Another goal of this experiment is to correlate aquaporin distribution with mitochrondria-rich (MR) cells (chloride cells in gill and enterocytes in intestine), the cells which are known to be the major site for ionoregulation. High level of Na^+-K^+-ATPase activity is found in these MR cells (Cutler et al., 2000; Hwang & Lee, 2007), and this ATPase plays a central role in active transport of Na^+ out of the animal cells with influx of K^+. In addition, Na^+-K^+-ATPase provides the driving force for many transporting systems in osmoregulatory important organs, including gills and kidney. Co-localisation of Na^+-K^+-ATPase and aquaporins can provide more information about the relationship between water and salt transport in these major osmoregulatory organs.

3.2 Materials and methods

3.2.1 Experimental animals

Adult silver sea bream (*Sparus sarba*) were purchased from local fish farms and transferred to recirculated one-tonne concrete tanks, where they were maintained in seawater for one week. The sea bream were adapted to either freshwater or seawater by gradual dilution of tap water or seawater for further four weeks at ambient temperature (18-25 °C). After salinity acclimation, sea bream were sacrificed and gills, intestine and kidney were dissected out. In order to study regional distribution of aquaporins, the intestines were further separated into oesophagus, stomach, anterior and posterior intestine.

3.2.2 Confocal laser scanning microscopy (CLSM)

The removed tissues were fixed either in Bouin's fixative or 4% phosphate-buffered (0.01M, pH 7.3) paraformaldehyde for 24 h in 4°C respectively. The fixed tissues were infiltrated with graded ethanol series and xylene and finally embedded in paraffin. Tissue sections (5 μm) were cut on a microtome and collected on poly-L-lysine-coated slides. The sections were firstly incubated in PBS-T (0.01

mmol l^{-1} Tween 20, 150 mmol l^{-1} NaCl in 10 mmol l^{-1} phosphate buffer, pH 7.3) for 10 minutes at room temperature. Then the sections were treated with 50 mmol l^{-1} NH_4Cl in PBS, pH 7.3, for 5 min. The sections were washed in PBS-T for 5 minutes and blocked by a blocking solution containing 1% bovine serum albumin (BSA) and 0.1% gelatin in PBS-T for 1 hour. The sections were incubated for 1 h at room temperature in a wet chamber with droplets (30µl) containing goat anti human AQP-1, 2 and 3 (Santa Cruz) at its optimal dilution in BS (1/100). Sections were also incubated with rabbit anti bream Na^+-K^+-ATPase α-subunit developed specifically for silver sea bream Na^+-K^+-ATPase by Deane & Woo (2005). Blank control was made by substituting antibodies with BS whereas negative control was carried out using 1% goat and 1% rabbit serum in BS.

The sections were washed in PBS-T (6 times x 15min). The sections and "control" sections were incubated in droplets of Fluorescein (FITC)-conjugated secondary antibody [1:200; AffiniPure donkey anti-goat IgG_{H+L}] or/and Cyanin-3(Cy3)-conjugated secondary antibody [1:200; AffiniPure donkey anti-rabbit gG_{H+L}] for 2 hours. All sections were then washed in PBS-T (6 x 5 min) and mounted with anti-fading mounting medium (Fluka). The prepared sections were observed using a confocal laser scanning microscope (Biorad MRC 600) equipped with a

krypton/argon laser in combination with a Nikon Diaphot microscope under excitation by blue light (488 nm), equipped with a 515nm emission barrier filter (BHS) and an A2 filter (blocking emission wavelengths below 600 nm). The pictures from each photomultiplier were subsequently merged in false colour to visualise the labels simultaneously (green colour: FITC-conjugates; red colour: Cy3-conjugates).

3.3 Results

3.3.1 Overview of immunohistochemical results

Immunoreactions with mammalian AQP-1 and AQP-3 antibodies were detected in gills, intestine and kidney in both seawater and freshwater adapted sea bream (Fig. 3.1 to 3.4, 3.6 to 3.14.). The mammalian AQP-2 antibody did not stain with the sea bream tissues examined. Na^+-K^+ATPase alpha subunit immunoreactivity was labeled in gills and kidney tissues (Fig. 3.5, 3.16). Figures 3.16-3.18 show non-specific binding in the tissue sections using pre-immune serum (control).

3.3.2 Immunolocalisation of aquaporins in the gill filaments

In both seawater and freshwater adapted sea bream, immunoreactivity to

mammalian AQP-1 and AQP-3 was mainly detected in the primary lamellae of the gill filament (Fig. 3.1-3.4). Figure 3.5 shows staining of Na^+-K^+ATPase alpha subunit being present at the junction of primary and secondary gill lamellae in seawater adapted sea bream. Mammalian AQP-1 and AQP-3 antibodies were also localized in cells of the interlamellar region on primary lamellae, with AQP-3 labeling being located closer to the basolateral side of cells (Fig. 3.3 and 3.4). Immunoreactivity to AQP-1 was primarily found in the junctions between the primary and secondary lamellae (Fig. 3.1 and 3.2). There are no apparent differences in the distribution pattern and intensity of AQP immunoreactivity in gill lamellae between seawater and fresh adapted sea bream.

3.3.3 Immunolocalisation of aquaporins in gastrointestinal tract

Among the different gastrointestinal tissues examined, immunoreactivity to mammalian AQP-1 and AQP-3 antibody was only detected in anterior and posterior intestines (Fig. 3.6-3.12). Both antibodies predominantly stained on the basal side of the epithelial cells in villi of anterior and posterior intestine. Fig. 3.6 also shows signals of mammalian AQP-1 being scattered among some goblet cells in the anterior intestine of a seawater adapted sea bream. Figure 3.13 shows staining of

Na^+-K^+ATPase alpha subunit being present at the cell surface of enterocytes in seawater adapted sea bream and signal intensity was fading towards the apical side.

3.3.4 Immunolocalisation of aquaporin in kidney

In both seawater and freshwater adapted sea bream, immunoreactivity to mammalian AQP-1 was clearly restricted to the apical membrane of some renal tubular cells (Fig. 3.13-3.14). In contrast to AQP-1, staining corresponding to the Na^+-K^+ATPase alpha subunit was found on the basolateral side of all tubular cells (Fig. 3.15).

3.4 Discussion

The present study characterized aquaporin water channels in various osmoregulatory organs of silver seabream by the application of mammalian aquaporin-specific antibodies. Similar approach had been successfully adopted by Tse et al. (2006) and Burnelli et al. (2010) to demonstrate distribution of AQP-3 in seawater acclimated eels (*Anguilla japonica*) and AQP-1 and AQP-3 in rainbow wrasse respectively, however, these positive results should be interpreted cautiously.

Since the polyclonal antibodies used in our experiment were specific to several epitopes of corresponding mammalian AQP homologues, the immunoreactivity detected in the sea bream tissues may represent the presence of the particular aquaporin and/ or its isoforms. In fact, immunoblotting using human AQP-1 anti-serum and kidney protein samples of sea bream resulted in two heterogenous bands, therefore any "AQP-1" immunoreativity detected in the microscopy may merely reflect the appearance of fish AQP-1 isoforms or other AQP homologues that share similar epitopes with the human AQP-1.

The mammalian AQP-2 antibody did not stain with the sea bream tissues examined. The vasopressin/vasotocin-dependent AQPs are only found in amphibians and mammals (Ogushi et al., 2007), and the presence of the AQP-2 type homologues in these land animals may be an adaptive change to conserve water in terrestrial life. Previous findings had demonstrated the potency of vasotocin to increase water transport in fish gills (Maetz et al., 1964; Marshall, 2003), so the possibility of vasotocin-dependent AQPs in fish should not be overlooked.

Immunoreactions of mammalian AQP-1 and AQP-3 antibodies were detected in gills, intestine and kidney in both seawater and freshwater adapted sea bream.

Na^+-K^+ATPase alpha subunit was labeled in gills, intestine and kidney tissues. Non-specific immunoreactivity was observed in the tissue sections using pre-immune serum (control). The remaining part of our discussion will be focused on the results from our immunolocalisation study on AQP-1 and AQP-3 in the gill, intestine and kidney of silver sea bream.

3.4.1 Gills

In gills of freshwater- and seawater-acclimated sea bream, the signals for immunoreactivity of antibodies against mammalian AQP-1 and AQP-3 homologues were relatively weak, however, these weak stainings were concentrated in the interlamellar spaces where the density of chloride cells is highest in the gill filament (Kelly & Woo, 1999). Chloride cells are known to play an important role in osmoregulation during hyperosmoregulation and hyporegulation of euryhaline fish (Hwang & Lee 2007).

Previous studies on AQP-3 in fish revealed the water channel was localized in the basolateral membrane of gill chloride cells (Lignot et al., 2003; Watanabe et al., 2005). Watanabe et al. (2003) suggested AQP-3 is involved in regulatory volume changes

and osmoreception, which could trigger functional differentiation of chloride cells. Indeed, osmotic water permeability of the gills (Motais & Isaia, 1972) and branchial expression of AQP-3 (Cutler & Cramb, 2003) in freshwater-acclimated fish is much higher than those of seawater-acclimated fish. It is also possible that AQP-3 may act as the pathway in the basolateral membrane for the exit of water to serosal fluid and the prevention of cell swelling (Cutler & Cramb, 2000; Matsuzaki et al., 2000).

Localization of AQP-1 and AQP-3 in gills of teleostean species had been lately reported in rainbow wrasse (Brunelli et al., 2010). In the study, both aquaporins were localized in the gill chloride cells and co-existed with the electrogenic Na^+-K^+-ATPAase. Similar co-localization of aquaporins and Na^+-K^+-ATPAase was also observed in the gills of silver sea bream, this implies possible linkage between water transport and ionoregulation in these chloride cells.

3.4.2 Intestine

In the intestines of seawater- and freshwater-adapted sea bream, the anti-AQP-1 staining was concentrated in the basolateral side of the epithelia and immunoreacted with goblet cells. Only trace level of AQP-1 immunoreactivity was detected in the

apical surface of posterior intestine of the freshwater-adapted sea bream.

In contrast to our findings, Aoki et al. (2003) reported that anti-eel AQP-1 was heavily stained in the apical surface columnar epithelial cells in the mucosa of posterior intestine in seawater-adapted Japanese eels. The AQP-1 immunoreactivity for freshwater-adapted eels, however, was relatively weak. Besides, AQP-1 immunoreaction was also detected in endothelial cells of the blood vessels distributed in the mucosa of the posterior intestine of Japanese eels (Aoki et al., 2003). Similar distribution patterns were observed in both intestine and rectum of European eels (Martinez et al., 2005b).

The results obtained from Japanese eels suggest a possible role of AQP-1 on facilitation of water entry from the apical side of epithelial cells, preferentially in the mucosa along the posterior intestine (Aoki et al., 2003). The authors also postulated further on the existence of another AQP water channel located on the basolateral surface of epithelial cells which would act as the exit pathway for the water absorbed from the lumen.

Recent studies have attempted to elucidate the possibility of arrangement of

different aquaporin isoforms for transcellular water transport in gastrointestinal tract. Several AQP-1 isoforms had been characterized in European eels (AQP-1 and AQP-1 dup, Martinez et al., 2005b) and gilthead sea bream (SaAQP-1a & SaAQP-1b, Raldua et al., 2008), where these isoforms exhibit their own distinctive tissue distribution patterns. Raldua et al. (2008) demonstrated that the location of the sea bream AQP-1 isoform, SaAQP-1a, is highly region-specific along the digestive tract. Immunoreactivity to the sea bream AQP-1 isoform was primarily located in the apical membrane of epithelial cells in the duodenum and hindgut. In the rectum, SaAQP-1a was predominantly localized in the perinuclear compartment, with much weaker signal being detected in the apical brush border and lateral plasma membrane. Immunoreactivity to another isoform, SaAQP-1b, is only restricted to the apical brush border (Raldua et al. 2008).

In the present study, the immunoreactions to mammalian anti-AQP-1 and anti-AQP-3 were found to be localized in the basolateral side of cytoplasm of the mucosal epithelial cells along the intestine. Indeed, the water absorption across teleost intestine is tightly linked to the absorption of Na^+ and Cl^- (Skadhauge, 1974; Usher et al., 1991) which is driven by basolateral Na^+-K^+-ATPase activity (Skou, 1990; Skou & Esmann, 1992). Our results had shown the immunoreativity of Na^+-K^+-ATPase alpha

subunit was stained on the surface of enterocytes and the staining became less intense in the apical side of the enterocytes. In contrast to most results regarding intestinal AQP-1 distribution in teleosts, the AQP-1 isoform or aquaporin homologues detected by the mammalian antibodies may serve as the outlet for releasing water to the serosal side of the intestine in the silver sea bream. In marine teleosts, intestinal water absorption is a crucial process for the fish to compensate for the osmotic water loss during seawater adaptation. This intestinal water absorption relies on net NaCl uptake that is driven by the electrogenic Na^+-K^+-ATPase located on the basolateral membrane of intestinal epithelium (Grosell, 2007). The water molecules may passively enter the epithelium through the apically located AQP-1 while the AQP homologue detected in the baslateral membrane could allow efficient removal of water from the epithelium as driven by electrogenic Na^+-K^+-ATPase. Taken together, these results indicate that intestinal water absorption may involve this paracellular pathway that is facilitated by apical and basolateral AQP water channels.

Immunoreactivity of mammalian AQP-1 antibody was also detected in goblet cells of the anterior intestine of seawater adapted sea bream, suggesting possible role of AQP in water trafficking during mucus secretion. In European eels, similar observation was also reported in goblet cells of rectum with intense staining of eel

AQP-3 specific antibody (Lignot et al., 2002). The mucus secreted from goblet cells generally provides a barrier that reduces the ability of pathogens to invade through the intestinal epithelium. In addition, a role of AQP for maintenance of "wetness" on the luminal surface was proposed by Lignot et al. (2002), however, the physiological significance of this role requires more evidence to verify.

3.4.3 Kidney

Immunoreactivity associated with anti-AQP-1 was predominantly localized in the apical membrane of epithelial cells of some renal tubules. The localization is in consistent fashion to the observation of AQP-1 distribution reported in the kidney of European eels (Martinez et al., 2005a). By using eel AQP-1 antibody, the immunoreactivity was detected at the apical surface in certain subset of renal tubules. In mammals, AQP-1 is located in the apical and basolateral membrane of proximal tubules and descending thin limb (Nielsen et al., 2002). In AQP-1 null mice, osmotic water permeability was significantly reduced in proximal tubule (Schnermann et al., 1998) and descending thin limb (Chou et al., 1999). Absence of AQP-1 results in defective isosmolar fluid absorption in proximal tubule and defective countercurrent exchange, hence the osmolality of the medullary interstitium is significantly lower

than normal level (Yang et al., 2001).

Nephron of marine teleosts is relatively simple and the proximal tubules constitute the major portion (Dantzler, 2003). The proximal tubule is basically divided into early proximal segments and late proximal segments with regard to their functions. Renal reabsorption of Na^+, Cl^-, divalent ions and fluid occur in the early proximal segment while preurine is modified by secretion of Na^+, Cl^- and water in the late proximal segment. In the present study, AQP-1 immunoreactivity was detected in apical brush border of a subset of renal tubules of seawater-adapted sea bream. AQP-1 has been reported to be present in both apical and basolateral plasma membranes of proximal tubules (Nielsen et al., 2002), likewise it is also possible for the AQP-1 to be localized in the apical membrane of proximal tubules of the silver sea bream. The presence of AQP-1 immunoreactivity may explain the apical pathway for water transport in proximal tubules, however, there is no functional evidence to show whether this apically located AQP-1 is involved in absorption or secretion in the kidney of sea bream.

Water exit and entry in various proximal segments are tightly coupled with the movement of Na^+, Cl^- and were driven by basolaterally-located electrogenic

Na^+-K^+-ATPase (Dantzler, 2003 ; Beyenbach, 2004). The activity and expressions of this sodium pump is influenced by external water salinity (Kelly & Woo, 1999; Deane & Woo, 2004) and plays a central role in renal osmoregulation of silver sea bream. By applying polyclonal sea bream-specific antibody for Na^+-K^+-ATPase (Deane & Woo, 2004), intense immunoreaction was detected in the basolateral side of all types of renal tubular cells, including the subset of renal tubules with mammalian anti-AQP-1 immunoreactivity. It is possible that Na^+-K^+-ATPase participates indirectly in water transport and functions of aquaporins in the sea bream kidney, however, further experiment is necessary to justify this hypothesis.

Since AQP-1 immunoreactivity was restricted to the apical side of a subset of proximal segment, water transport across the basolateral membrane and other renal tubules must be facilitated by other alternative pathways. This suggests the possible existence of other types of aquaporins in order to complete the water transport pathway across the renal epithelia. Further research has revealed two other aquaporin homologues, AQP-1dup and AQPe, which were highly expressed within the kidney (Martinez et al., 2005b).

3.5 Conclusion

Since the antibodies used in our immunolocalisation study were raised against the mammalian-type of aquaporin homologues, the results obtained should be interpreted cautiously and were not subjected to quantitative analysis. Besides, these mammalian antibodies cannot distinguish different isoforms of fish aquaporin homologues, such as AQP-1 and its duplicate form (AQP-1 dup), that makes precise localization of specific fish aquaporin impossible.

Among the four aquaporin homologues examined, AQP-1 and AQP-3 are shown to present in osmoregulatory organs in silver sea bream. Colleagues of our group have recently isolated partial cDNA clone of an aquaporin from silver sea bream which shares high homology to reported sequences of AQP-3 in other teleosts and higher vertebrates (Deane & Woo, 2006).

In order to further our understandings of aquaporin in the marine teleost, the next step of the present study attempts to isolate silver sea bream AQP-1 cDNA sequence and use it as a tool in mRNA expression to elucidate the effect of environmental salinity and hormonal modulation on this aquaporin water channel.

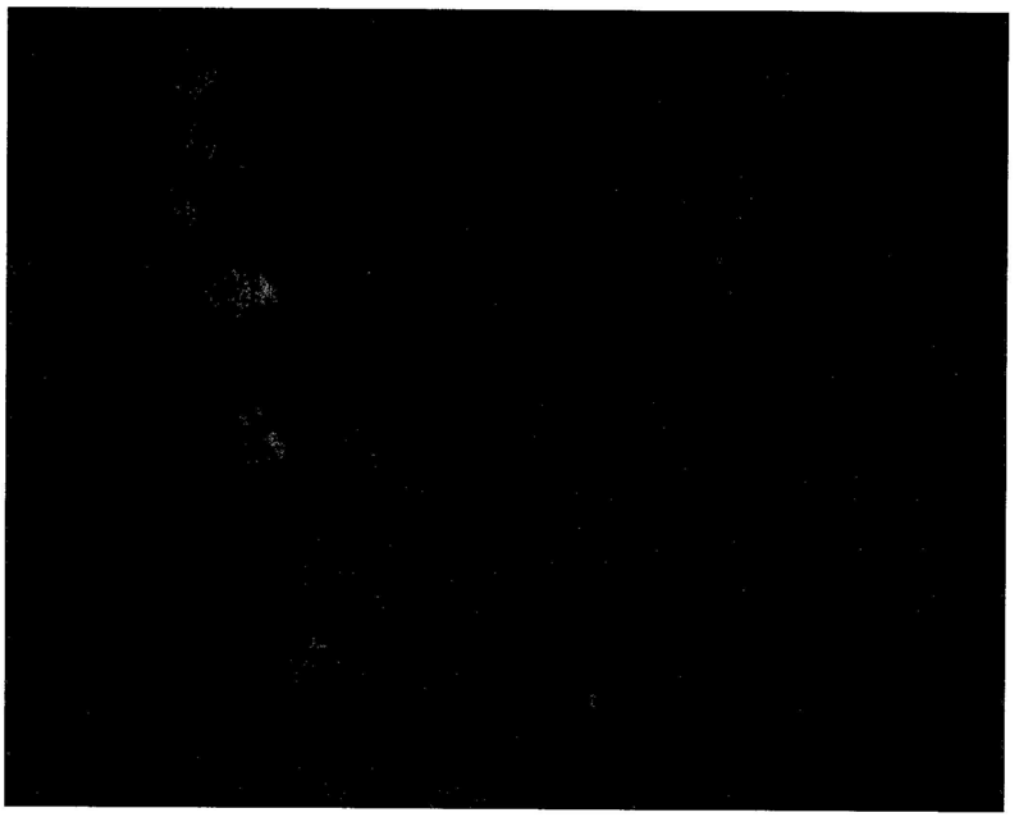

Figure 3.1 Immunolocalisation of AQP-1 in gill filament of seawater adapted sea bream using mammalian AQP-1 antibody (L.S.).

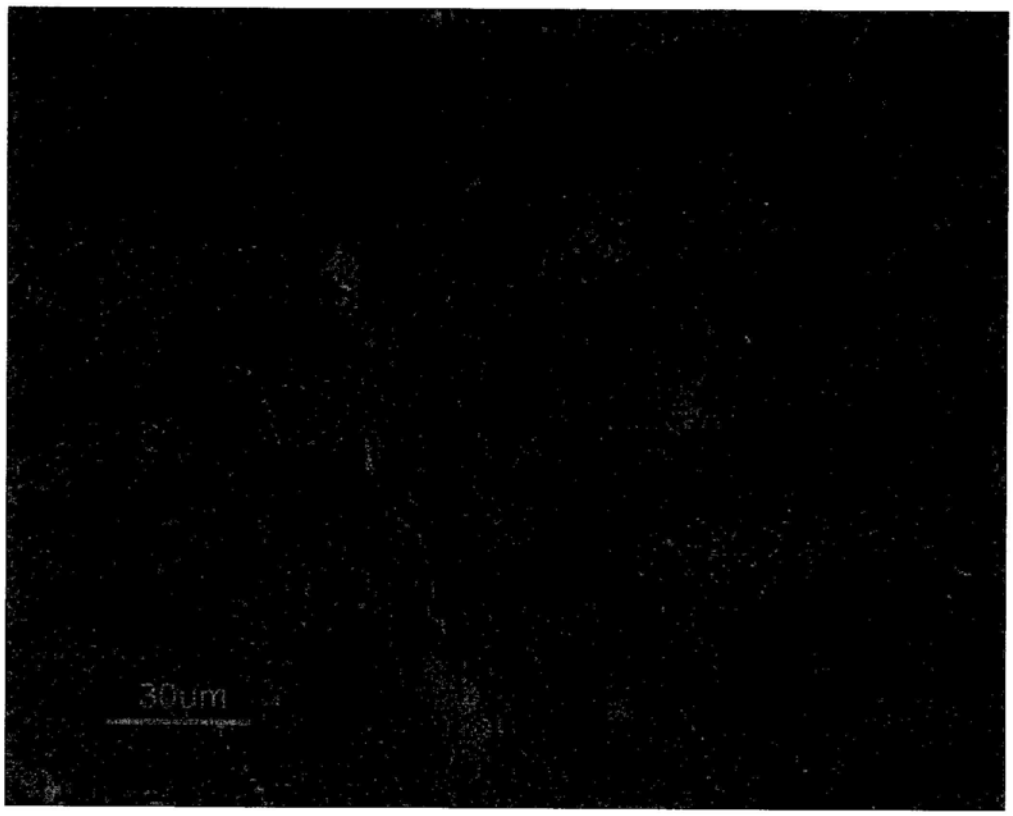

Figure 3.2 Immunolocalisation of AQP-1 in gill filament of freshwater adapted sea bream using mammalian AQP-1 antibody (L.S.).

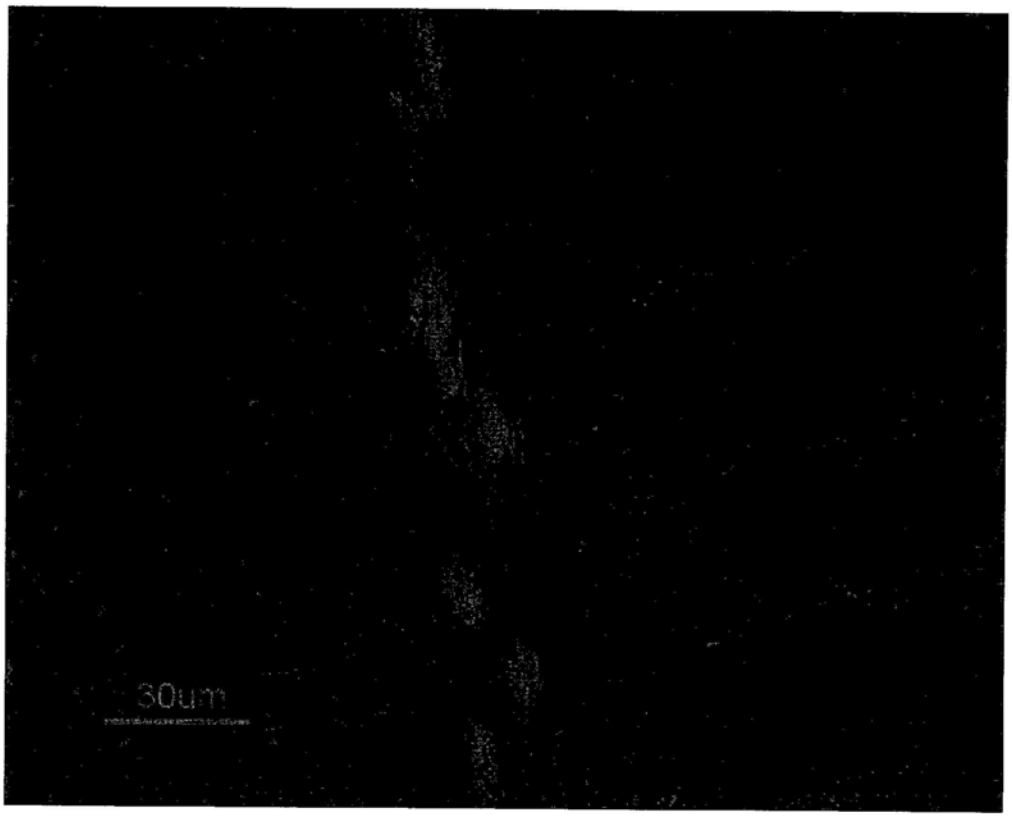

Figure 3.3 Immunolocalisation of AQP-3 in gill filament of seawater adapted sea bream using mammalian AQP-3 antibody (L.S.).

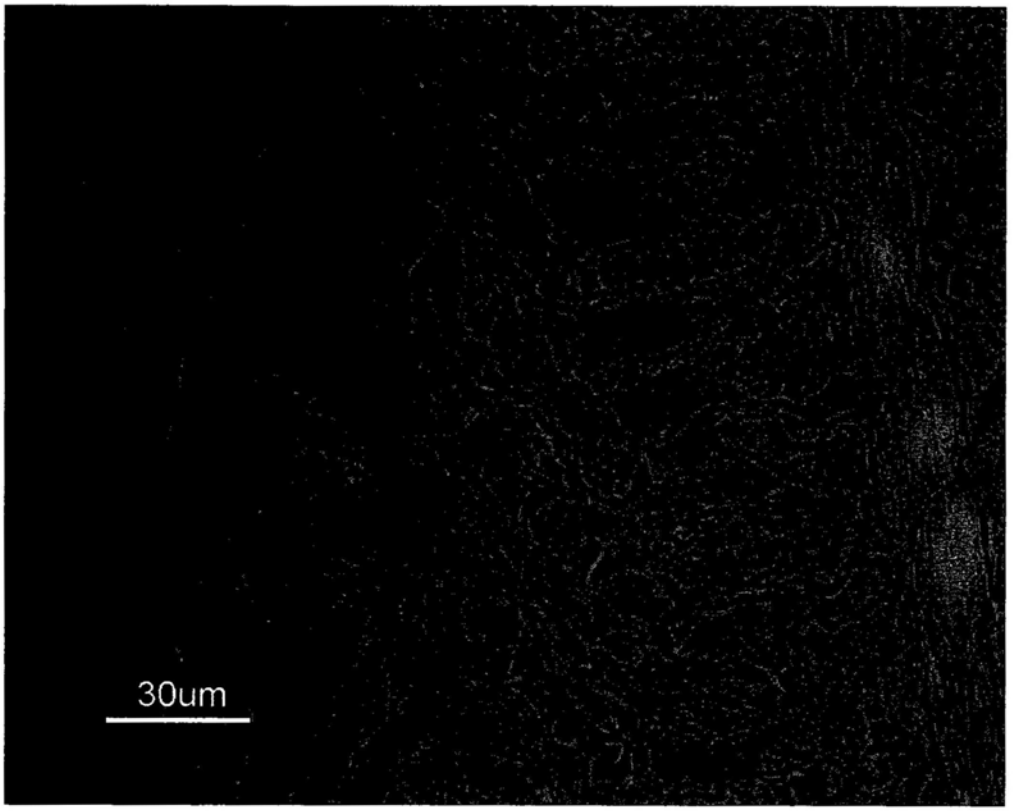

Figure 3.4 Immunolocalisation of AQP-3 in gill filament of freshwater adapted sea bream using mammalian AQP-3 antibody (L.S.).

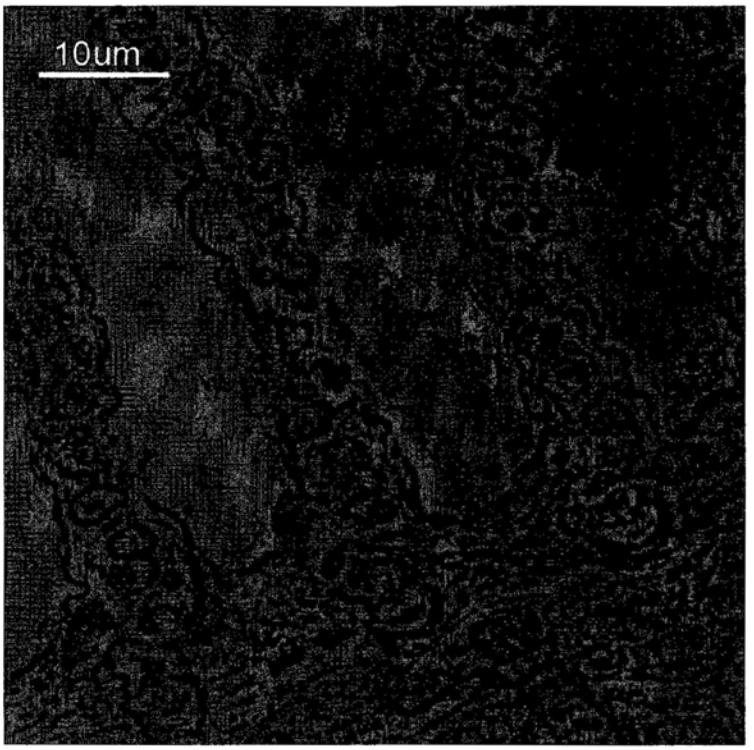

Figure 3.5. Immunolocalisation of Na^+-K^+ATPase alpha subunit in gill filament of a seawater adapted sea bream (L.S.).

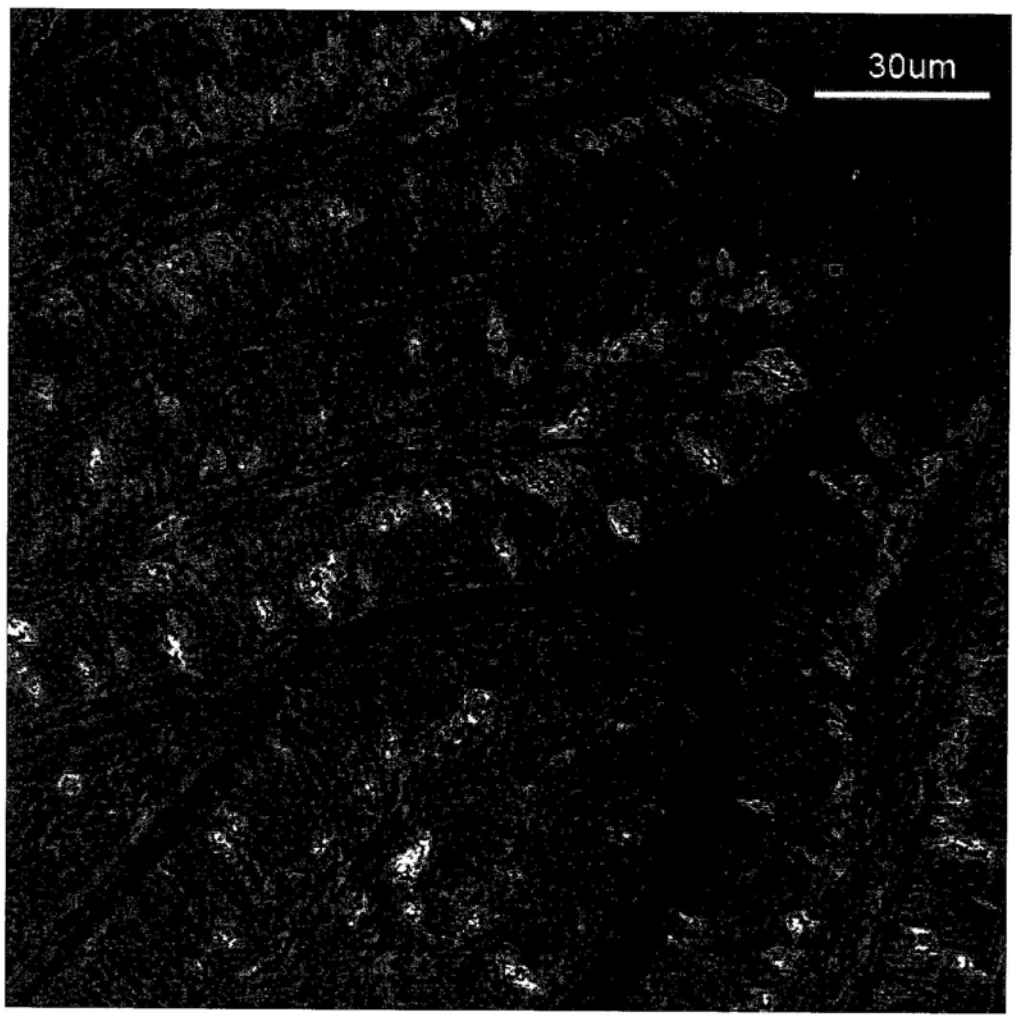

Figure 3.6. Immunolocalisation of AQP-1 in villus of anterior intestine of a seawater adapted sea bream using mammalian AQP-1 antibody (X.S.).

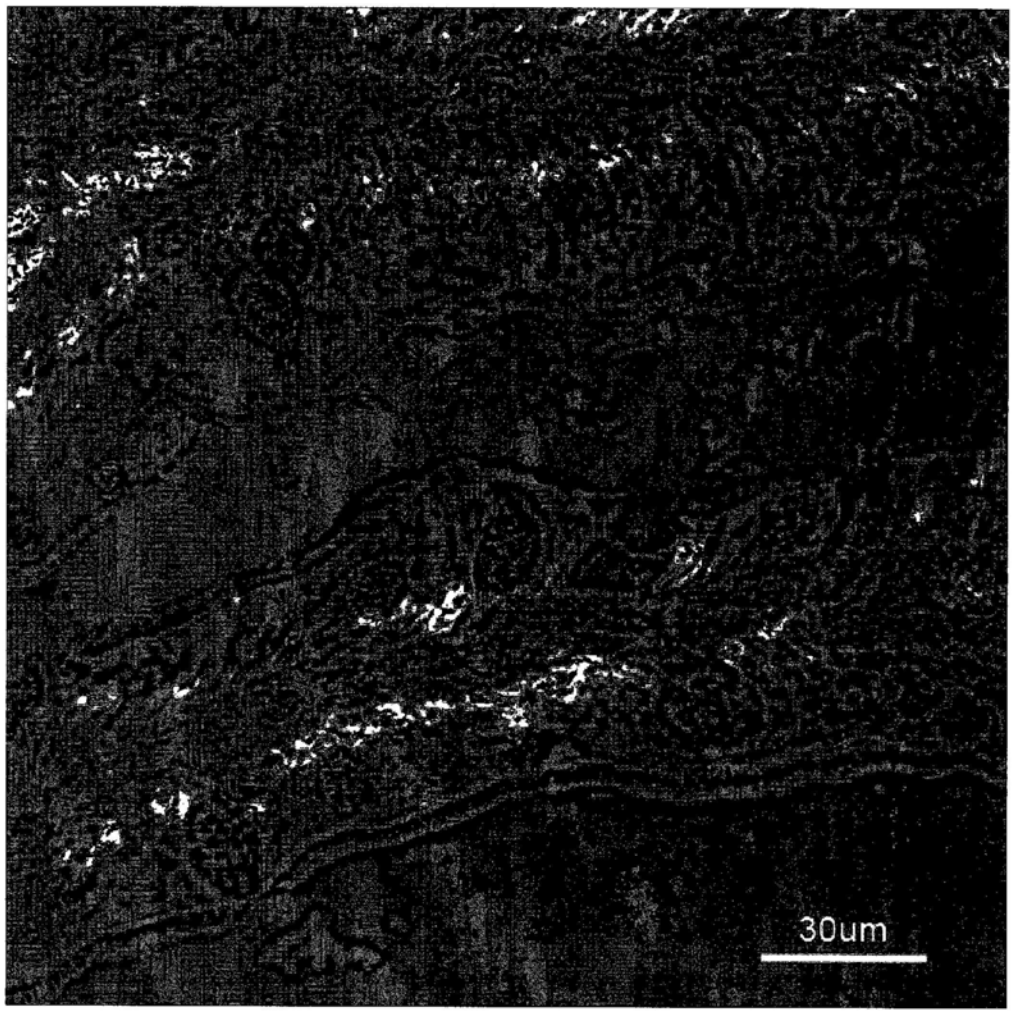

Figure 3.7. Immunolocalisation of AQP-1 in villi of anterior intestine of a freshwater adapted sea bream using mammalian AQP-1 antibody (X.S.).

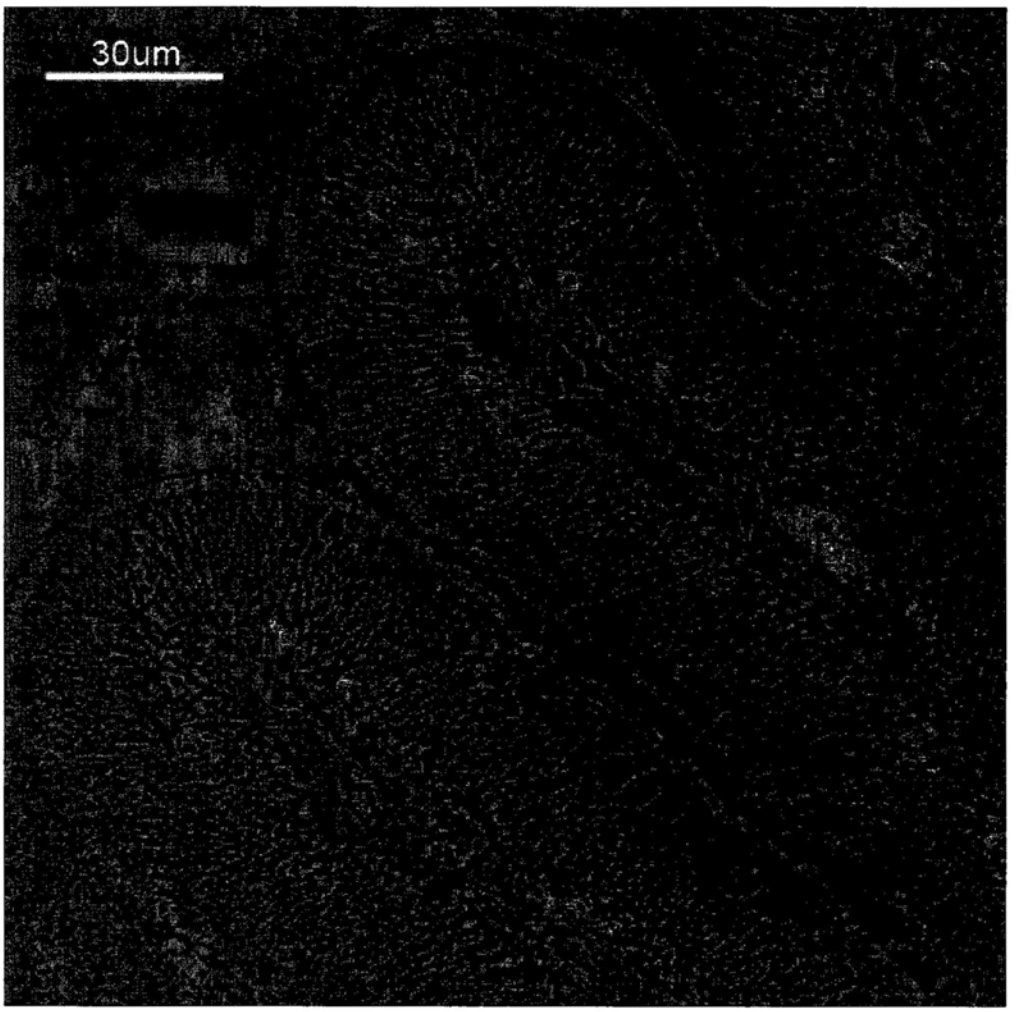

Figure 3.8. Immunolocalisation of AQP-1 in villi of posterior intestine of a seawater adapted sea bream using mammalian AQP-1 antibody (X.S.).

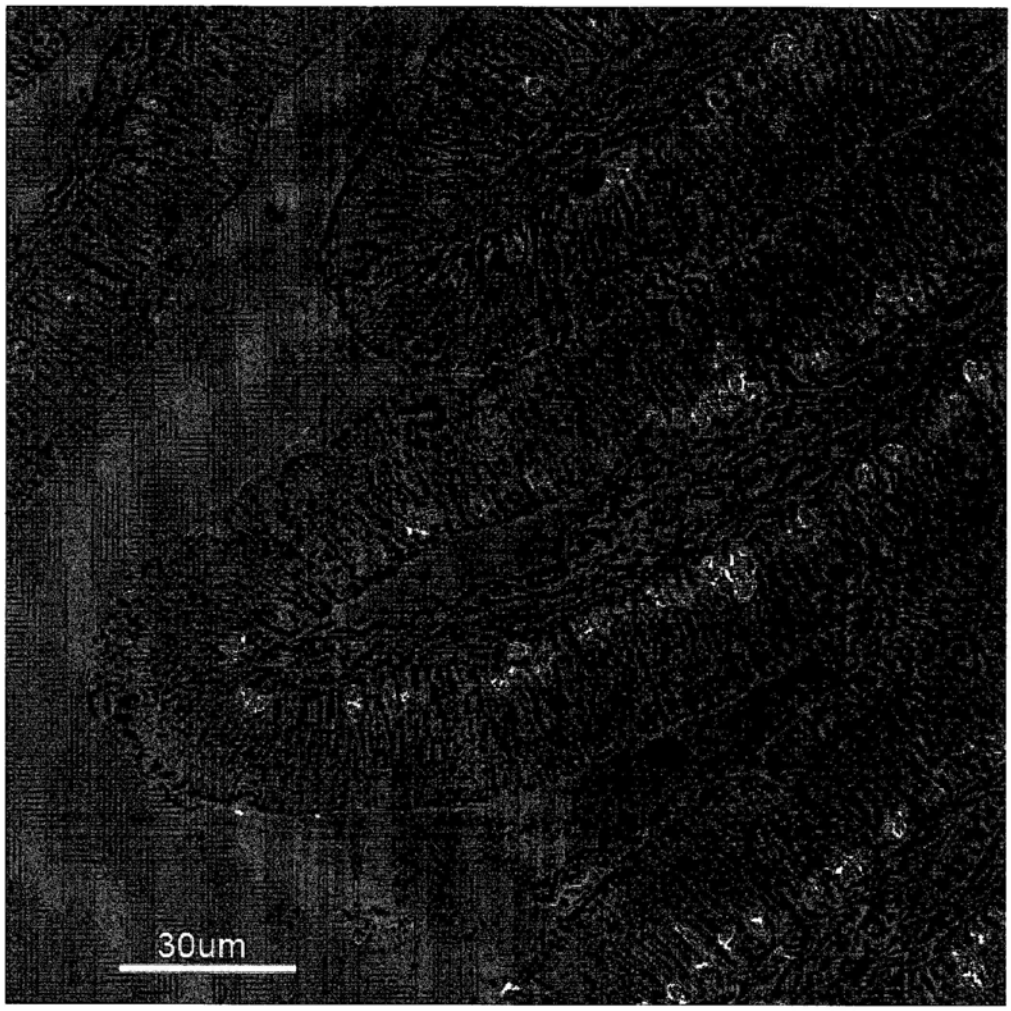

Figure 3.9. Immunolocalisation of AQP-1 in villi of posterior intestine of a freshwater adapted sea bream using mammalian AQP-1 antibody (X.S.).

Figure 3.10. Immunolocalisation of AQP-3 in villi of anterior intestine of a seawater adapted sea bream using mammalian AQP-3 antibody (X.S.).

Figure 3.11. Immunolocalisation of AQP-3 in villi of anterior intestine of a freshwater adapted sea bream using mammalian AQP-3 antibody (X.S.).

Figure 3.12. Immunolocalisation of AQP-3 in villi of posterior intestine of a seawater adapted sea bream using mammalian AQP-3 antibody (X.S.).

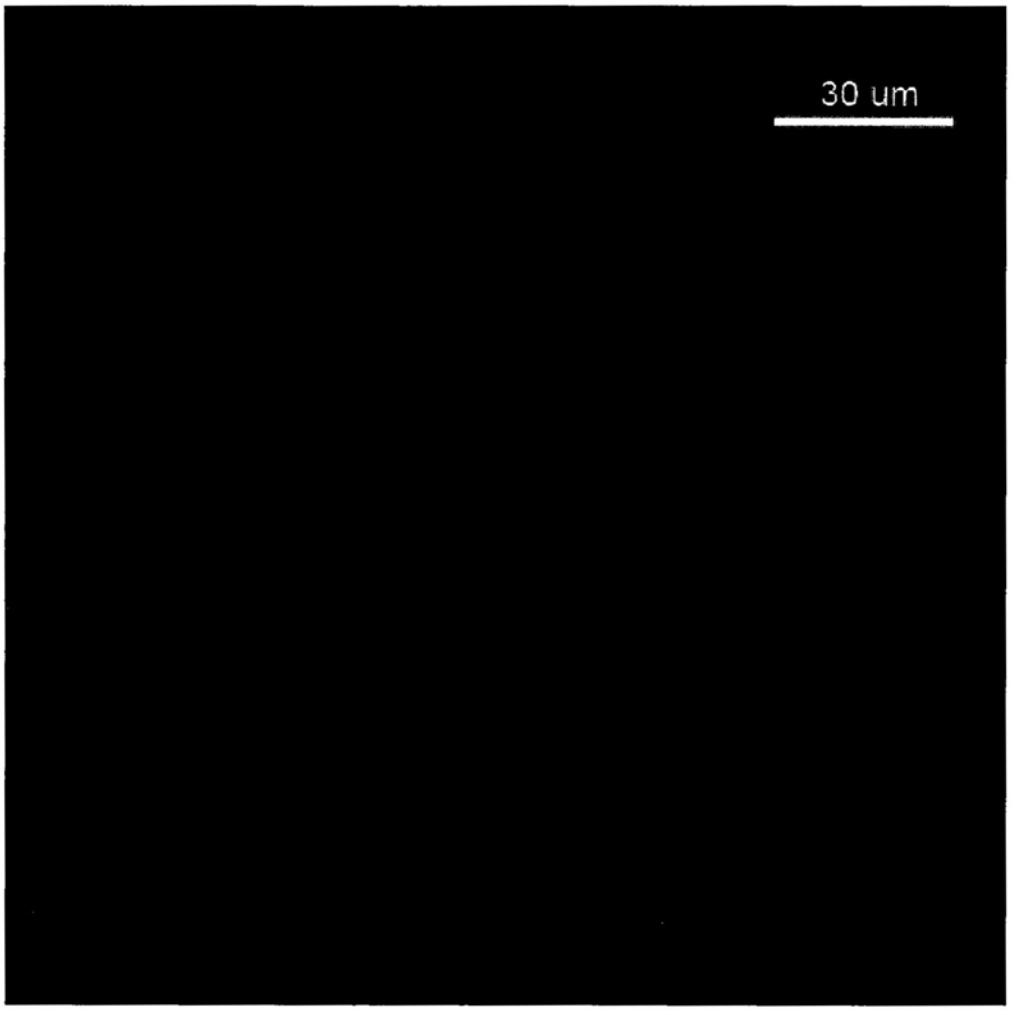

Figure 3.13. Immunolocalisation of Na^+-K^+ATPase alpha subunit in villi of anterior intestine of a seawater adapted sea bream using mammalian AQP-3 antibody (X.S.)

Figure 3.14. Immunolocalisation of AQP-1 in renal tubules of a seawater adapted sea bream using mammalian AQP-1 antibody (X.S.).

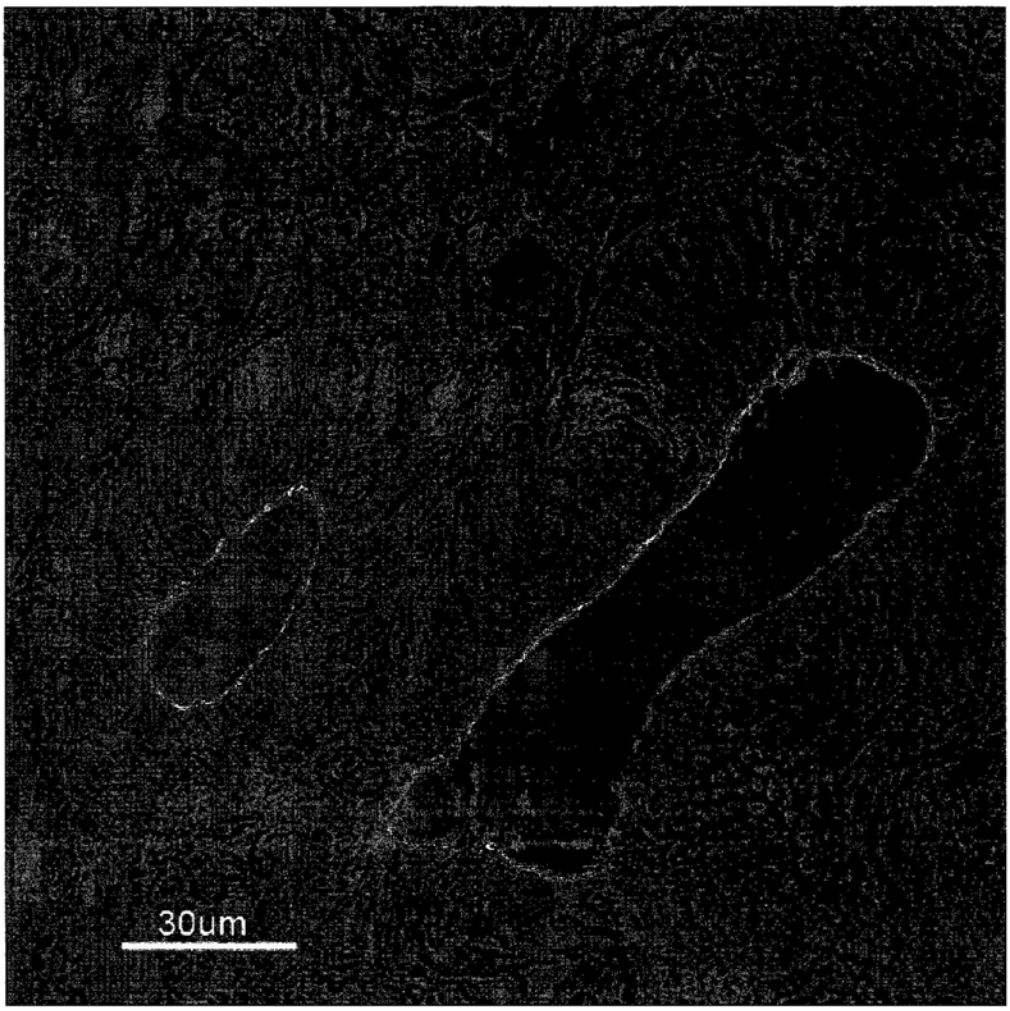

Figure 3.15. Immunolocalisation of AQP-1 in renal tubules of a freshwater adapted sea bream using mammalian AQP-1 antibody (X.S.).

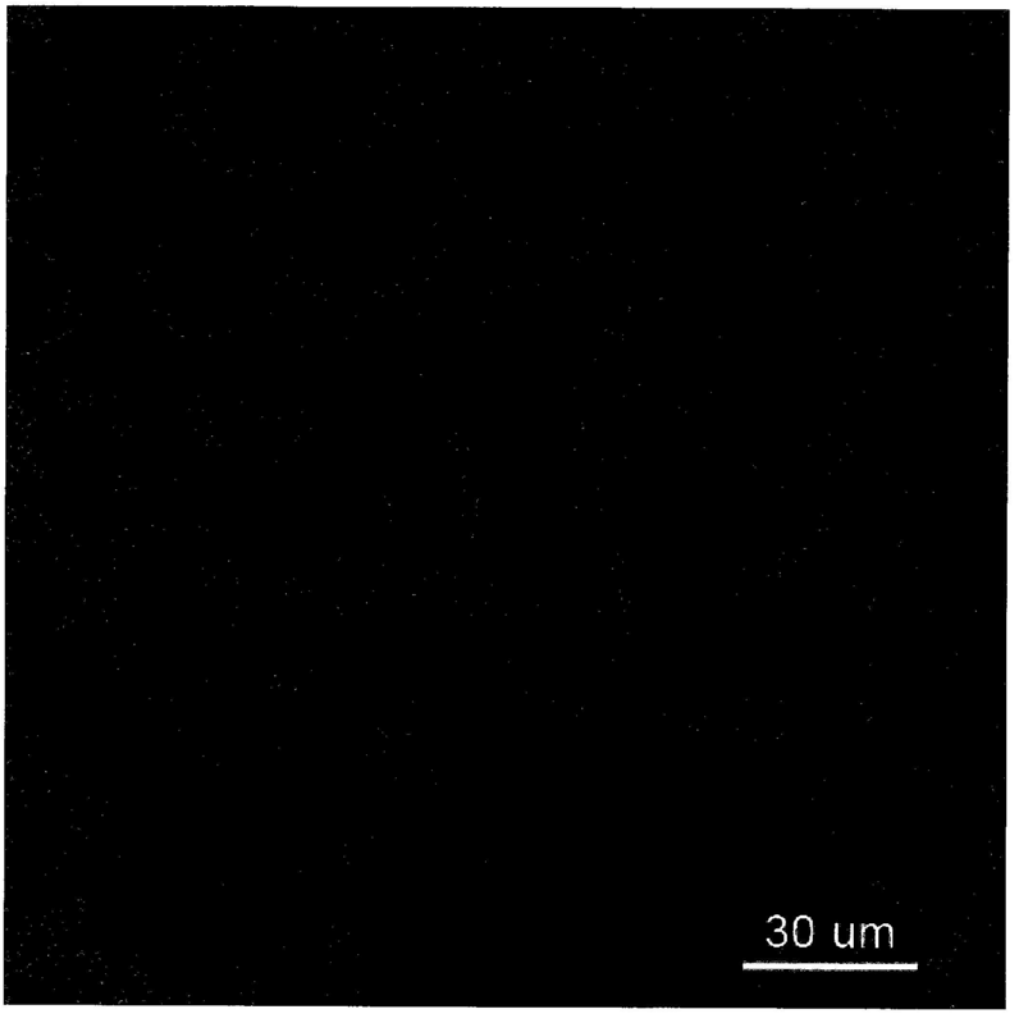

Figure 3.16. Immunolocalisation of Na^+-K^+ATPase alpha subunit in renal tubules of a freshwater adapted sea bream (X.S.).

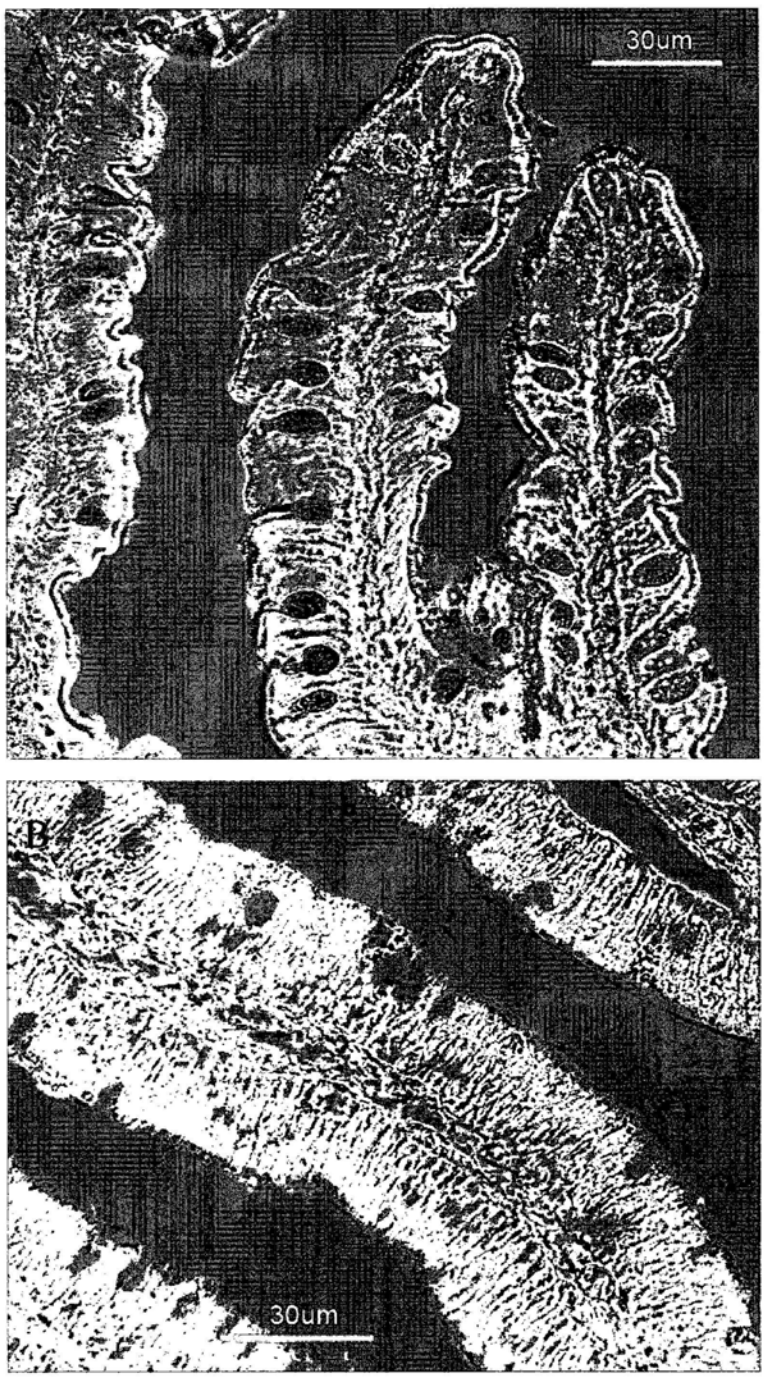

Fig. 3.17. Non-specific binding in sections of (A) anterior intestine and, (B) posterior of a freshwater adapted sea bream after incubation with pre-immune goat serum (X.S.).

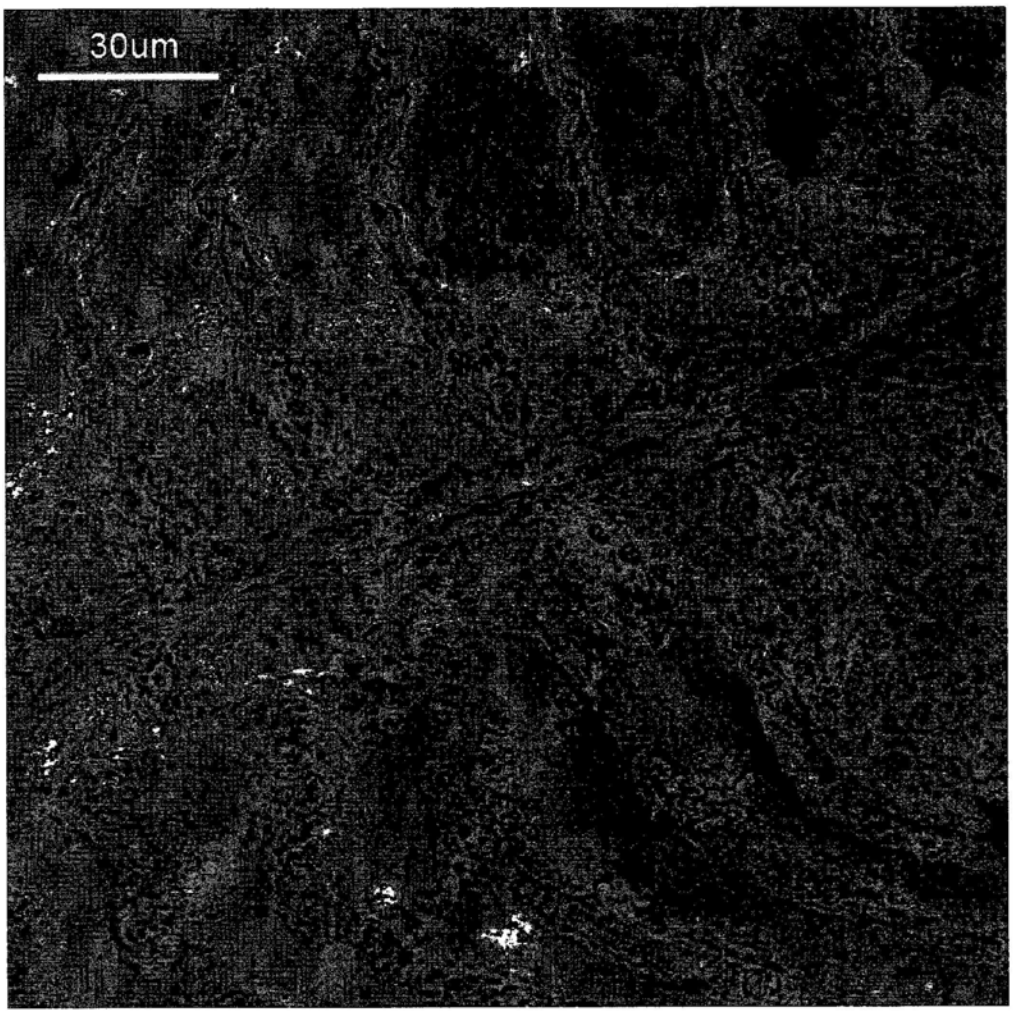

Fig. 3.18. Non-specific binding in sections of gill filaments of a freshwater adapted sea bream after incubation with pre-immune goat serum (L.S.).

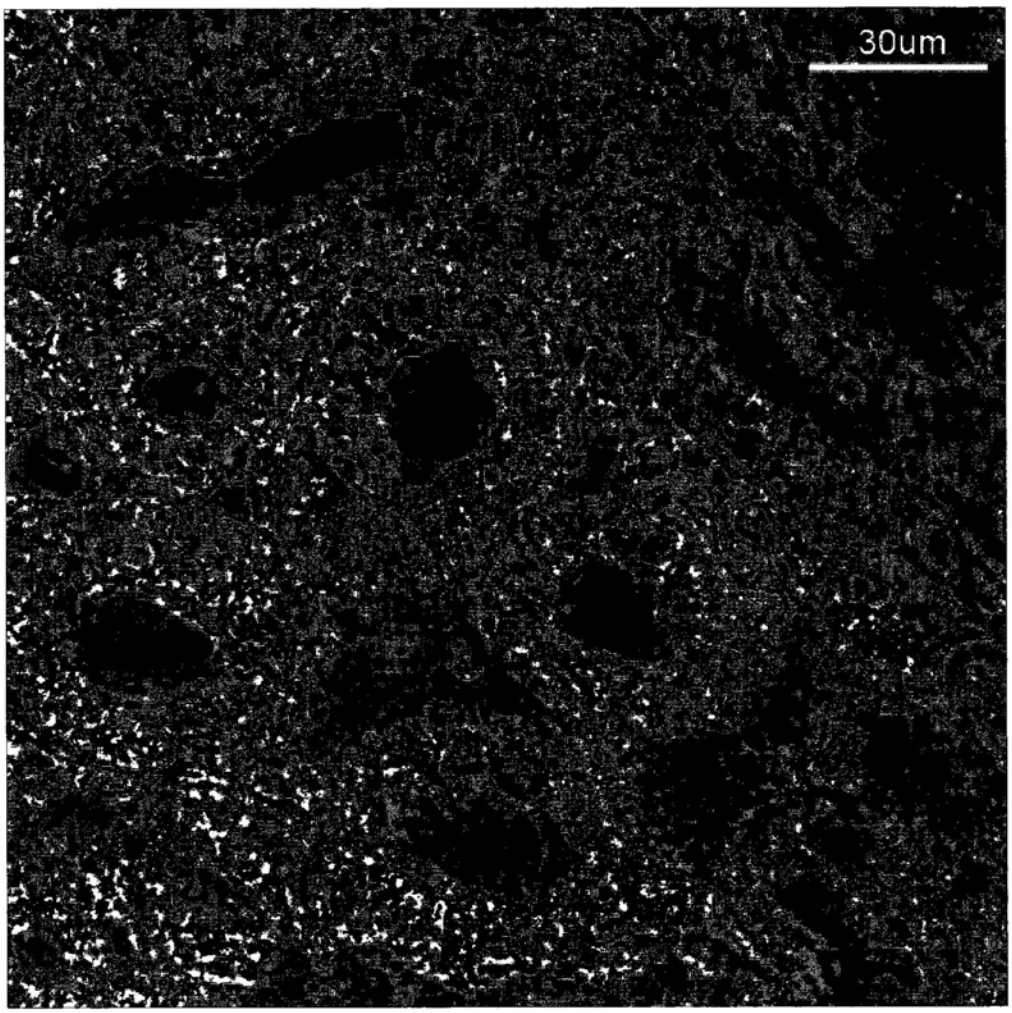

Fig. 3.19. Non-specific binding in sections of renal tissues of a freshwater adapted sea bream after incubation with pre-immune goat serum (X.S.).

Chapter 4

Isolation of cDNAs of aquaporin-1 and provasotocin from the silver sea bream

4.1 Introduction

Researchers had spent decades to identify and characterize the molecular processes and channels responsible for water transport across biological membranes. Biochemical purification and functional reconstitution techniques have successfully enriched the understanding of active transport processes, such as the Na^+-K^+-ATPase, however, the use of these techniques may easily result in loss of activity due to protein denaturation. More recently, development of cloning techniques and expression systems allow direct assessment of the functions of various ion transporters and water channels.

In the previous part of my experiment, immunohistochemical studies using mammalian aquaporin antibody have demonstrated the distribution of AQP-1 in gills, intestine and kidney of silver sea bream. The AQP-1 homologues of teleosts have been recently isolated and their role for osmoregulation in these organs was studied by several research groups (Aoki et al., 2003; Martinez et al., 2005a, 2005b, 2005c; Raldúa et al., 2008).

At least two isoforms of AQP-1 were found in teleost species (Martinez et al.,

2005a; Raldúa et al., 2008; Tingaud-Sequeira et al., 2008) and they exhibit different patterns of tissue distribution and are involved in controlling water balance in specific organs or tissues. The AQP-1 isoforms in teleosts are now named as AQP-1 (now also known as AQP-1 dup and AQP-1a) and AQP-1b (also known as AQP-1 dup and AQP-1o). AQP-1a is ubiquitously distributed in many organs (Fabra et al., 2006; Raldúa et al., 2008), including gills, intestine and kidney. However, AQP-1b is predominantly expressed in oesophagus, kidney and ovary of seawater-acclimated fish and oocytes (Fabra et al., 2006; Martinez, et al., 2005a; Raldúa et al., 2008).

Another focus of the present study is to reveal the influence of arginine vasotocin on the function of aquaporin-1 in the sea bream. It is clear that administration of arginine vasotocin at higher physiological levels reduces glomerular filtration rates in perfused trout kidney (Amer & Brown, 1995), however, effect of the neuropeptide hormone on aquaporin is unknown. Attempts had been made to demonstrate the putative relationship between mRNA expression levels of vasotocin receptor and aquaporin-1 in the black porgy (*Acanthopagrus schlegeli*; An et al., 2008). In this study, the trend of mRNA expression of AQP-1 is coherent with that of vasotocin receptor in the kidney of black porgies among the salinity treatment groups.

In the present study, in order to investigate the possible osmoregulatory role of AQP-1, cDNA of sea bream AQP-1 will be isolated from the fish. In addition, pro-vasotocin (arginine vasotocin precursor) will be cloned and the sequence information will be used for mRNA expression study to investigate the influence of the pro-vasotocin-vasotocin- receptor axis on aquaporin-1 expression.

4.2 Materials and methods

4.2.1 Fish culture and tissue sampling

Silver sea breams, weighing 100-150 g, were kept in a one-ton tank supplied with recirculating seawater (33 ‰) and fed *ad libitum* with fish meal pellets according to Woo & Kelly (1995). The fish were held for a week and the water temperature was kept between 17 - 20 °C. Feeding was terminated 24 hours before fish were sacrificed. Fish were removed from the water and were sacrificed by spinal transection. Hypothalamus, gill filaments and kidney tissues were immediately dissected from the sea bream and were immediately added into 1ml Tri-reagent solution (Applied Biosystems) in 4°C for RNA extraction.

4.2.2 Total RNA extraction and first strand cDNA synthesis

Procedures for total RNA extraction and first strand cDNA synthesis were modified from those described by Wong et al. (2006). Hypothalamus, intestine and kidney tissues were homogenized separately for 30-60 sec in 1 ml of Tri-reagent solution (Applied Biosystems). 0.2 ml chloroform was then added into the homogenate and the mixture was shaken vigorously for 15 seconds. After 15-minute incubation at room temperature, the mixture was centrifuged at 8 000 g. The upper aqueous layer was transferred to a new Eppendorf tube and 0.5 ml isopropanol was added. The mixture was incubated at room temperature for 10 minutes followed by 15-minute centrifugation at 12000 g. The supernatant was discarded and the RNA pellet was vortexed and washed with 1 ml 75% ethanol. The pellet was spun by a brief centrifugation at 7500 g and stored in DEPC-treated water. Concentration and purity of RNA were measured by spectrophotometer (Eppendorf Biophotometer plus, Germany), and quality was confirmed by running 1.5% agarose gel with ethidium bromide.

To remove DNA contamination, 3 μg RNA extract was firstly incubated with DNAse in 5 x DNAse buffer (InVitrogen) for 15 min at room temperature, followed by

addition of 5 mM EDTA and incubation at 65°C for another 15 min. The product was mixed with 0.5 μg oligo-dT primer (Proligo) at 72 °C for 10 minutes. First strand cDNA was synthesized after incubation with M-MLV reverse transcriptase at 42 °C for two hours.

4.2.3 Cloning and characterization of aquaporin-1 gene

4.2.3.1 Amplification of partial fragments of silver sea bream aquaporin-1

The design of silver sea bream aquaporin-1 primers was based on existing aquaporin-1 sequences isolated from the black porgy (*Acanthopagrus schlegeli*, GenBank accession no. EF451961, An et al. 2008), gilthead sea bream (*Sparus aurata*; GenBank accession no. AAV34610; Fabra et al. 2005) and Japanese eel (*Anguilla japonica*; GenBank accession no. BAC82110; Aoki et al. 2003). Comparative analysis on these sequences revealed there are two completely conserved regions, approximately 58 amino acids upstream from the first NPA motif and 44 amino acids downstream from the second NPA motif, and the two nucleotide sequences were selected for sense and antisense primer synthesis. Due to the high conservancy of nucleotide sequences in these regions, three sense and two antisense specific primers were designed and they had the following sequences:

Table 4.1 Specific primers designed for amplification of silver sea bream aquaporin 1

Primer synthesized	Oligonucleotide sequences
Sense	
A1S1	5'-CTTCTGGAGGGCCGTTCT-3'
A1S2	5'-TGTTCAAGGCGGTCATGTA-3'
Antisense	
A1AS1	5'-ATGTACATGACCGCCTTGA-3'
A1AS2	5'-CACCCAGTACACCCAGTGGT-3'
A1AS3	5'-GGGAAGTCGTCGAATTTGG-3'

cDNAs of intestine and kidney tissues were used in isolation of a cDNA fragment encoding aquaporin 1. For each primer amplification, each PCR was carried out in a volume of 25 μl (1 × PCR buffer, 0.5 mM each dNTP, 2.5 mM $MgCl_2$, 0.8 mM each primer, 0.5 U of *Taq* polymerase) on an Eppendorf 9600 Thermal Cycler (Eppendorf, Germany) for 33 cycles using a profile of 30 seconds at 94°C, 30 seconds at annealing temperature and 60 s at 72°C. A gradient of annealing temperatures were tested in order to find out the optimal temperature for each possible combination of primers. After 33 cycles, an additional 10 min at 72°C was allowed for final extension. By using agarose gel electrophoresis, a single DNA fragment of about 690 bp was

obtained in the resolved PCR product with primer combination of A1S1 and A1AS3 under an optimal annealing temperature of 60°C. The isolated DNA fragment was purified using NucleoTrap® gel purification protocols (BD Biosciences Clontech, U.S.) and cloned into pCR®4-TOPO® plasmid using TOPO TA Cloning Kit for sequencing (Invitrogen, U.S.A.). Cloned plasmid DNA was prepared with NucleoSpin Plasmid isolation kit (Macherey-Nagel, Duren, Germany) for DNA sequence analysis.

4.2.3.2 Rapid amplification of sea bream aquaporin 1 cDNA 5' and 3' ends (5'-RACE and 3'-RACE)

The 5' and 3' ends of the cDNA were isolated by 5' and 3' RACE system for Rapid Amplification of cDNA Ends (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. For 3'-RACE and 5'-RACE reverse transcription, dT-adaptor primer (5'-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3') and antisense gene specific primer A1AS3 were used respectively.

Antisense primer AUAP (5'-GGCCACGCGTCGACTAGTAC-3') and sense gene specific primer A1S1 were used in first round PCR for 3'-RACE. A nested PCR was carried out by using Antisense primer AUAP and sense gene specific primer A1S2.

For 5'-RACE, antisense gene specific primer A1AS3 and sense primer AAP (5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3') were used for first round PCR, followed by a nested PCR by substitution of antisense gene specific primer A1AS3 by A1AS2. The amplified 3' and 5' cDNA ends were purified using NucleoTrap® gel purification protocols (BD Biosciences Clontech, U.S.) and cloned into pCR®4-TOPO® plasmid using TOPO TA Cloning Kit for sequencing (Invitrogen, U.S.). Cloned plasmid DNA was prepared with NucleoSpin Plasmid isolation kit (Macherey-Nagel, Duren, Germany) for DNA sequence analysis.

4.2.4 Tissue distribution analysis of aquaporin 1 mRNA

Seawater-acclimated silver sea bream was firstly anesthetized by MS-222 (Sigma, U.S.A.), in order to study the distribution of AQP-1 in blood cells, 1 ml of blood was withdrawn into a heparinized syringe from the caudal vein and the blood cells were spun down at 6500 G for 10 minutes. The supernatant was discarded and cells were resuspended and washed twice in Hanks buffered saline. Perfusion was used to remove the blood from gills, liver and kidneys. A PE-50 polyethylene cannula (Clay-Adams, USA) filled with heparinized physiological saline (composition in g/L modified from Rankin and Maetz (1971): NaCl, 8.36; KCl, 0.25; Na_2HPO_4, 0.20; $MgSO_4 \cdot 7H_2O$, 1.01;

$CaCl_2$, 0.14; $(NH_4)_2SO_4$, 0.04; KH_2PO_4, 0.04; glucose, 1.00] into the first afferent branchial artery and the set up was connected to a peristaltic pump. The aorta slightly anterior to bulbous arterious was tied off by silk suture and an incision was made between the bulbus arteriosus and the heart to allow the blood/perfusate mixture to run out. The saline was kept in an ice-bath throughout the perfusion operation. Perfusion was also performed via the dorsal aorta on several occasions when blood inside the kidney had not been removed properly by the branchial afferent artery perfusion. The perfusion operation was deemed complete when the red color of the organs disappeared, and brain, gill filament, heart, kidneys, liver, intestine, rectum, and urinary bladder were dissected and put into 1 ml Tri-reagent (Molecular Research Center, USA). All samples were then stored at -70 °C.

Extraction of total RNA from each tissue and reverse transcription were performed using the protocols described in previous section. RT products of negative control for each tissue were also prepared with the same amount of RNA without M-MLV reverse transcriptase. Each PCR was carried out in a volume of 25 µl (1 × PCR buffer, 0.5 mM each dNTP, 2.5 mM $MgCl_2$, 0.8 mM each primer, 0.5 U of *Taq* polymerase) on an Eppendorf 9600 Thermal Cycler (Eppendorf, Germany) using sense primers A1S1 (5'-CTTCTGGAGGGCCGTTCT-3') and antisense primer

A1AS3 (5'-GGGAAGTCGTCGAATTTGG-3'). To determine the optimal cycles for tissue distribution study, PCR was performed for 5 minutes at 94 °C and various cycles with the profile of 30 seconds at 94 °C, 30 seconds at 60 °C and 60 seconds at 72 °C, followed by 10 minutes at 72 °C and 5 minutes at 4 °C. The PCR products were loaded to 2.0 % agarose gel with ethidium bromide for separation and the band intensities were quantified with Gel-Doc 1000 system (Bio-Rad, USA) and analyzed with Quantity One Molecular Analyst Software (Bio-Rad, USA).

The optimum cycle number for carrying out the PCR was obtained by plotting an amplification profile, with the optimum cycle number being the mid cycle number of the exponential phase in the amplification profile for all organs. The optimal cycle number used for tissue distribution analysis was 30 cycles as it falls at the exponential phases of most tissue tested. After validation, PCR was performed with the same profile for 30 cycles. The PCR products were loaded to 2.0 % agarose gel with ethidium bromide for separation and the band intensities were quantified

4.2.5 Amplification of partial fragment of silver sea bream provasotocin

To study the role of vasotocin in osmoregulation, a sea bream provasotocin partial

fragment was isolated from cDNAs of hypothalamus. Degenerate primers were designed from comparison of existing cDNA sequences of piscine pro-vasotocin and pro-isotocin. Nucleotides encoding for the hormone portion of pro-vasotocin was selected for the design of sense primer due to the higher variations between the fish pro-vasotocin and pro-isotocin sequences.

Table 4.2 Degenerate primers designed for amplification of silver sea bream provasotocin

Primer synthesized	Oligonucleotide sequences
Sense	
Pro-v S1	5'-CBGBCTGYTACATCCAGAA-3'
Pro-v S2	5'-AGGGGATCCGCSTGYTACATHCARAAYTGYCC-3'
Antisense	
Pro-V AS1	5'-ATCCACARGGTCTCCCTCCT-3'
Pro-V AS2	5'-GGGTGRGCAGGTARTTCTC-3'
Pro-V AS3	5'-WGRGCAGGTAGTTCTCCTCCA-3'

Each primer amplification was carried out similar to the aforementioned for aquaporin 1 under a gradient of annealing temperatures. After 37 cycles with

annealing temperature at 58°C, an additional 10 min at 72°C was allowed for final extension. After agarose gel electrophoresis, a single DNA fragment of about 180 bp was obtained in the resolved PCR product with primer combination of ProVS1 and ProVAS3. The isolated DNA fragment was purified using NucleoTrap® gel purification protocols (BD Biosciences Clontech, U.S.) and cloned into pCR®4-TOPO® plasmid using TOPO TA Cloning Kit for sequencing (Invitrogen, U.S.). Cloned plasmid DNA was prepared with NucleoSpin Plasmid isolation kit (Macherey-Nagel, Duren, Germany) for DNA sequence analysis.

4.3 Results

4.3.1 Nucleotide and amino acid sequence of sea bream AQP-1

The DNA sequencing results obtained from the isolated clones of intestine and kidney tissue are matched and the sea bream AQP-1 cDNA consists of 904 bp containing 51 bp of a 5'-untranslated region (UTR), 774 bp of an open reading frame (ORF) and 79 bp of 3'-UTR (Figure 4.1). The nucleotide sequence encodes a protein with 258 amino acids with calculated mass of 27.1 kDa.

The two channel-forming NPA (asparagine-proline-alanine) signature motifs are

located in 67-69 and 181-183 of the amino acid sequence. Hydropathy analysis predicted (Fig. 4.2) six transmembrane regions (TM1-6) with an N terminus and a C terminus localized in the cytoplasm. Sequence comparison of the encoded protein with known AQP-1 proteins of human and other teleosts indicated that teleost AQP-1 NPA motifs, and the residues of the pore-forming region (Phe56, His180, and Arg195; human AQP-1 numbering) in TM2, TM5 and Loop E. The amino acid sequences of TM2, TM5, Loop B and E are conserved among these water-selective AQPs. A Cys residue is located before the second NPA motif (Cys178 for sea bream AQP-1), which is the potential site responsible for the inhibition of water permeability by mercurial compounds.

The silver sea bream AQP-1 protein shared highest levels of amino acid identity with the Sparidae, gilthead sea bream AQP-1a (97.7%) and black porgy (95.8%), and relatively lower identity with European eel (83.6%) AQP-1 proteins. The cloned silver sea bream AQP-1 amino acid sequence shares relatively low identity to AQP-1b of the gilthead sea bream (56%) which is slightly lower than the identity to human AQP-1 protein (57.8%).

4.3.2 Tissue distribution of mRNA expression of sea bream AQP-1

Perfusion procedures successfully removed the blood from gills and liver tissues, nevertheless, it was more difficult to obtain blood-free samples of kidney. RT-PCR revealed the mRNA of sea bream AQP-1 is ubiquitously expressed in most tissues examined. No band was detected in MMLV-negative controls of these tissues, thus the possibility of false positive results caused by genomic contamination is eliminated (Fig. 4.4). Expression of the water channel transcript was present in the gills, liver, intestine, rectum, kidney, heart and urinary bladder (Fig. 4.4). The expression of AQP-1 mRNA was not detected in the brain tissue, while significant level of expression was present in blood cells.

4.3.3 Partial sequence of sea bream pro-vasotocin cDNA

The partial fragment of sea bream pro-vasotocin cDNA consists of 184 bp, including the regions encoding for the processing and amidation signal (Gly-Lys-Arg), vasotocin hormone and intial part of the neurophysin (Fig. 4.5a). The deduced amino acid sequences for these two regions are fully conserved with other teleostean pro-vasotocin (Fig. 4.5b). In common with other pro-vasotocins, glutamine and

arginine are located at position 4 and 8 of the hormone peptide (Fig. 4.5b). In comparison between the sea bream pro-vasotocin with the flounder pro-isotocin, lower conservancy is found in the nucleotides encoding for the hormone itself despite high homology in other parts of the compared sequences.

4.4 Discussion

4.4.1 Amino acid sequence of sea bream AQP-1

A cDNA of AQP-1 homologue was isolated from intestine and kidney tissues of silver sea bream. Hydropathy analysis (Kyte & Dolittle, 1982) predicted the sea bream AQP-1 consists of six transmembrane domains, which is a key characteristic of members of a major intrinsic protein (MIP) family and AQP-like family (Bognia et al., 1999).

The amino acid sequence of TM2 and TM5, as well as of loops B and E of the sea bream AQP-1, is highly conserved with respect to human AQP-1 and other teleostean AQP-1 (Fig. 4.3). The loops B and E of the sea bream AQP-1 are consistent with other AQP-1 homologues which exhibit high hydrophobicity. These loops were sequence-related repeats and considered to be functionally important for water

permeability in the postulated "hourglass model" for human AQP-1 (Bognia et al., 1999; Jung et al., 1994; Shi et. al., 1994) in which they overlap midway between the leaflets of the bilayer and create a narrow aqueous pathway.

The two signature NPA motifs of sea bream AQP-1 are also present in Loop B and E. The motifs are juxtaposed to each other to create a single aqueous channel spanning the bilayer (Jung et al., 1994) and acts mainly as a size-exclusion selectivity filter (de Groot & Grubmuller, 2001). The residues Phe-56, His-180 and Arg-195 in the human AQP-1 was conserved in the sea bream and other teleost AQP-1 (Fig. 4.3). Molecular dynamic simulation has revealed water permeation through AQP-1, with these residues forming a aromatic/arginine region, which acts as a proton filter (de Groot et al., 2001). Arg-195 in loop E provides a functionally positive charge at the narrowest segment of the channel. His-180 in TM5 is uncharged at neutral pH, but becomes protonated at lower pH, providing a second positive charge. The hydrophobic Phe-56 side chain orients the water molecules such as to enforce strong hydrogen bonds to Arg-195 and His-180. Together with short α-helices with partial positive charges in the terminal part of loops B and E, these residues create strong repelling positive charges to resist passage of protons and ultimately provide selectivity against ions and protons (Agre et al., 2002; de Groot et al., 2001; de Groot & Grubmuller,

2001).

The cysteine residue (at 178) is positioned in loop E which is located in a position two amino acid preceding the second signature NPA motif of the sea bream AQP-1. This position is in similar to the position observed in AQP-1 homologues of human and other teleosts (Fig. 4.3). Mercurials can reversibly inhibit water transport in mammalian red blood cells and this cysteine residue is the putative mercurial-inhibiting site present in a subgroup of mammalian AQPs (Bognia et. al., 1999; Shi et. al. 1994). The structural characteristics of the sea bream AQP-1 strongly suggested that the deduced protein might be a functional water channel and could be classified as one of the mammalian AQP-1 homologues.

Similar to other fish AQP-1a, the silver sea bream AQP-1 shares relatively low homology with fish AQP-1b proteins and the most divergent region between the amino acid sequences of two fish AQP-1 isoforms was the C-terminus. Expression study of chimeric and mutated C-terminus of gilthead sea bream AQP-1b proteins in *Xenopus* oocytes indicated the C-terminus of AQP-1b, unlike that of AQP-1a, contains specific residues involved in the control of AQP-1b intracellular trafficking through phosphorylation-independent and –dependent mechanisms (Tingaud-Sequeira et al.,

2008).

4.4.2 Tissue distribution of AQP-1

RT-PCR analysis demonstrated the sea bream AQP-1 is transcribed in gills, heart, intestine, rectum, kidney, liver and urinary bladder. This tissue distribution pattern is similar to those reported in other teleost and mammal AQP-1 (Borgnia et al., 1999; An et al., 2008; Ma & Verkman, 1999; Martinez et al., 2005a) except for the absence of mRNA expression in the sea bream brain tissues. In mammals, AQP-1 was found in brain, eye, salivary gland, respiratory tract, heart, liver, pancreas, spleen, gastrointestinal tract, kidney, ovary, muscle and erythrocyte (Ishibashi et al., 2009). The mammalian aquaporin is involved in osmoregulation by facilitating water transport across the epithelium of proximal tubules and descending thin limbs of Henle in kidney (Nielsen & Agre, 1995). AQP-1 is also abundant in capillary epithelium in which it contributes to vascular permeability (Nielsen et al., 1993b). Its presence in descending vasa recta defines the pathway for transfer of large quantities of water from tubular lumen to the interstitium and then into the vascular space (Pallone et al., 1997).

Recent researches on fish AQP-1 suggested the aquaporin channel plays an

osmoregulatory role in intestine (Aoki et al., 2003; Raldua et al., 2008), gills (An et al., 2008) and kidney (Martinez et al., 2005a) of teleosts in response to salinity perturbations. The distribution of AQP-1 mRNA expression in the sea bream gills, kidney, intestine and rectum is consistent with the observations obtained from immunohistochemical methods in previous chapter.

In the present study, blood cells of the silver sea bream express significant levels of AQP-1 mRNA, a phenomenon which has not been observed in studies on the AQP-1 of other fish species. In contrast to most mammals, fish red blood cell is nucleated and is capable to synthesize protein. Since red blood cells constitute to the major portion of blood cells, expression of AQP-1 mRNA in red blood cells may mimic the gene expression results of AQP-1 from studies on other organs, especially in those organs which are enriched with blood. Therefore, removal of blood by perfusion is necessary in order to obtain more accurate results.

AQP-1 mRNA is expressed in the brain of other teleosts studied (An et al., 2008; Martinez et al., 2005a), however, no AQP-1 transcript was detected in the sea bream brain. In fact, a significant role of AQP-1 in cerebrospinal fluid secretion by choroid plexus of the brain has been demonstrated in rats from prenatal stage to maturity

(Bondy et al., 1993). It is possible that other AQP-1 isoforms or types of AQPs may take over the role of water homeostasis in the brain of sea bream. AQP-4, a more abundant aquaporin in the mammalian brain (Ishibashi et al., 2009), is involved in more pronounced role in cerebral water balance, as well as astrocyte migration and neural signal transduction (Verkman et al., 2006). Curiously, transcriptional analysis showed the presence of AQP-1b transcript in the brain of zebrafish but the same result was not detectable in other teleosts (Tingaud-Sequeira et al., 2008). This divergent expression of AQP-1b in the brain of teleosts may give clues to the absence of AQP-1 transcript in some tissue of silver sea bream, these observations suggest different expression pattern of AQP-1 isoforms among different fish species.

4.4.3 Partial cDNA of sea bream pro-vasotocin

The partial fragment of sea bream pro-vasotocin was isolated from hypothalamus and it shares high identity with other reported sequences of teleostean pro-vasotocin. The hormone portion of the sea bream pro-vasotocin resembles the amino acid sequence of pro-vasotocin of other teleosts, with glutamine and arginine residues in position 4 and 8 respectively. Sequence comparison also revealed a high homology between sea bream pro-vasotocin and flounder pro-isotocin, especially in the regions

of neurophysins.

In the flounder, sequences of pro-vasotocin and pro-isotocin are highly similar at both amino acid (97%) and nucleotide levels (97%), and a very high homology exists between the two mRNAs found in the central portions of neurophysins (Warne et al., 2000). The flounder pro-isotocin differs from its pro-vasotocin with replacement of glutamine and arginine residues by serine and isoleucine in positions 4 and 8 of the hormone portion respectively.

With reference to the high homology between flounder pro-vasotocin and pro-isotocin mRNAs, similar level of homology between the two neuropeptides could be observed in the sea bream. In fish, pro-vasotocin and isotocin are synthesized in the preoptic neurons of the hypothalamus (Duarte et al., 2001; Cerdá-Reverter & Canosa, 2009). Hence the specific primers for the measurement of hypothalamic pro-vasotocin gene expression must be carefully designed to avoid any false measurements of pro-isotocin gene expression.

4.5 Conclusions

Amino acid sequence analysis of sea bream AQP-1 full cDNAs indicated the protein shares common structural characteristics of the AQP-1 homologues in other vertebrates. The water channel is ubiquitously present in major osmoregulatory organs of the sea bream in which its role in water regulation among these organs can be further studied by RT-PCR. Expression of AQP-1 mRNA in blood cells implied removal of blood in these organs is necessary to minimize any errors incurred by simultaneous measurement of AQP-1 expression in blood cells. Together with the sequence information obtained from the partial clone of pro-vasotocin, design of specific primers is now possible for studying gene expression of sea bream AQP-1 and pro-vasotocin.

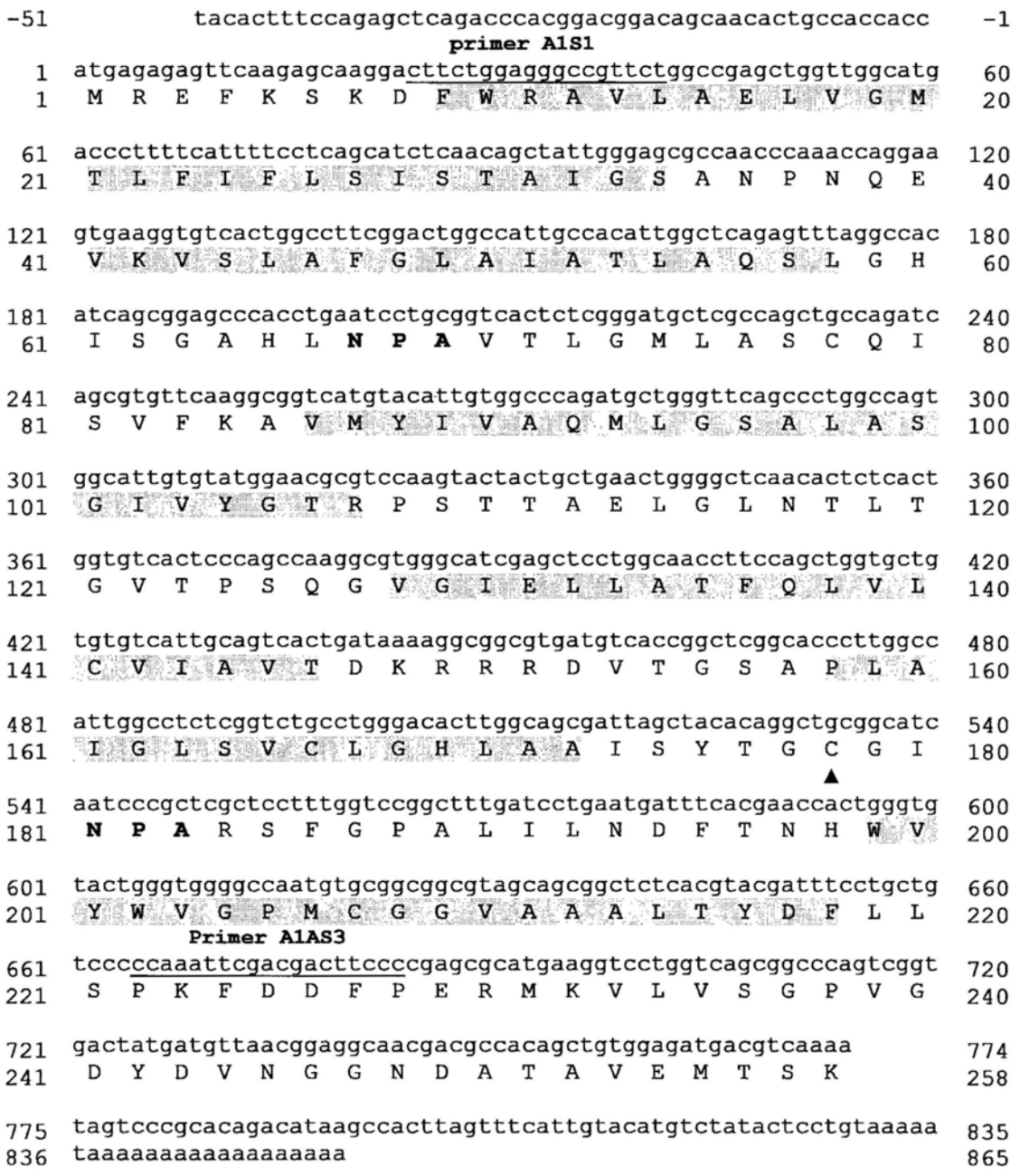

Figure 4.1 Sea bream AQP-1 cDNA and deduced amino acid sequence. Two MIP family signatures (NPA) were identified and are indicated in bold at positions 67 and 181. Solid triangle indicates the Cys residue located before the second NPA motif.

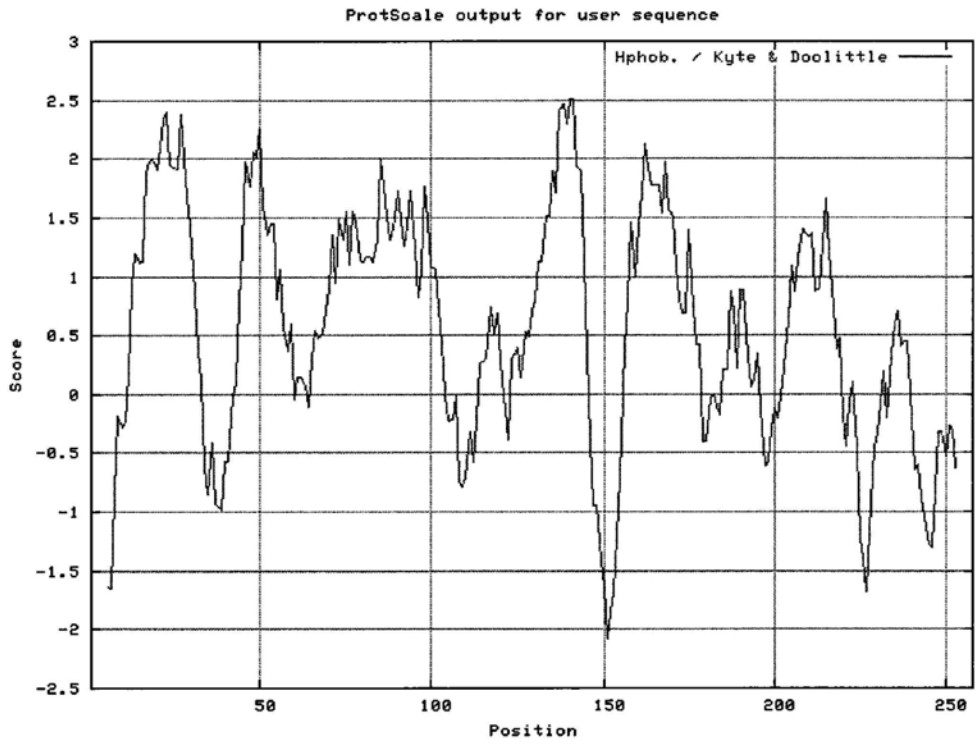

Figure 4.2. Kyte-Doolittle hydropathy profile (window 11) of the deduced sea bream AQP-1 amino acid sequence **(ProtScale,** http://www.expasy.ch/cgi-bin/protscale.pl?1)

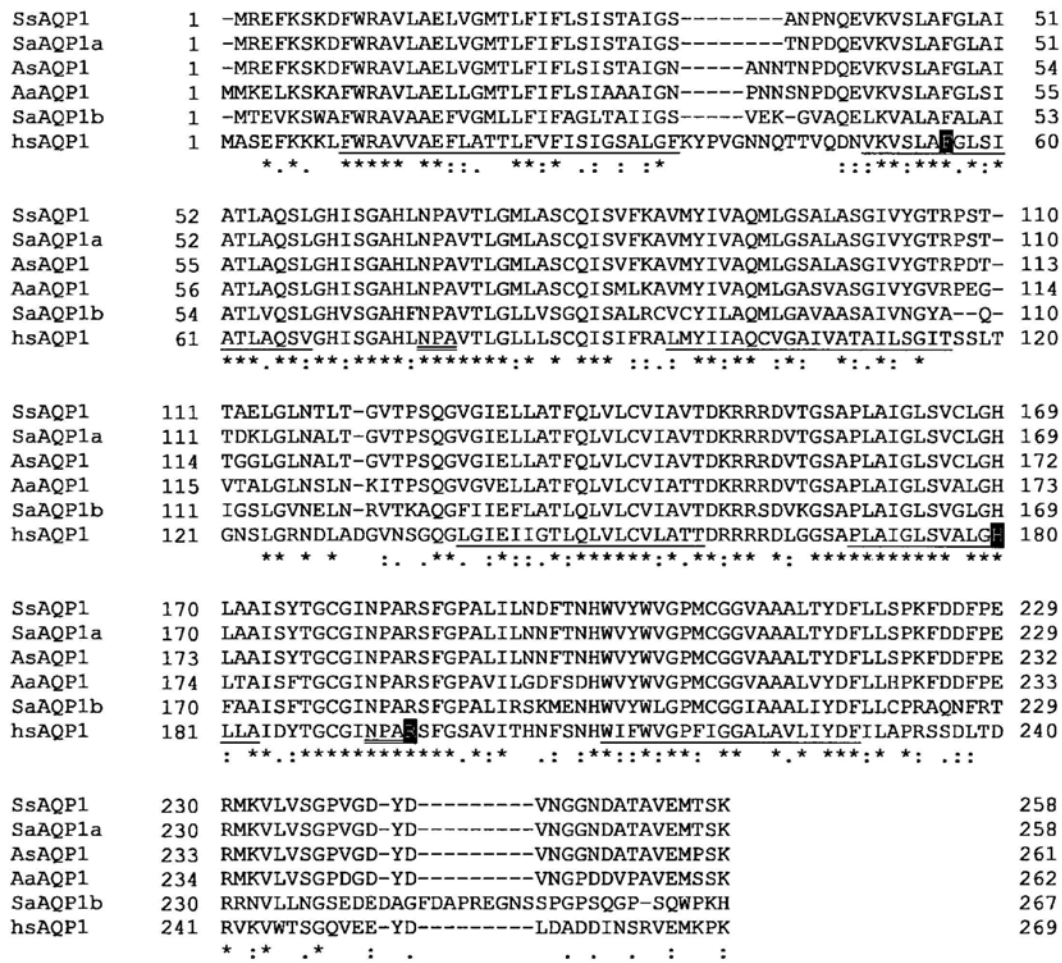

Figure 4.3 Comparison of amino acid sequences of the AQP-1 of silver sea bream (SsAQP1), gilthead sea bream (SaAQP1a), black porgy (AsAQP1), European eel (AaAQP1), human (hsAQP1), and AQP-1b of gilthead sea bream (SaAQP1b). Approximate locations of the six transmembrane domains are underlined and two signature NPA motifs are double underlined. Asterisks denote identical amino acid residues among different AQP-1 sequences.

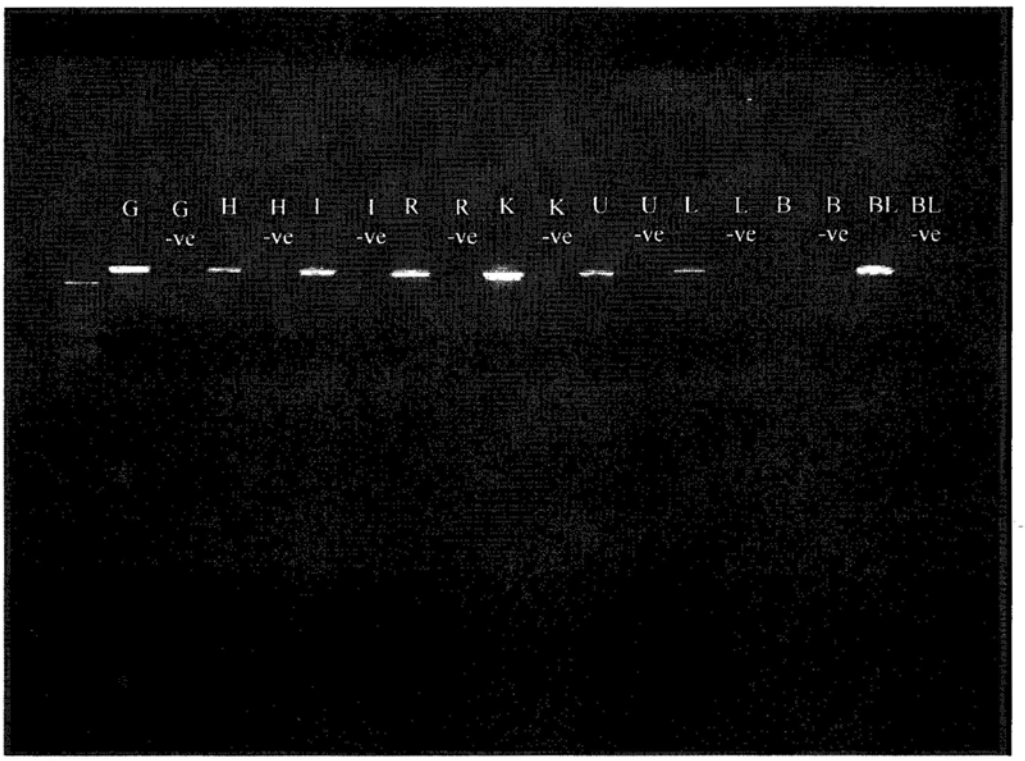

Figure 4.4. Tissue distribution of AQP-1 in the seawater adapted silver sea bream by using RT-PCR. Abbreviations: -ve: MMLV negative control for each examined tissue; G: Gill ; B: Brain; H: Heart; I: intestine; R: Rectum; K: Kidney ; U: Urinary bladder; L: Liver; B: Brain; and BL: Blood.

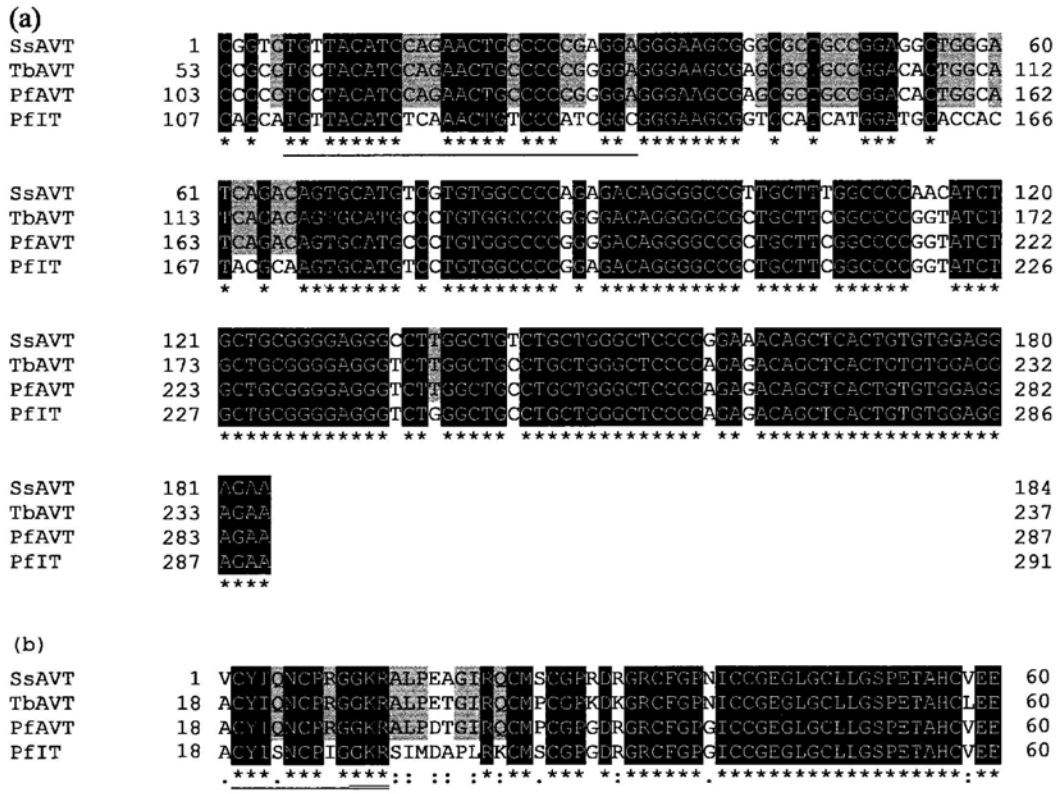

Figure 4.5. (a) Alignment of cDNA sequence and (b) alignment of deduced amino acid sequence of provasotocin from silver sea bream (SsAVT), *Thalassoma bifasciatum* (TbAVT, Genbank: AY167033.1), *Platichthys flesus* (PfAVT, Genbank: AB036517.1), and pro-isotocin of *P. flesus* (PfIT, Genbank: AB036518.1). Identical bases and amino acid residues among the four compared sequences are indicated in black. Identical bases and amino acid residues among provasotocin sequences are indicated in grey. cDNA and amino acid sequences for the hormones are underlined.

Chapter 5

Influence of environmental salinity on the expression of hypothalamic provasotocin and AQP-l in gills, intestine and kidney of the silver sea bream

5.1 Introduction

Responsiveness of AQP-1 to environmental salinity change has been studied in piscine osmoregulatory tissues (gills, kidney and intestine) by several research groups (An et al., 2008; Aoki et al., 2001; Giffard-Men et al., 2007; Martinez et al., 2005a, 2005b, & 2005c; Raldúa et al., 2008). Although significant alterations of AQP-1 expression had been detected in fishes after hyposmotic or hyperosmotic adaptation, some inconsistent results were found from these studies.

In eels, mRNA expression of AQP-1 was upregulated in intestine of seawater-adapted eels (Aoki, et al., 2003; Giffard-Men et al., 2007; Martinez et al., 2005b) and gilthead sea bream (Raldúa et al. 2008), however, this AQP-1 upregulating response was found in black porgy adapted from seawater to freshwater (An et al., 2008). Besides, renal mRNA expression of AQP-1 was higher in seawater-adapted eels than freshwater-adapted eels (Martinez et al., 2005a) while a reverse trend was observed in black porgy (An et al., 2008), with highest AQP-1 expression level being observed in porgy adapted to 10% seawater. The salinity acclimation periods in these experiments vary from 24 hours to as long as 21 days. These inconsistent results may

simply represent the transient responses of AQP-1 at different time points during hyper- or hypo-osmoregulation.

A recent study has also extended their interest to the relationship of vasotocin receptors and AQP-1 expression of teleosts during salinity acclimation (An et al., 2008). In fish, vasotocin is essential for water conservation and vasotocin administration results in a decrease in the number of filtering nephrons and urine production in vivo and kidney preparations in situ (Amer & Brown, 1995; Henderson & Wales, 1974). There are also suggestions that vasotocin can directly influence gill ion and water transport (Maetz et al., 1964; Marshall, 2003). Hypothalamic pro-vasotocin mRNA expression and plasma vasotocin levels significantly increased in flounder at eight hours following transfer from freshwater to seawater (Warner, et al., 2005). These evidences suggest the vital role of vasotocin in salinity acclimation process, especially during the early stages during adaptation.

In the present study, two approaches were used to investigate the changes in AQP-1 and hypothalamic pro-vasotocin mRNA expressions during salinity acclimation process: (1) chronic gradual and long-term salinity acclimation (0 ‰, 6 ‰, 12 ‰, 33 ‰, 50‰ and 70 ‰) for 4 weeks, and (2) abrupt hyposmotic acclimation

(direct salinity change from 33 ‰ to 6 ‰) for 2 hours to seven days. After these salinity acclimation processes, various osmoregulatory organs (gills, intestine, and kidney) were removed for the extraction of RNA and used for the subsequent determination of gene expression profiles. Prior to organ removal, the osmoregulatory organs were perfused to remove the blood (containing high AQP-1 expression) so as to obtain precise expression levels of the organs examined.

5.2 Chronic effect of salinity on the expression of hypothalamic pro-vasotocin, and aquaporin-1 in gills, intestine and kidney

5.2.1 Materials and methods

5.2.1.1 Fish

Silver sea bream weighing 100-150 g, were purchased from local fish farm, and were kept in a recirculating seawater (33 ‰) system in Simon F. S. Li Marine Science Laboratory, Chinese University of Hong Kong for not less than three weeks to allow recovery from transportation stress. The fish were then divided into six groups randomly and put into six one-ton seawater (33 ‰) tanks with individual recirculating filter systems. Salinity of each tank was adjusted to 0, 6, 12, 33, 50 or 70 ‰ gradually by flushing tap water or hypersaline water daily. These salinity adjustments were

completed within seven days and the fish were acclimated for further 28 days in their respective final salinities. The fish were fed *ad libitum* with fish meal pellets according to Woo & Kelly (1995) throughout the experiment and feeding was terminated 24 hours before fish were sacrificed.

5.2.1.2 Tissue sampling

Silver sea bream were removed from the tanks and were firstly anesthetized by MS-222 (Sigma, U.S.A.). Blood was withdrawn into a syringe from the caudal vein and the blood samples were centrifuged to obtain serum. Hematocrit was determined immediately after blood collection.

In order to remove the blood from gills and kidney, perfusion was performed through the first branchial afferent artery as described in the Materials and Methods section in Chapter 4.2.4. Perfusion was also performed via the dorsal aorta on several occasions when blood inside the kidney had not been removed properly by the branchial afferent artery perfusion. The perfusion operation was deemed complete when the red color of the organs disappeared. Gills, kidneys and intestine were dissected and put into 1 ml Tri-reagent (Molecular Research Center, USA). Muscle

tissues were removed for determination of muscle moisture content. All samples were then stored at -70 °C.

5.2.1.3 Serum electrolytes and muscle moisture content

Serum Na^+ concentration was determined using atomic absorption spectrophotometry (Hitachi) after dilution of 5 µl of serum sample with 10 ml of ultrapure water. Serum Cl^- concentration was determined using a chloridometer (Corning-eel 920) by addition of 20 µl serum sample to acid buffer. To determine muscle moisture content, the muscle tissues was weighed and dried in an oven at 100 °C overnight. The dried muscle tissue was weighed and muscle moisture content was calculated from the difference between the dried weights and wet weight of muscle tissues.

5.2.1.4 RNA extraction and first strand cDNA synthesis

The methods for RNA extraction and first strand cDNA synthesis followed exactly the same procedures as previously described in the Materials and Methods section in Chapter 4.2.2.

5.2.1.5 Validation for semi-quantitative RT-PCR

The abundance of AQP-1 and pro-vasotocin transcripts was detected by semi-quantitative RT-PCR. The specific primers used for cloning of sea bream AQP-1 gene (A1S1 and A1AS3) continued to be used as the primers for AQP-1 in semi-quantitative RT-PCR. Primers for pro-vasotocin was designed based on the sequence results as described in Chapter 4.3. Primers for the housekeeping gene, β-actin were adopted according to Deane et al. (2002). The sequence information of the primers is summarized as follow:

	Oligonucleotide sequences	
AQP-1	5'-CTTCTGGAGGGCCGTTCT-3'	Sense
(660 bp)	5'-GGGAAGTCGTCGAATTTGG-3'	Antisense
Pro-vasotocin	5'-AGAACTGCCCCCGAGGA-3'	Sense
(180 bp)	5'-CCTCCACACAGTGAGCTGTTTC-3'	Antisense
β-actin	5'-TCACCAACTGGGATGACATG-3'	Sense
(800 bp)	5'-ATCCACATCTGCTGGAAGGT-3'	Anti-sense

Each PCR was carried out in a volume of 25 μl (1 × PCR buffer, 0.5 mM each dNTP, 2.5 mM $MgCl_2$, 0.8 mM each primer, 0.5 U of *Taq* polymerase) on an Eppendorf 9600 Thermal Cycler (Eppendorf, Germany) using respective primer sets.

To determine the optimum cycle number in all tissues and each primer set, PCR was performed for 5 minutes at 94 °C and various cycles with the profile of 30 seconds at 94 °C, 30 seconds at 60 °C and 60 seconds at 72 °C, followed by 10 minutes at 72 °C and 5 minutes at 4 °C. The PCR products were loaded to 2.0 % agarose gel with ethidium bromide for separation and the band intensities were quantified with Gel-Doc 1000 system (Bio-Rad, USA) and analyzed with Quantity One Molecular Analyst Software (Bio-Rad, USA). The optimum cycle number for carrying out the PCR was obtained by plotting an amplification profile, with the optimum cycle number being the mid cycle number of the exponential phase in the amplification profile. The optimal cycle number used for tissue distribution analysis was 30 cycles as it falls at the exponential phases of most tissue tested. For the optimum annealing temperature, PCR was performed with optimum cycle numbers with a gradient of annealing temperatures.

5.2.1.6 Semi-quantitative PCR

After validation, the optimum cycle numbers for all combinations of tissues and primer sets and optimal temperature for all primer sets were listed as follows:

	Tissue	Optimal temperature	Optimum cycle number
AQP-1	Gills	60 °C	29
	Intestine		25
	Kidney		26
Pro-vasotocin		62 °C	34
β-actin	Gills	60 °C	26
	Intestine		24
	Kidney		24
	Hypothalamus		27

PCR products of target gene and β-actin were electrophoresed on the same well of 2.0% agarose gel. Expression of target gene was expressed as a ratio of intensity between target gene and β-actin.

5.2.1.7 Statistical analysis

All data were expressed as mean values ± standard error of the mean (S.E.M.). Data from each group were then subjected to a one-way analysis of variance (ANOVA), followed by a Tukey's test (SPSS) with $p<0.05$ to delineate significance.

5.2.2 Results

5.2.2.1 Serum electrolytes, muscle moisture and hematocrit

Serum Na^+ concentration was not significantly different among the fish acclimated to intermediate salinities (6‰ to 50‰), whereas lowest and highest serum Na^+ were found in the 0‰- and 70‰-acclimated sea bream respectively (Figure 5.1). Acclimation to 0‰ and 70‰ resulted in approximately 11% reduction and 27% elevation in serum Na^+ concentration.

In addition, serum Cl^- concentration (Fig. 5.2) exhibited a declination from the highest salinity group (70‰) to the lowest salinity group (0‰). Similar to serum Na^+, highest level of serum Cl^- was detected in the 70‰-acclimated fish which is 15% higher than the seawater group. The serum Cl^- concentration of isosmotic (12‰) and hypo-osmotic (6‰) were significantly lower than those of all the hyperosmotic groups (33‰, 50‰, 70‰). The 0‰-acclimated fish possessed the lowest serum Cl^- level among different salinity groups.

Fig. 5.3 and 5.4 illustrated the muscle moisture content and hematocrit of different salinity treatment groups respectively. Muscle moisture was increased

significantly as the salinity decreased. The muscle was most hydrated in the 0‰-acclimated fish. No significant difference was observed in the hematocrit values among fish of different salinity groups.

5.2.2.2 Relative expression of AQP-1 in intestine, gills and kidney

In intestine, the expression of AQP-1 was found to be the highest in intestines of 70‰-acclimated fish and it was about 2.1-7.9 fold higher than those for fish adapted to other salinities (Fig. 5.5). The intestinal expression of AQP-1 was also upregulated to a smaller extent in isosmotic salinity (12‰) which was significantly higher than the 0‰, 6‰ and 33‰ groups. Branchial AQP-1 expression was found to be the highest in 0‰-acclimated fish, whereas no significant difference in the expression levels was observed among the other salinity groups (Fig. 5.6). In kidney, the expression level for AQP-1 was not significantly different over the salinity range tested despite a descending trend of the expression levels from freshwater to hypersaline salinity was evident (Fig. 5.7).

5.2.2.3 Relative expression of hypothalamic provasotocin

Transcription of hypothalamic provastocin was significantly downregulated in 0‰-acclimated fish (Fig. 5.8). The expression levels of hypothalamic pro-vasotocin were maintained at higher levels at hypo-osmotic (6‰), isosmotic, hyperosmotic (33‰) and hypersaline (50‰ and 70‰) conditions.

5.2.3 Discussion

5.2.3.1 Chronic effect of salinity acclimation on serum electrolyte levels, muscle moisture content and hematocrit

5.2.3.1.1 Serum electrolyte levels

Circulating electrolytes and muscle water content are basic indices to reflect the effectiveness of fish adapting to a new environment with different salinity. Long-term acclimation of marine fish to low salinity regimes usually results in minor differences between pre- and post-transfer electrolyte levels in many teleosts (Woo & Fung, 1981; Woo & Wu, 1982; Dutil et al., 1992; Mancera et al., 1993; Woo & Chung, 1995). However, in American plaice (*Hippoglossoides platessoides*), after 46-day adaptation to 7‰, plasma Na^+ concentrations had dropped by 28% below those of the controls

(28‰) (Murnro et al., 1995), probably reflecting relatively inefficient ionoregulatory mechanisms of this species to low salinities.

From the results of the present and previous studies, it appears that *Sparus sarba* is an excellent osmoregulator. In the present study, acclimation to a salinity range of 50-6‰ had no significant effect on serum Na^+ in *Sparus sarba* but the levels are only significantly changed in the two extreme salinities (0 or 70‰). The reduction in serum Na^+ after 0‰ acclimation agreed with the extent of 13% that was previously reported by Luk (2001). Higher serum Na^+ concentration in 70‰-acclimated fish indicated the salt loading in the extreme hypersaline medium may have exceeded the hypo-osmoregulatory capacity of the silver sea bream. In spite of these, it is noteworthy that the osmoregulatory functions of silver sea bream are highly efficient when compared to those other marine teleosts. For instance, the black sea bream (*Mylio macrocephalus*) exhibited an approximately 19% reduction in Na^+ level after a 3-week acclimation to 0‰. Furthermore, the silver sea bream is a better osmoregulator as far as serum Na^+ is concerned, as compared to euryhaline eels with a nearly 29% reduction in plasma Na^+ levels after acclimation from seawater to freshwater (Utida et al., 1972).

Serum Cl^- levels appeared to be less stable than serum Na^+ during salinity acclimation. The Cl^- level significantly declined as the ambient salinity decreased, however, the extent of perturbations in the two extreme salinities were similar to those observed in serum Na^+.

5.2.3.1.2 Muscle moisture

Changes in muscle water content during salinity acclimation had been amply studied in many teleosts, the response of which to osmotic adaptation varies in different species. In the American plaice (*Hippoglossoides platessoides*) adapted to 7‰ for 42 days, muscle water content had increased by 3% (Munro et al., 1994).

In the 13 marine species examined by Wu & Woo (1983), 12 out of them, including two sparid species (*Rhabdosarga sarba*; silver sea bream and *Chrysophrys major*), exhibited higher muscle water content when exposed to lower salinity regimes. After acclimation to dilute media (5‰ and 3‰) for more than two weeks, *C. major* showed a significant muscle hydration (4-6%), however, *Mylio macrocephalus*, the black sea bream, exhibited muscle dehydration. A more recent study reassured that the black sea bream exhibited stable muscle moisture content where significant tissue

hydration was only found in 0‰-acclimated fish (Kelly et al., 1999).

In the present study, the muscle moisture content of silver sea bream exhibited relatively stable muscle moisture, with 1-2% hydration after exposure to two hyposmotic salinity regimes (0‰ and 6‰). The silver sea bream also showed excellent ability to adjust to extreme salinities in hypersaline (50‰ and 70‰) media, with only 0.7-1.3% reduction in muscle water content. The ability to maintain a steady state of muscle water content reflects the actual capacity of the sea bream to adapt to wide range of salinity regimes.

5.2.3.1.3 Hematocrit

The change in hematocrit values reflects a change in the number or the volume of the erythrocyte or a variation of the circulating blood volume (Soivio et al., 1973). Decrease in external salinity usually results in elevations of hematocrit value, as reported in many marine teleosts (Oikari, 1978; Woo & Fung, 1981; Woo & Wu, 1982; Wu & Woo, 1983; Roche et al., 1989). Wu & Woo (1983) had demonstrated the increase in hematocrit values in 13 species of adult marine teleosts in response to hypo-osmotic exposure.

Hematocrit values did not significantly change among silver sea bream acclimated to different salinities, although it was apparently lowest in 12% and 33%, and with an increasing trend being apparent towards the salinity extremes. These results imply there is no net change in circulating volume and red blood cells volume and numbers among different salinity groups and concurs with the euryhalinity of the fish.

5.2.3.2 Chronic effect of salinity acclimation on intestinal AQP-1 expression

The present study is the first to profile the AQP-1 expression in osmoregulatory organs of silver sea bream adapted to hypo-osmotic, isosmotic, hyperosmotic and hypersaline environments. Long-term hypo-osmotic acclimation did not significantly cause any variation in AQP-1 expression levels between the sea bream acclimated to seawater and hypo-osmotic salinity.

In contrast, studies on intestinal AQP-1 of other teleosts had shown that in general, seawater-adapted fish exhibited a higher AQP-1 expression than those adapted to lower salinities except for a completely opposite trend of AQP-1 expression being reported in a study on the intestine of black porgy (An et al., 2008). In European

eels, the expression levels of AQP-1 mRNA were highly upregulated (5 to 23 fold) in the different regions of intestines of both seawater yellow and silver eels (Martinez et al., 2005b). Aoki et al. (2003) reported the intestinal water absorption rate and AQP-1 expression levels of seawater eels were higher than freshwater Japanese eels. Taken these evidences together, it is widely accepted that AQP-1 expression level is upregulated after hyperosmotic adaptation and this elevated expression may contribute to higher water permeability which had been previously observed in many teleosts (Oide & Utida, 1967; Kirsch & Meister, 1982).

Indeed in the present study, several fold of upregulation of AQP-1 transcript was detected in intestine of silver sea bream acclimated to an extreme salinity of 70‰. We speculate intestinal AQP-1 was not upregulated in the silver sea bream under mild hypertonic stress until the osmotic stress exceeds the fish's capacity during long-term salinity acclimation. In most of the past studies on intestinal AQP-1, the salinity acclimation period did not exceed 21 days. It is possible that upregulation of AQP-1 was a transient osmoregulatory strategy in the early phase of salinity acclimation. Alternately, it may not only be AQP-1, but other aquaporins homologues or pathways may also contribute to water absorption in intestine. Recently, Cutler et al. (2008) reported that significant level of AQP-8 transcript was detected in intestine of

European eels and its expression was elevated during salinity acclimation.

Curiously, high level of AQP-1 expression was also found in intestine of silver sea bream acclimated to isosmotic salinity of 12‰. Although the osmoregulatory response of kidney may be different from intestine, elevated level of renal AQP-1 expression was also reported in black porgies acclimated to 10‰ (An et al., 2008). The authors suggested the fish may suffer from the most osmotic stress in a salinity of 10‰, however, this explanation is unlikely to fit into a majority of observations in which teleosts have been shown to have minimal osmotic stress with lowest biochemical activities for osmoregulation during isosmotic acclimation (Deane et al., 2004; Jensen et al., 1998; Kelly et al., 1999; Lin et al., 2003). Indeed, pituitary growth hormone and hepatic IGF-I transcripts were highest in silver sea bream adapted to isosmotic salinity, however, the transcription levels progressively decreased towards hypersaline salinity (Deane et al., 2004). With the use of immunocytochemical and ultrastructural methods, Mancera et al. (1995) demonstrated there is an activation of pituitary growth hormone cells in the brackish water adapted gilthead sea bream when compared to those adapted to seawater. Apart from growth promoting action, growth hormone has been proven to increase the hypo-osmoregulation capacity of teleosts during seawater adaptation. Exogenous growth hormone induced 'seawater morphology' in the

mucosa of the midgut of *Salmo salar* prior to smoltification (Nonnotte et al., 1995) and the drinking response in *S. salar* pre-smolts after seawater transfer (Fuentes & Eddy, 1997). In the present study, elevated AQP-1 transcriptional levels in 12‰-adapted sea bream could be influenced by higher availability of growth hormone as usually observed in fish adapted to this isomotic salinity, hence converting the intestine functionally toward maintaining hypo-osmoregulation and water absorption. Nonetheless, more studies in other teleost species will be necessary to elucidate the significance of this observation in isosmotic salinity.

5.2.3.3 Chronic effect of salinity acclimation on branchial AQP-1 expression

In the present experiment, freshwater adaptation significantly upregulated AQP-1 expression in gills of silver sea bream whereas hypersaline salinities did not affect the expression levels compared to fish acclimated to 33‰. These results concur with previous studies on fish AQP-1 (An et al., 2008) and AQP-3 (Cutler & Cramb, 2002; Lignot et al., 2002) in gills, mostly focused on the homologues of mammalian AQP-3, which demonstrated higher levels of aquaporin expression in freshwater-adapted fish. The disparity of branchial aquaporin expression levels between freshwater and seawater teleosts may provide explanatory clues to the existence of three- to 11-fold

higher levels of osmotic water permeability found in the gills of freshwater fish (Motais & Isaia, 1972).

The exact role for higher osmotic permeability in gills of freshwater fish was unclear, however, Cutler & Cramb (2002) proposed that it is a consequence of differential expression of one or more apically located solute transporters, thus increasing the coupling of water uptake across the apical membrane into the branchial epithelial cells. To prevent the cells from swelling, the water may exit through the basolaterally located AQP-3 water channels in chloride cells of eels (Lignot et al., 2002). Similarly, immunohistochemical study in the present study has revealed localization of AQP-1 in chloride cells of gills and AQP-1 may also serve as the pathway to release water through the basolateral membrane of these cells to the serosal fluid in order to prevent cell swelling during freshwater adaptation in silver sea bream. Nevertheless, the extent of upregulation of branchial AQP-1 transcription in the silver sea bream is notably lower than those reported for AQP-3 in gills of freshwater-adapted European eels and sea bass with as high as 33-fold and 8-fold of elevations respectively (Culter & Cramb, 2002; Giffard-Mena et al., 2007). Apparently, AQP-1 has a less important role in gills during long-term hypo-osmotic acclimation.

5.2.3.4 Chronic effect of salinity acclimation on renal AQP-1 expression

When euryhaline fish acclimate to new salinity regimes, adjustments of urine production are primarily regulated via changes in the glomerular filtration rate in order to maintain water balance in hyposmotic and hyperosmotic adaptation (McDonald, 2007). The contribution due to changes in water reabsorption in the kidneys was generally thought to be far less important for both freshwater and seawater teleosts (Cutler & Cramb, 2000).

In the present study, RT-PCR analysis did not show any significant difference in renal transcription abundance for AQP-1 among silver sea bream exposed to various salinities. Martinez et al. (2005a) reported differential expression levels between yellow eels and silver eels after seawater adaptation. In yellow eels, there is a marked downregulation of AQP-1 transcript after seawater transfer. In contrast, no significant difference could be detected for the AQP-1 transcript in silver eels. The authors postulated that the different expression responses may be attributed to the 'silvering' process that has not occurred in yellow eels, and this process tightens the water movement as well as overall permeability in body surfaces in preparation for seawater entry in silver eels. As a result, osmotic water loading from the freshwater

environment is reduced and relieves the need to maintain the high glomerular filtration rate and corresponding tubular ion and water transport for diuresis in the kidney. This 'tightening' process may also partly explain comparable expression levels of AQP-1 mRNA in silver sea bream among various salinity groups.

In fact, the percentage of filtered water that has been reabsorbed is markedly higher in nephrons of marine teleosts than freshwater teleosts (Wood & Patrick, 1994), thus it is likely that other types of aquaporins or cellular pathways may operate to account for this disparity. Martinez et al. (2005a) also reported the existence of one AQP-1 isoform (AQP-1a) and an aquaglyceroporin (AQPe) in kidney, both of which exhibit markedly higher transcription levels than AQP-1 in kidney, however, whether stimulated expression of these aquaporins exists in freshwater yellow eels is still unclear.

In most teleosts, the nephrons of kidney are connected to a pair of mesonephric ducts and the ducts unite and open into the urinary bladder (Hickman & Trump, 1969; Nishimura & Imai, 1982). Reabsorption of water in the urinary bladder contributes significantly to the overall osmoregulatory homeostasis of marine teleosts (Lahlou et al., 1969; Howe & Gutknecht, 1978; McDonald & Grosell, 2006) and the rate of fluid

reabsorption in urinary bladder of seawater acclimated flounder, *Platichthys stelatus*, is double that of freshwater acclimated flounder (Demarest, 1984). Recently, Chau (2009) reported elevated levels of AQP-1 expression in urinary bladder of silver sea bream adapted to hyperosmotic salinities. It appears that the teleostean urinary bladder may share part of the role for water reabsorption from the urine which is used to be mostly carried out in the kidney of tetrapod vertebrates.

5.2.3.5 Chronic effect of salinity acclimation on pro-vasotocin expression

The present study provides insight into the influence of salinity on vasotocin synthesis in the hypothalamus of fish. Inconsistency abides in the existing data showing changes in levels of provasotocin expression of teleost species in response to salinity acclimation. In situ hybridization showed hybridization signals of pro-vasotocin mRNA had been significantly lower in the nucleus preopticus magnocellularis of rainbow trout for two weeks after transfer from freshwater to 80% seawater (Hyodo & Urano, 1991). Reverse transfer of trout from 80% seawater to freshwater stimulated provasotocin expression which returned to the initial freshwater level (Hyodo & Urano, 1991). Contrary to the results of Hyodo & Urano (1991), abundance of hypothalamic pro-vasotocin transcript was not significantly changed in

the euryhaline flounders *P. flesus* transferred from freshwater to seawater for three days, however, the abundance was apparently higher in the seawater transfer fish (Warne et al., 2000).

In silver sea bream, expression levels of pro-vasotocin transcripts were similar among 6‰ to 50‰ whereas synthesis of the vasotocin precursor was significantly downregulated in fish acclimated to freshwater. The results generally agree with utilization of pituitary vasotocin reserve and rapid secretion of the hormone observed in many euryhaline teleosts adapted to hyper-osmotic regimes (Carlson & Holmes, 1962; Haruta et al., 1991; Warne et al., 2005). The higher level of pro-vasotocin expression in higher salinity regimes reflects the need for replenishment of the pituitary vasotocin storage where vasotocin is continuously released to the bloodstream.

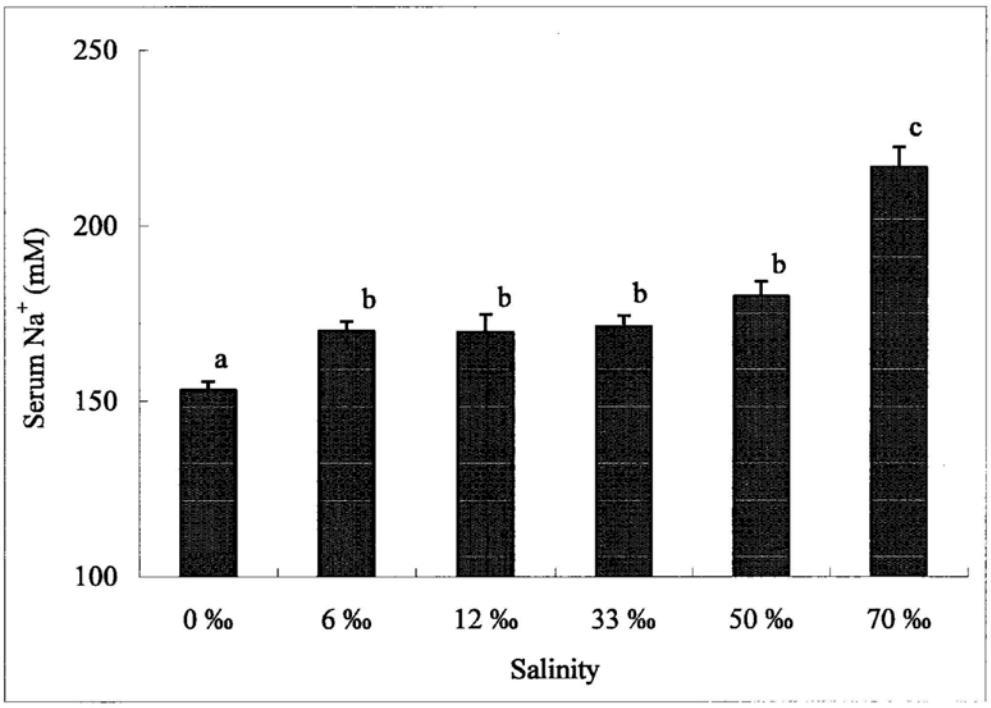

Figure 5.1. Changes in serum sodium concentrations (mM) of silver sea bream adapted to different salinities (n=7). Values are means ± S.E.M. Different alphabets denote significant differences among different groups (One way ANOVA, Tukey's test, $p<0.05$).

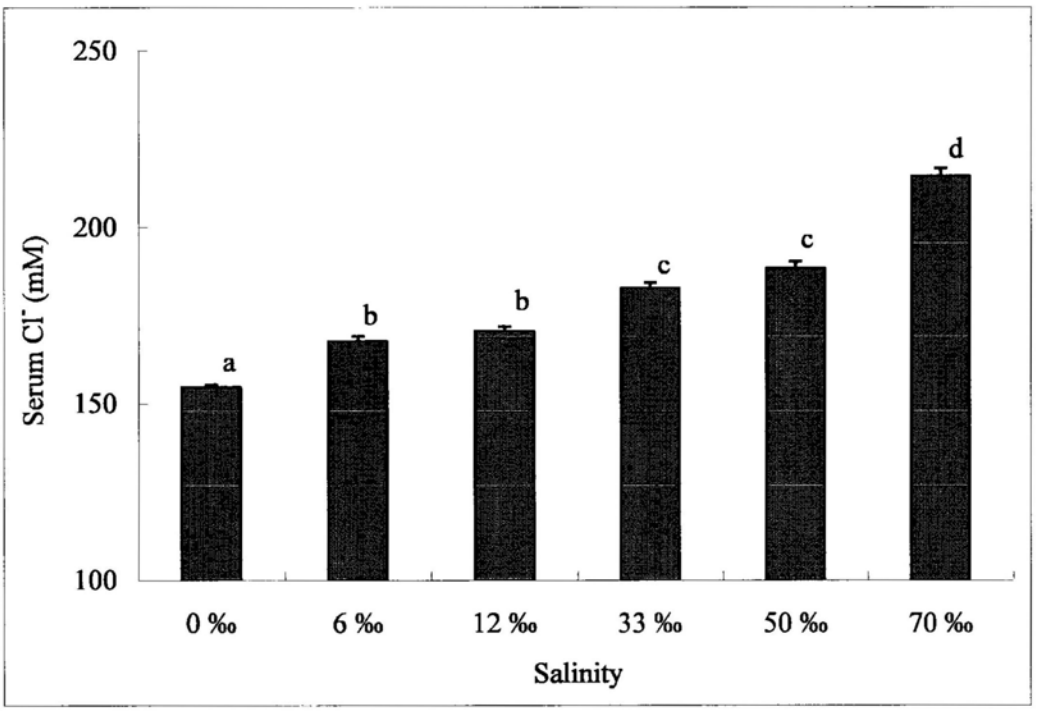

Figure 5.2. Changes in serum chloride concentrations (mM) of silver sea bream adapted to different salinities (n=7). Values are means ± S.E.M. Different alphabets denote significant differences among different groups (One way ANOVA, Tukey's test, $p<0.05$).

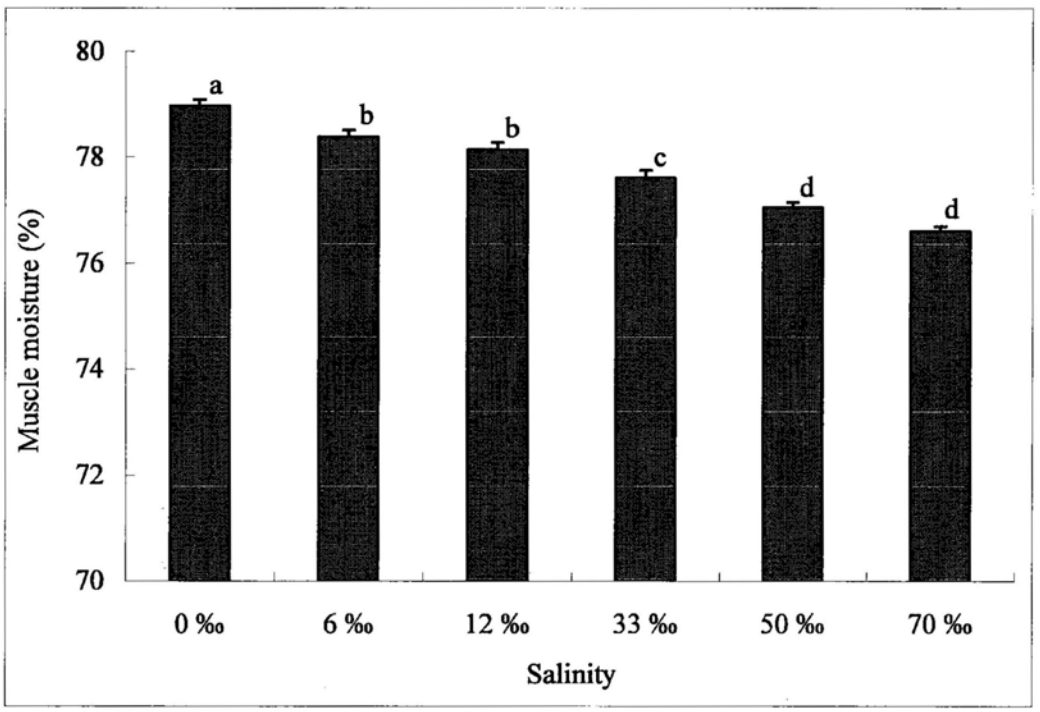

Figure 5.3. Changes in muscle moisture (%) of silver sea bream adapted to different salinities (n=7). Values are means ± S.E.M. Different alphabets denote significant differences among different groups (One way ANOVA, Tukey's test, $p<0.05$).

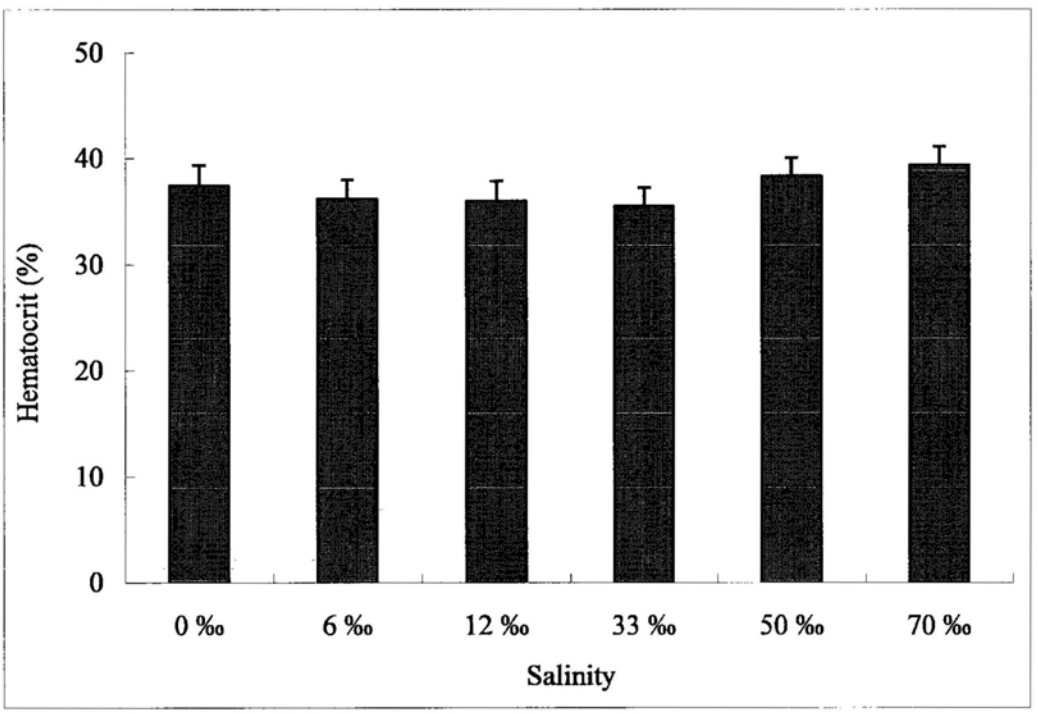

Figure 5.4. Changes in hematocrit (%) of silver sea bream adapted to different salinities (n=7). Values are means ± S.E.M. No significant difference was found among different groups (One way ANOVA, Tukey's test, $p>0.05$).

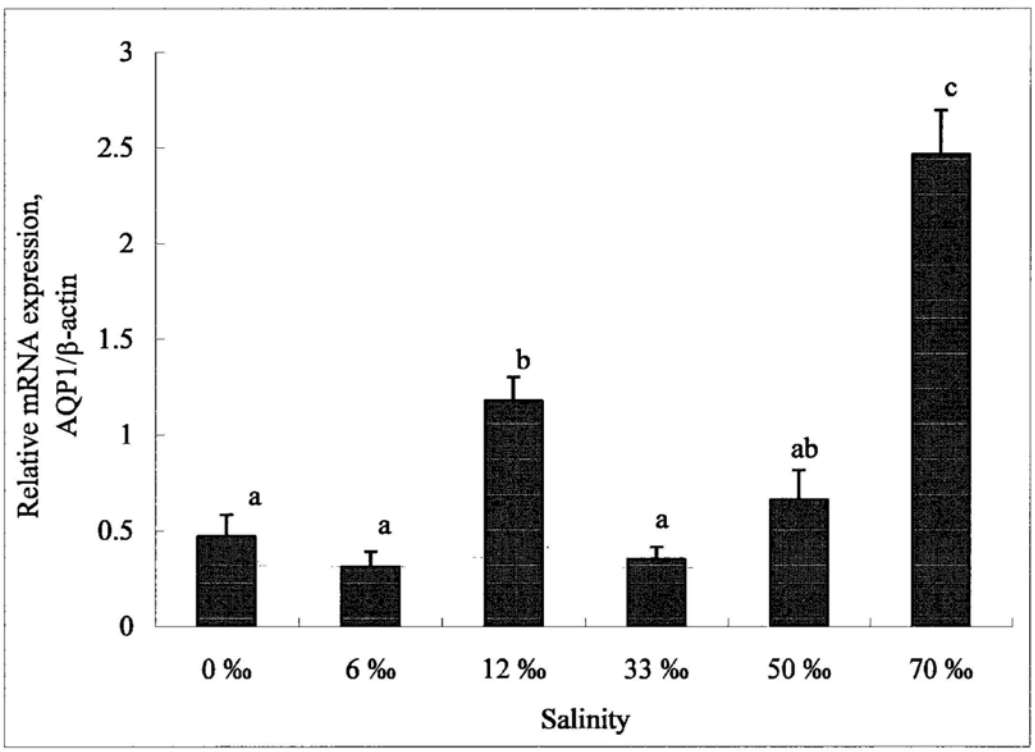

Figure 5.5. Expression of AQP-1 in intestine of sea bream adapted to different salinities. Expression was expressed as a ratio of AQP-1 to β-actin expression (n=7). Values are means ± S.E.M. Different alphabets denote significant differences among different groups (One way ANOVA, Tukey's test, $p<0.05$).

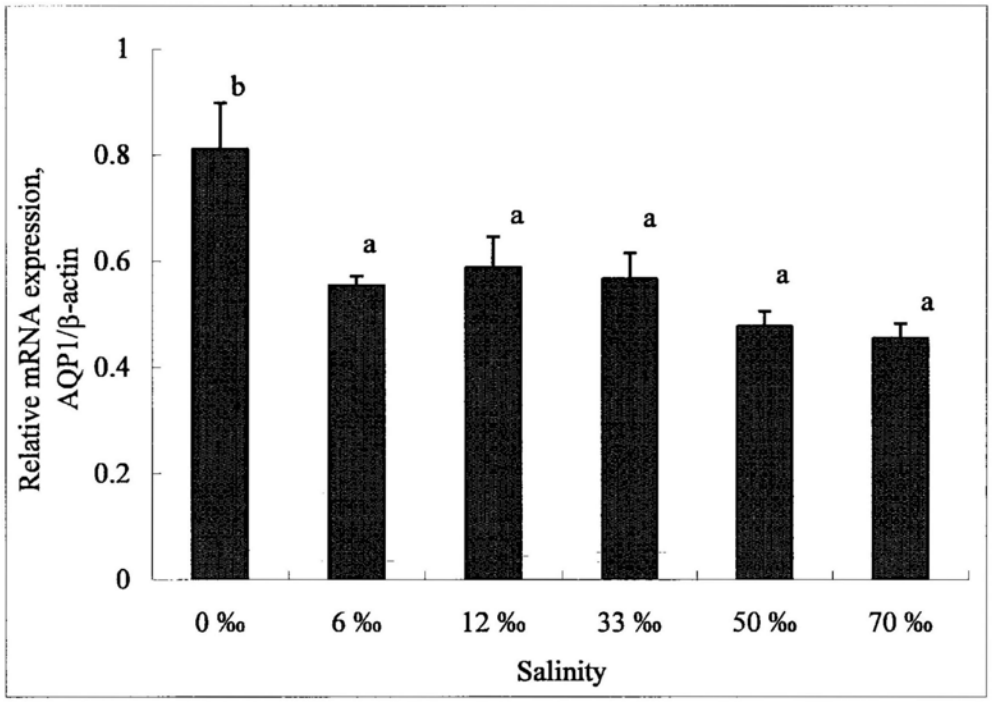

Figure 5.6. Expression of AQP-1 in gills of sea bream adapted to different salinities. Expression was expressed as a ratio of AQP-1 to β-actin expression (n=7). Values are means ± S.E.M. Different alphabets denote significant differences among different groups (One way ANOVA, Tukey's test, $p<0.05$).

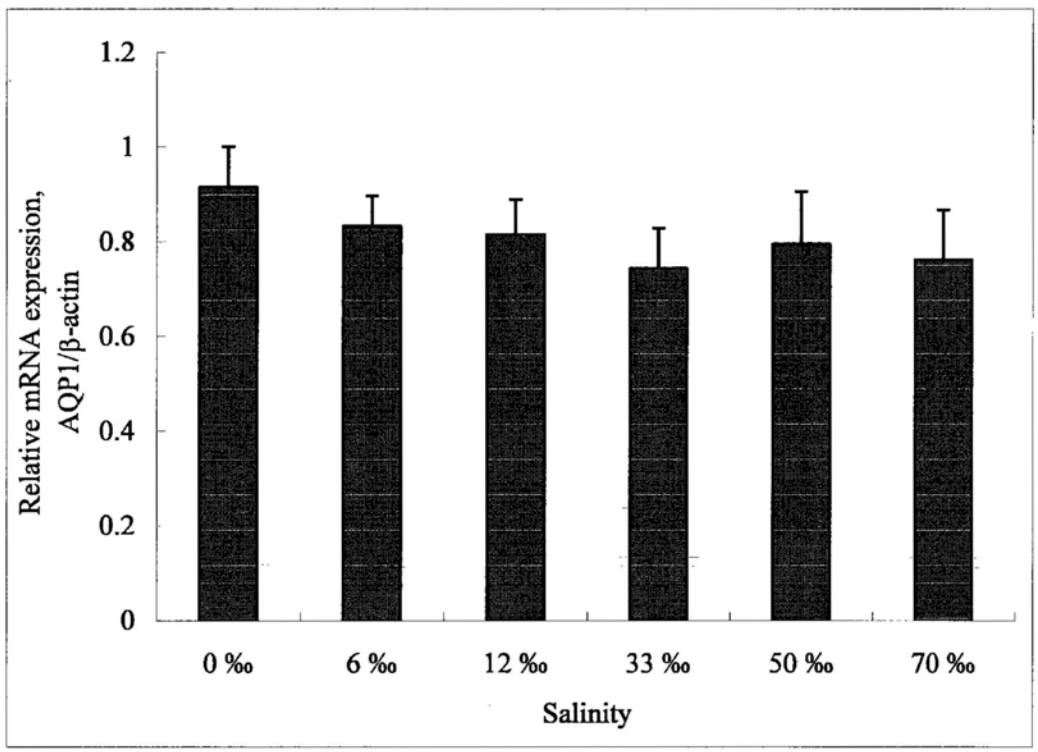

Figure 5.7. Expression of AQP-1 in kidney of sea bream adapted to different salinities. Expression was expressed as a ratio of AQP-1 to β-actin expression (n=4). Values are means ± S.E.M. No significant difference was found among different groups (One way ANOVA, Tukey's test, $p>0.05$).

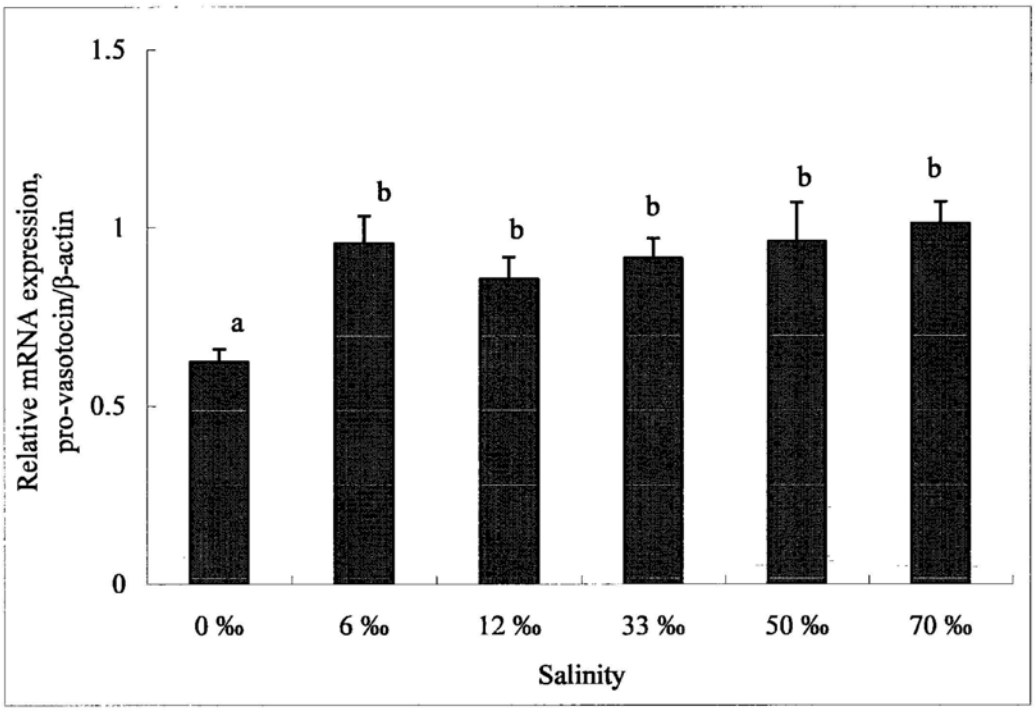

Figure 5.8. Expression of hypothalamic pro-vasotocin of sea bream adapted to different salinities. Expression was expressed as a ratio of pro-vasotocin to β-actin (n=7). Values are means ± S.E.M. Different alphabets denote significant differences among different groups (One way ANOVA, Tukey's test, $p<0.05$).

5.3. Effect of abrupt hypo-osmotic exposure on the expression of hypothalamic pro-vasotocin, and aquaporin-1 in gills, intestine and kidney

5.3.1 Materials and methods

5.3.1.1 Fish

Silver sea bream weighing 100-150 g, were purchased from local fish farm, and kept in a recirculating seawater (33‰) system in Simon F. S. Li Marine Science Laboratory, The Chinese University of Hong Kong for not less than three weeks to allow recovery from transportation stress. The fish were then divided into 13 groups randomly. Seven groups of fish (33‰-33‰ groups) were transferred into seven one-ton seawater (33‰) tanks with independent recirculating filter systems. The remaining six groups (33‰-6‰ groups) of fish were abruptly transferred to six one-ton tanks pre-adjusted to a hypo-osmotic salinity of 6‰. Fish of the 33‰-6‰ groups were sacrificed at 2, 6, 12, 24, 72 and 168 hours after transfer. Pre-transferred fish were used as time=0 group. Fish of the control transfer groups (33‰-33‰ groups) were sampled at the same time intervals as the 33‰-6‰ groups. Fish were fed *ad libitum* with fish meal pellets according to Woo & Kelly (1995) throughout the experiment and feeding was terminated 24 hours before fish were sacrificed.

5.3.1.2 Tissue sampling

Silver sea bream were removed from the tanks and was firstly anesthetized by MS-222 (Sigma, U.S.A.). Blood was withdrawn into a syringe from the caudal vein and the blood samples were centrifuged to obtain serum. Hematocrit was determined immediately after blood collection.

In order to remove the blood from gills and kidney, perfusion was performed through the first branchial afferent artery as described in the Materials and Methods section in Chapter 4.2.4. Perfusion was also performed via the dorsal aorta on several occasions when blood inside the kidney had not been removed properly by the branchial afferent artery perfusion. The perfusion operation was deemed complete when the red color of the organs disappeared Gills, kidneys and intestine were dissected and put into 1 ml Tri-reagent (Molecular Research Center, USA). Muscle tissues were removed for determination of muscle moisture content. All samples were then stored at -70 °C.

5.3.1.3 Serum electrolytes and muscle moisture content

Analysis of serum Na^+ and Cl^-, muscle moisture content followed exactly the same procedures as previously described in the Materials and Methods section in Chapter 5.2.1.3.

5.3.1.4 RNA extraction, first strand cDNA synthesis and semi-quantitative RT-PCR

RNA extraction, first strand cDNA synthesis and semi-quantitative RT-PCR followed exactly the same procedures as previously described in the Materials and Methods section in Chapter 4.2.2 and Chapter 5.2.1.5.

5.3.1.5 Statistical analysis

All data were expressed as mean values ± standard error of the mean (S.E.M.). The groups were then subjected to a one-way analysis of variance (ANOVA), followed by a Tukey's test (SPSS) with $p<0.05$ to delineate significance. For comparison of same time point between two groups, unpaired Student's t-test was

used with $p<0.05$ to delineate significance.

5.3.2 Results

5.3.2.1 Serum electrolytes, muscle moisture and hematocrit

In the 33‰-6‰ group, serum Na^+ levels significantly fell two hours after hypo-osmotic exposure, then it started to elevate after six-hour exposure and gradually returned to a steady level (Fig. 5.9). Serum Cl^- levels also significantly fell two hours after hypo-osmotic exposure, however, it continued to fall until six hours (Fig. 5.10). Serum Cl^- levels started to elevate 12 hours after abrupt exposure to 6‰ and became stabilized between 24 and 168 hours. Serum Na^+ and Cl^- levels were significantly different from the 33‰-33‰ control group at every time point and did not return to pre-exposure levels 168 hours after hyposmotic exposure.

Muscle moisture content was significantly elevated in the 33‰-6‰ group during 12 to 24 hours after abrupt hypo-osmotic exposure, but the differences were narrowed down and were not statistically significant at the final stage of the experimental period (Fig. 5.11). On the other hand, a transient drop was found in hematocrit two hours after low salinity exposure. Then the hematocrit levels of 33‰-6‰ group mostly returned

to levels similar to the 33‰-33‰ control group six-hour after the transfer (Fig. 5.12).

5.3.2.2 Relative expression of AQP-1 in intestine, gills and kidney, and hypothalamic pro-vasotocin

Intestinal AQP-1 expression exhibited transient perturbations in 33‰-6‰ group and 33‰-33‰ control group (Fig. 5.13). The AQP-1 expression levels exhibited 2-hour and 12-hour post-exposure peaks in both 33‰-6‰ and 33‰-33‰ groups. The transcription levels of AQP-1 were significantly downregulated at six hours and 168 hours after low salinity exposure. In addition, the level of AQP-1 transcription did not return to post-exposure level until, at most, 168 hours (Fig. 5.13).

In gills, AQP-1 expression levels appeared to fall two hours after exposure to 6‰ and continued to fall until 12 hours (Fig. 5.14). The expression level of AQP-1 transcript was lowest 12 hours after low salinity exposure and the amount of AQP-1 mRNA was less than half of those of 33‰-33‰ control group at the same time point. The AQP-1 expression began to elevate after 24-hour exposure and returned to pre-exposure levels thereafter.

There is no significant difference or trend observed in the expression levels of renal AQP-1 (Fig. 5.15) and hypothalamic provasotocin (Fig. 5.16) after abrupt hyposmotic exposure.

5.3.3 Discussion

5.3.3.1 Effect of abrupt hypo-osmotic exposure on serum electrolyes, muscle moisture and hematocrit

5.3.3.1.1 Serum electrolytes

Responses of silver sea bream to abrupt low-salinity exposure had been studied by Kelly & Woo (1999) and the study demonstrated uncoupled trends of serum Na^+ and Cl^- perturbations after the exposure. Serum Na^+ concentrations were relatively stable throughout the low salinity exposure whilst serum Cl^- concentration dropped markedly. Woo & Wu (1982) reported a steady elevation in the serum Na^+ levels of *Mylio macrocephalus* when adapted from seawater to 7‰ water.

In general, abrupt hypo-osmotic exposure elicited hyponatraemia in other teleost species (Dutil et al., 1992; Munro et al., 1994). Mancera et al. (1993) also reported a

transient decrease in Na^+ levels of gilthead sea bream (*Sparus aurata*) 12 hours after hypo-osmotic exposure (seawater to 7‰) and the Na^+ levels returned to normal values after 24 hours.

In contrast to the results of Kelly & Woo (1999), both serum Na^+ and Cl^- levels of silver sea bream were significantly reduced in early stage of abrupt exposure to 6‰ and recovered to steady concentrations slightly lower than seawater control levels after 24 hours. However, serum Na^+ levels started moving in the upward channel six hours after hypo-osmotic exposure and this occurred six hours earlier than serum Cl^-. The fish appeared to exhibit its higher capability to retain Na^+ with an earlier occurrence of refractory period.

5.3.3.1.2 Muscle water content

Woo & Wu (1982) reported the presence of muscle dehydration in *Mylio macrocephalus* after abrupt transfer from 33‰ to 7‰. In the present study, muscle moisture levels of silver sea bream slightly elevated during 12 to 24 hours after abrupt exposure to 6‰ and then returned to pre-exposure levels. The muscle water content was relatively stable throughout the transfer and was consistent with the results of a

similar study reported by Kelly & Woo (1999) on the same fish. Contrary to these sea bream species, abrupt hypo-osmotic exposure to 7‰ induced significant muscle hydration in other marine teleosts (Dutil et al., 1992; Munro et al., 1994). The silver sea bream demonstrated its tolerance to low salinity challenge by limiting the extent of muscle hydration within a short period of time post-exposure to 6‰.

5.3.3.1.3 Hematocrit

As mentioned in section 5.3.2.1.3, variations in hematocrit value generally indicate changes in number or volume of red blood cells, or circulating volume. It is generally accepted that the hematocrit value of marine teleosts increases after long-term acclimation to low salinity environments.

In contrast to long-term hypo-osmotic acclimation, abrupt hypo-osmotic exposure elicit a significant fall in hematocrit of silver seabream two hours post exposure before it rebounded back to the levels of seawater control group at six hours. Taken together with results of serum Na^+ and Cl^- levels, the decline in hematocrit indicated a net gain in the total circulatory volume, most likely due to the rapid water influx immediately after the low salinity challenge. However, rapid restoration of hematocrit and serum electrolytes in silver sea bream suggested there is an efficient

osmoregulatory mechanism taken place to prevent further passive water entry and to get rid of excessive water out of the body.

5.3.3.2 Effect of abrupt hypo-osmotic exposure on intestinal AQP-1 expression

To date, there is no time-course experiment performed to elucidate the transient response of aquaporins to hypo-osmotic stress in gills, kidney and intestine of fish. Chau (2009) had recently reported abrupt hypo-osmotic exposure study on the function of the urinary bladder of the silver sea bream and the AQP mRNA expression level was initially elevated followed by a final downregulated expression level. Studies on temporal changes of AQP-1 expression would allow us to understand the sequential events following salinity acclimation and may explain the contradictory results of AQP reported in fish.

Transcription level of AQP-1 was upregulated in intestine after transfer from freshwater to seawater for 7 days to 21 days in a number of euryhaline teleost species (Aoki et al., 2003; Raldua et al., 2008; Martinez et al., 2005b). In contrast, intestinal mRNA expression level of AQP-1 in seawater-adapted was significantly lower than those of black porgy adapted to brackish water (10‰) and freshwater after a 20-day

acclimation period (An et al., 2008). These results suggest that there are two opposite responses of AQP-1 towards salinity adaptation in fish, the explanation of which still remains obscure.

After abrupt hypo-osmotic exposure, marked fluctuations of AQP-1 mRNA expression were found in both 33‰-33‰ and 33‰-6‰ fish in the initial phase (from 2 to 12 hours) and when AQP-1 expression reached final level at 168 hours, it was significantly lower than the corresponding value for the hypo-osmotic transferred fish. Taken together with the expression results of 0‰- and 6‰-fish obtained in the 4-week salinity acclimation experiment, intestinal AQP-1 expression did not show an immediate response to abrupt low salinity exposure, but it was downregulated at about one-week after 6‰ transfer and finally recovered to expression levels of 33‰-sea bream in the long-run. The sequential responses during hypo-osmotic acclimation suggested AQP-1 plays a role in hyerosmoregulation at early stages but its role in the intestine become less important and possibly replaced by other aquaporins or other water transporting pathways during long-term adaptation.

5.3.3.3 Effect of abrupt hypo-osmotic exposure on branchial AQP-1 expression

During the first six hours after abrupt hypo-osmotic transfer, branchial AQP-1 expression significantly declined and was lower than levels of 33‰-33‰ group, then it gradually elevated back to pre-transfer and control levels. The immediate post-exposure downregulation of AQP-1 indicated a shut-down of water permeation across the gill epithelium to prevent further osmotic water load entry. Few studies have reported rapid changes in osmotic water permeability after seawater or freshwater transfer (Ogasawara & Hirano, 1984; Gallis et al., 1979). In a study on the isolated gill preparation of Japanese eels, it was demonstrated that the water permeability decreased to a low level three hours after transfer from seawater to freshwater (Ogasawara & Hirano, 1984).

Ogasawara & Hirano (1984) reported a decline in both chloride cell numbers and apical pit diameter in freshwater transferred eels within the first six hours, these observations concurred with the rapid decrease of branchial osmotic water permeability in these fish. For silver sea bream, a transient reduction in branchial chloride cell number was observed with a large increase in both apical area and fractional area after a six-hour hypo-osmotic exposure period (Kelly et al., 1999).

Morphology of these chloride cells also started to change from invaginated to co-existing on the same plane or protruding well above the pavement cells. Immunohistochemistry had shown that AQP-1 was primarily located in chloride cells in gills, coinciding with decreased availability of chloride cells and modification of cell morphology (Kelly & Woo, 1999) may contribute to the reduced expression level of AQP-1 during the early period of abrupt 6‰ transfer.

5.3.3.4 Effect of abrupt hypo-osmotic exposure on renal AQP-1 expression

In accordance with long-term acclimation, renal AQP-1 expression levels of silver sea bream did not change significantly after abrupt hypo-osmotic exposure and this result indicates the lack of responsiveness of AQP-1 to rapid hydration of the fish. The glomerular filtration rate in freshwater teleost is relatively high in order to get rid of excessive water loads (Nishimura & Imai, 1982). Freshwater adaptation induced initial diuresis in the euryhaline teleost, *Fundunlus kansae,* which reached a peak approximately two hours after experimentation (Fleming & Stanley, 1965). Further to this, 45% of the glomeruli are perfused and actively filtering in rainbow trout adapted to freshwater whereas only 5% of the glomeruli are filtering in the kidneys of seawater- adapted trout (Brown et al., 1978, 1980; Amer & Brown, 1995). Wong &

Woo (2006) also reported higher percentage of expanded glomeruli and increased glomerular diameter in the kidney of silver sea bream after abrupt 6‰ transfer, suggesting a rapid change in glomerular function in response to the hypo-osmotic exposure. Rapid alterations in glomerular filtration rate and glomerular intermittency appeared to be more rapid and effective strategy during salinity adaptation in fish.

5.3.3.5 Effect of abrupt hypo-osmotic exposure on hypothalamic pro-vasotocin expression

Hypothalamic vasotocin synthesis did not change significantly after abrupt transfer of the silver sea bream to 6‰ in the present study. Warne et al. (2005) reported rapid alterations of hypothalamic pro-vasotoicn expression in flounders after acute seawater transfer where the expression level was higher in seawater-acclimated fish. The expression level of pro-vasotocin returned to freshwater control level 24 hours after the seawater transfer in the flounder (Warne et al. 2005).

By comparison, expression levels of provasotocin mRNA between the chronic salinity treatment and abrupt hyposmotic exposure groups of sea bream, adjustment of vasotocin synthesis appears to be a strategy during long-term salinity acclimation. In

contrast, silver sea bream may utilize other parts of the vasotocin hormone system to exert its influences on target tissues upon acute salinity perturbations. Release of vasotocin from the pituitary reservoir could be the most effective way to elevate circulatory hormone levels to stimulate response in target tissues. In fact, pituitary vasotocin content falls and plasma vasotocin levels are raised after flounder were transferred from freshwater to seawater (Warne et al., 2005).

In addition to adjustments in availability of hormone, Warne (1999) suggested the vasotocin receptors in the target tissues could have a major role in modulating hormone function. Vasotocin V1 receptor transcription was downregulated at 24 hours following transfer of flounders from seawater to freshwater (Balment et al., 2006) and this altered level of receptors eventually lowered the sensitivity of target tissue to vasotocin. A positive relationship between vasotocin receptor and AQP-1 expressions was established in gills, intestine and kidney of black porgy (An et al., 2008). Partial clones of two subtypes of vasotocin V1 receptors (V1a and V1b) had been recently isolated from the silver sea bream (Luk & Woo, unpublished data) and preliminary tissue distribution analysis by RT-PCR revealed transcripts of the V1a receptors were expressed in gills, intestine and kidney of the fish. Further study in these receptors may provide a more complete picture on the action of the neuropeptide hormone in these

target tissues.

5.4 Conclusion

A pronounced effect of hypo-osmotic adaptation was observed on the AQP-1 mRNA expression levels in gills of silver sea bream, with the results showing an initial downregulation during abrupt 6‰ exposure and an increase of expression during long-term freshwater acclimation. In general, expression levels of AQP-1 was not significantly different within the intermediate salinities (6‰-33‰) in the osmoregulatory tissues examined and significant higher AQP-1 expression was only observed in intestine of fish acclimated to 70‰. These results suggested that AQP-1 may be involved with physiological roles other than osmoregulation under normal conditions and was only stimulated or suppressed upon acute salinity acclimation.

Downregulation of hypothalamic pro-vasotocin transcription was observed in fish acclimated to freshwater for four weeks, however, there was no significant change detected after abrupt 6‰ exposure. During abrupt hypo-osmotic exposure, it is likely that the other part of the vasotocin system, such as changes in receptor abundance in target tissues and circulatory hormone levels, may play a more rapid and promising

influence to regulate the action of vasotocin on osmoregulatory organs of the silver sea bream. In the present study, the decline in vasotocin synthesis correlates well with upregulation of AQP-1 trancription in freshwater-adapted sea bream, however, further investigation on the relationship of the hormone and the water channel is much needed.

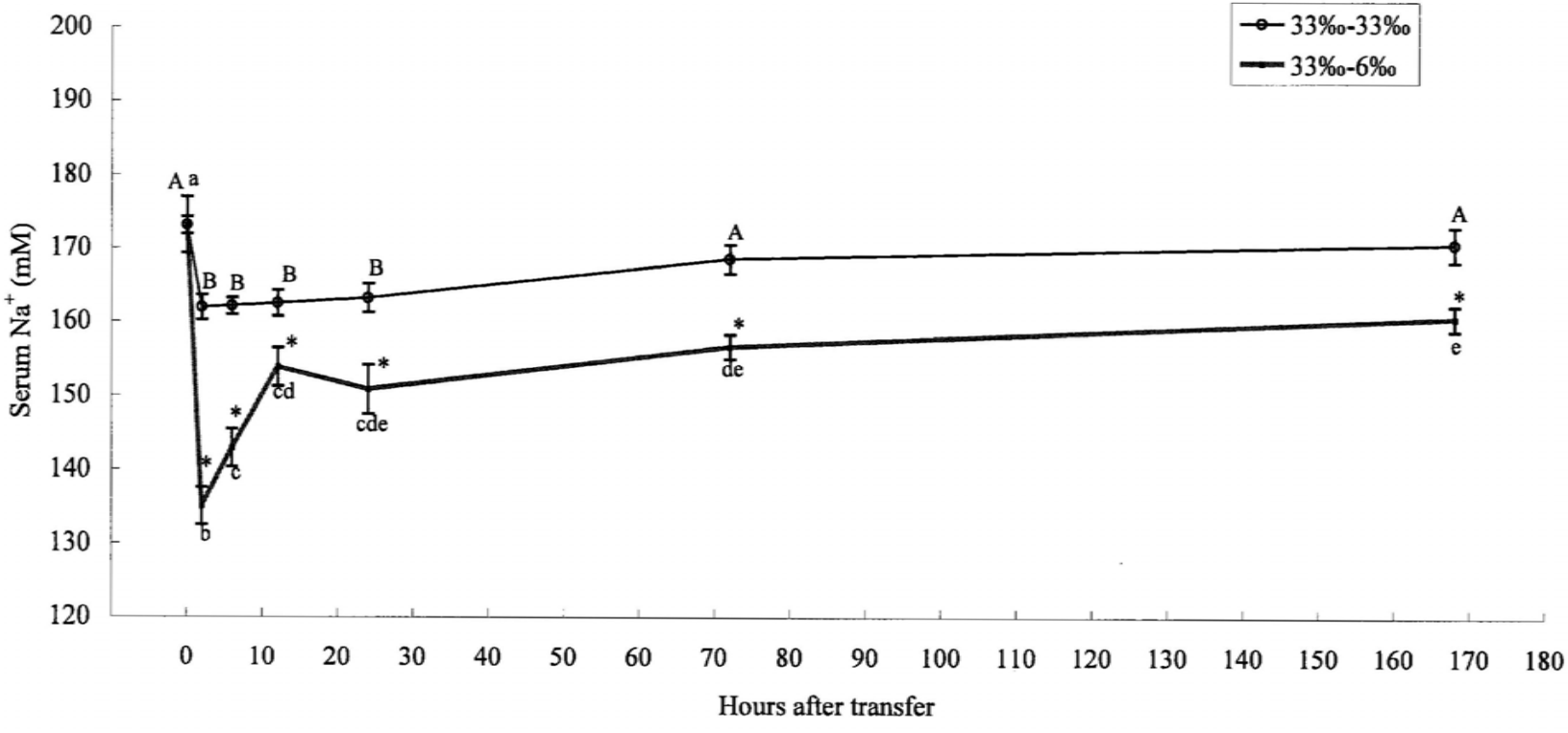

Figure 5.9. Changes in serum sodium concentrations (mM) of silver sea bream after hypo-osmotic transfer (n=7). Values are means ± S.E.M. Capital letters denote significant difference was found among 33‰-33‰ and small letters denote significant differences among 33‰-6‰ groups (One way ANOVA, Tukey's test, $p<0.05$). * denotes significant differences between groups at the same time point (unpaired t-test, $p<0.05$).

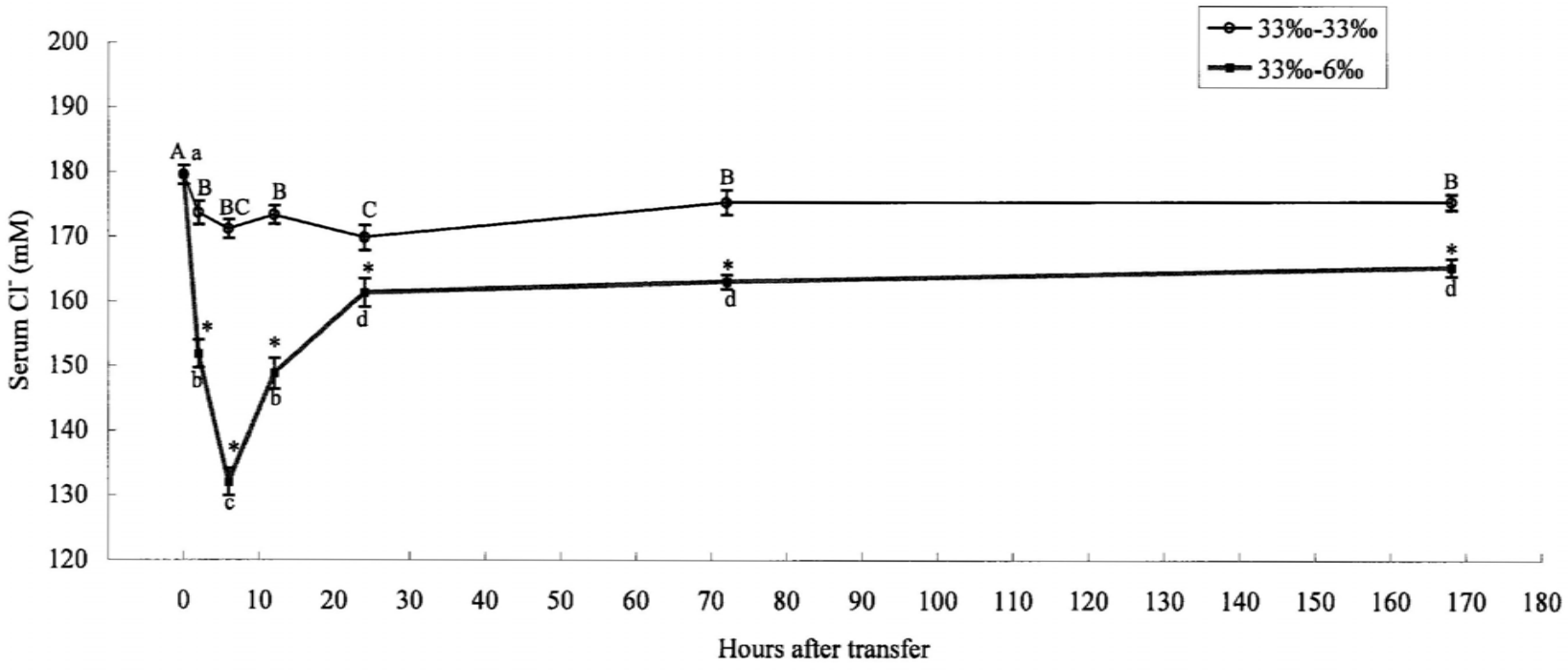

Figure 5.10. Changes in serum chloride concentrations (mM) of silver sea bream after hypo-osmotic transfer (n=7). Values are means ± S.E.M. Capital letters denote significant difference was found among 33‰-33‰ and small letters denote significant differences among 33‰-6‰ groups (One way ANOVA, Tukey's test, $p<0.05$). * denotes significant differences between groups at the same time point (unpaired t-test, $p<0.05$).

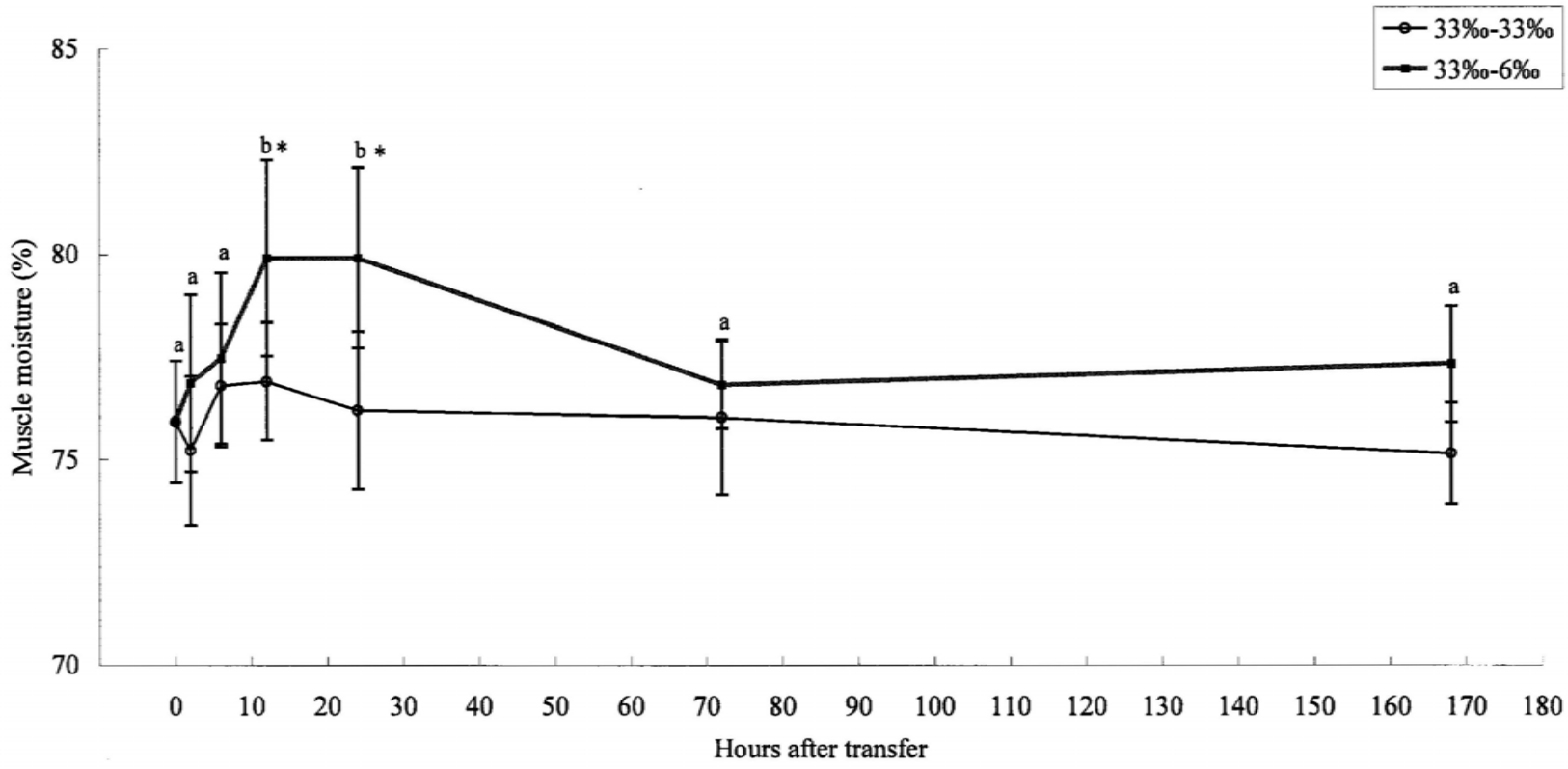

Figure 5.11. Changes in muscle moisture content (%) of silver sea bream after hypo-osmotic transfer (n=7). Values are means ± S.E.M. No significant differences was found among 33‰-33‰ and small letters denote significant differences among 33‰-6‰ groups (One way ANOVA, Tukey's test, $p<0.05$). * denotes significant differences between groups at the same time point (unpaired t-test, $p<0.05$).

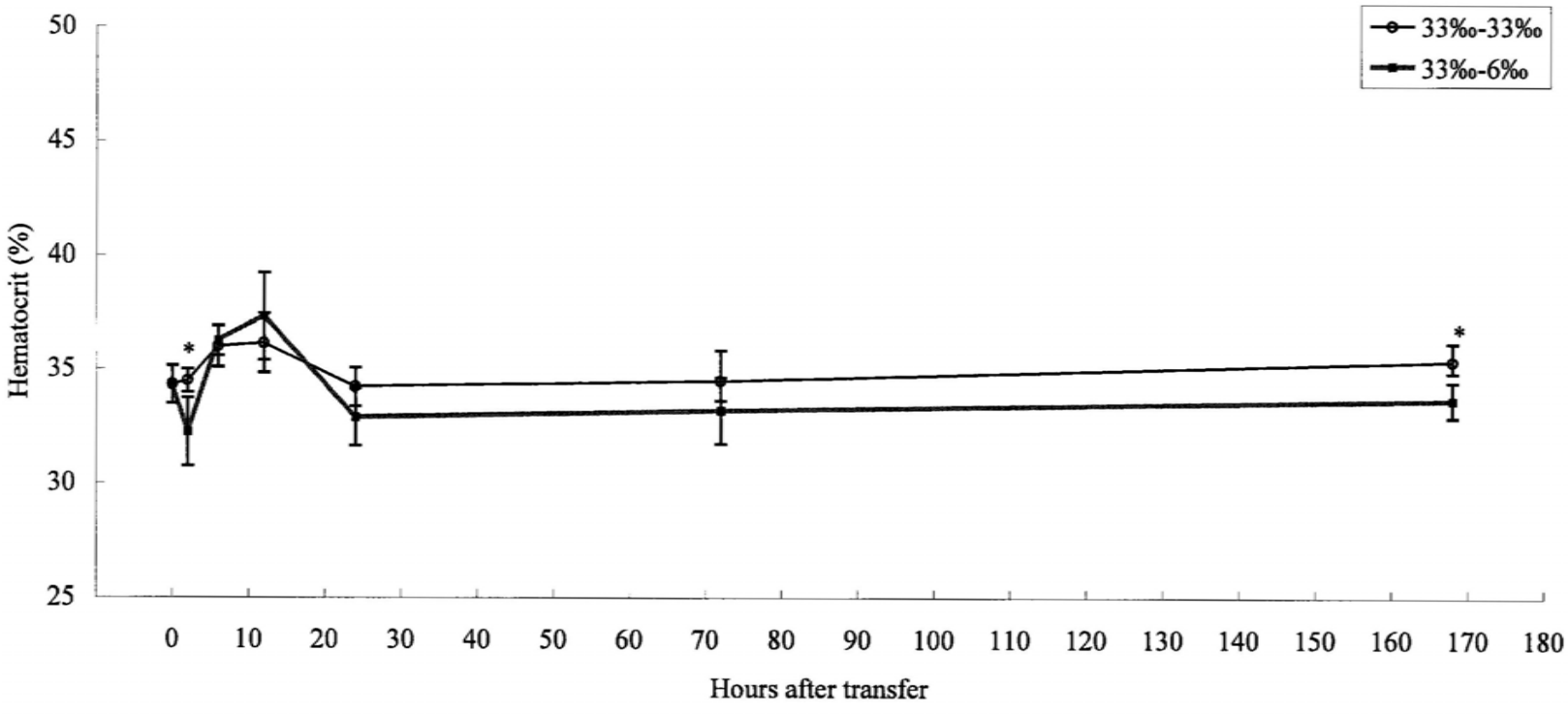

Figure 5.12. Changes in hematocrit (%) of silver sea bream after hypo-osmotic transfer (n=7). Values are means ± S.E.M. No significant differences was found among 33‰-33‰ and 33‰-6‰ groups (One way ANOVA, Tukey's test, $p>0.05$). * denotes significant differences between groups at the same time point (unpaired t-test, $p<0.05$).

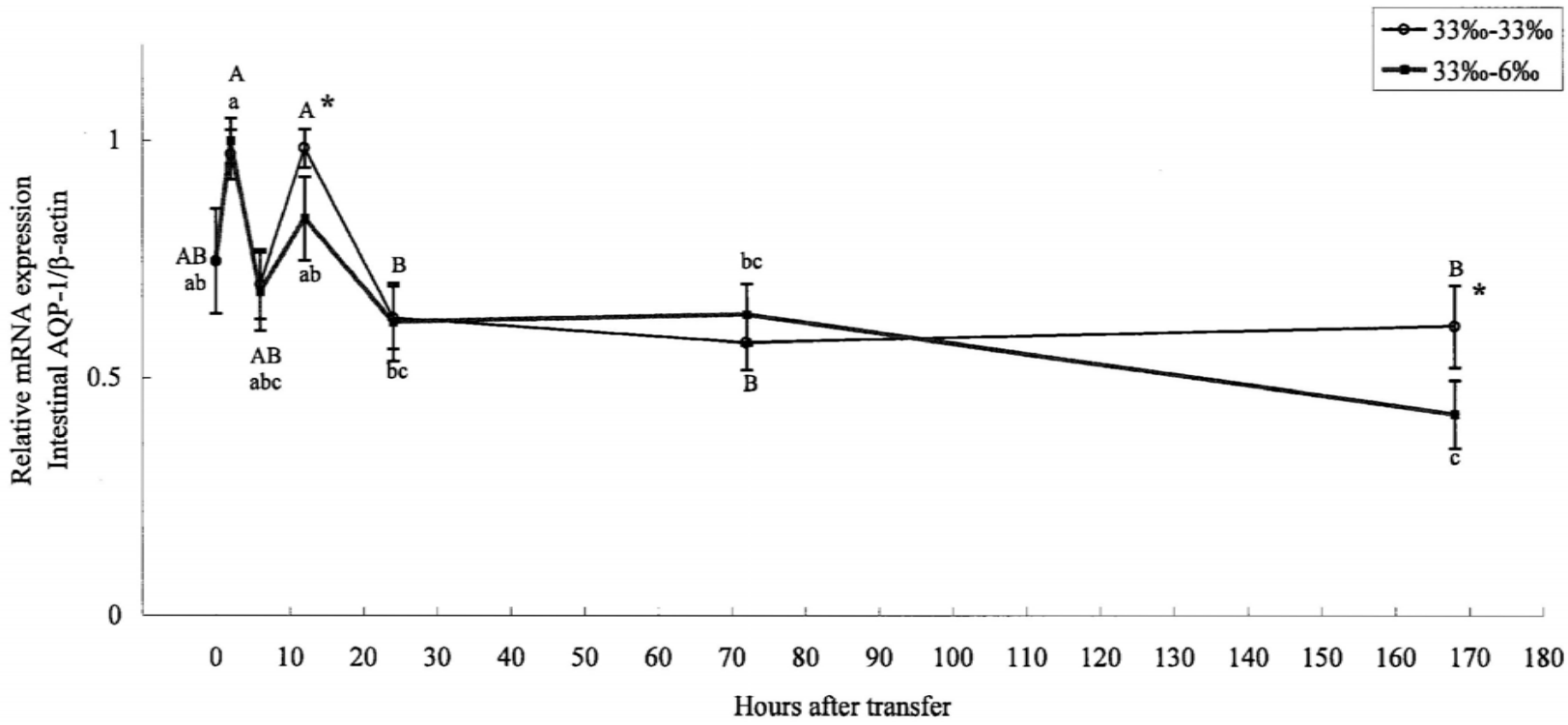

Figure 5.13. Expression of AQP-1 in intestine of silver sea bream after hypo-osmotic transfer. Expression was expressed as a ratio of AQP-1 to β-actin expression (n=7). Values are means ± S.E.M. Capital letters denote significant difference was found among 33‰-33‰ and small letters denote significant differences among 33‰-6‰ groups (One way ANOVA, Tukey's test, $p<0.05$). * denotes significant differences between groups at the same time point (unpaired t-test, $p<0.05$).

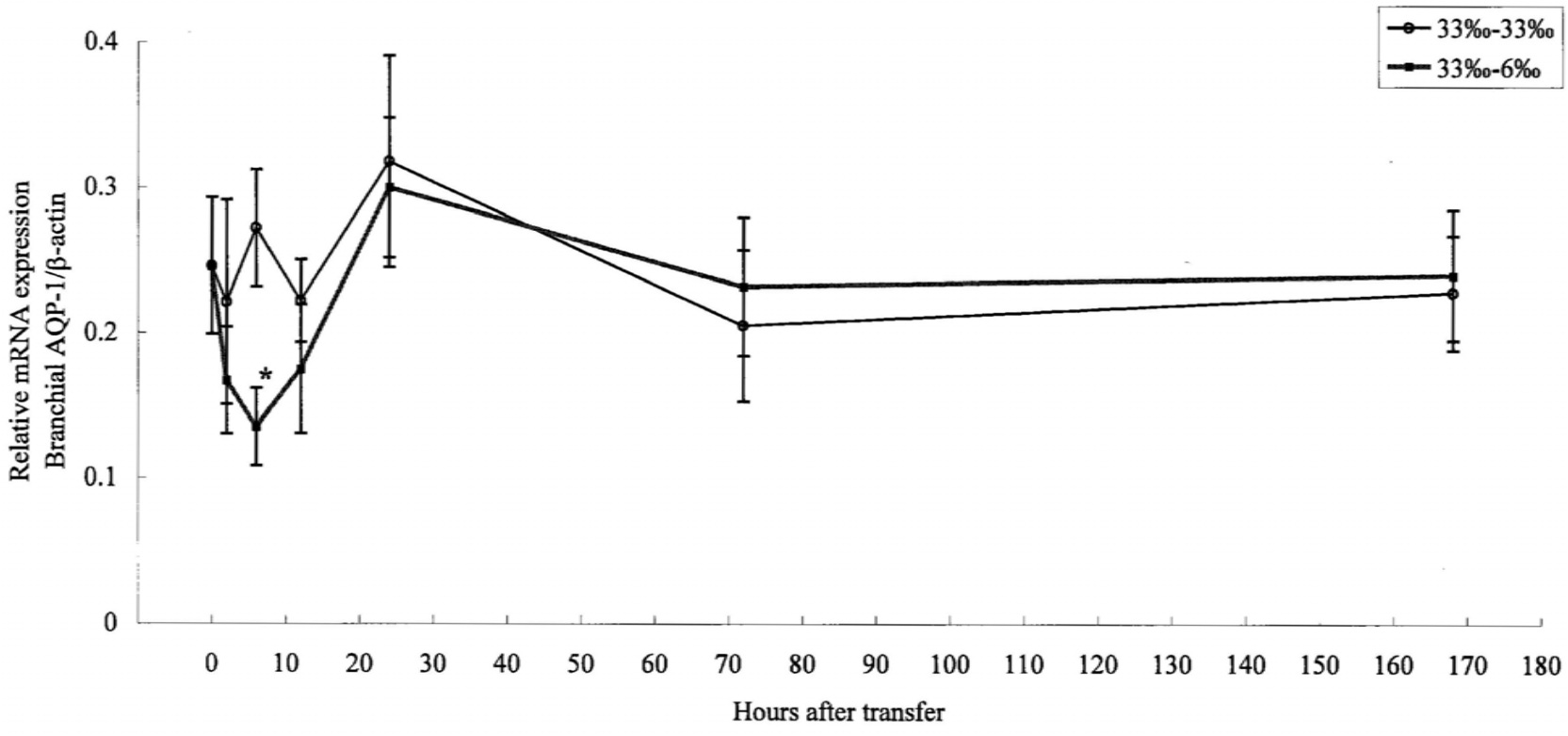

Figure 5.14. Expression of AQP-1 in gills of sea bream after hypo-osmotic transfer. Expression was expressed as a ratio of AQP-1 to β-actin expression (n=7). Values are means ± S.E.M. No significant differences was found among 33‰-33‰ and 33‰-6‰ groups (One way ANOVA, Tukey's test, $p>0.05$). * denotes significant differences between groups at the same time point (unpaired t-test, $p<0.05$).

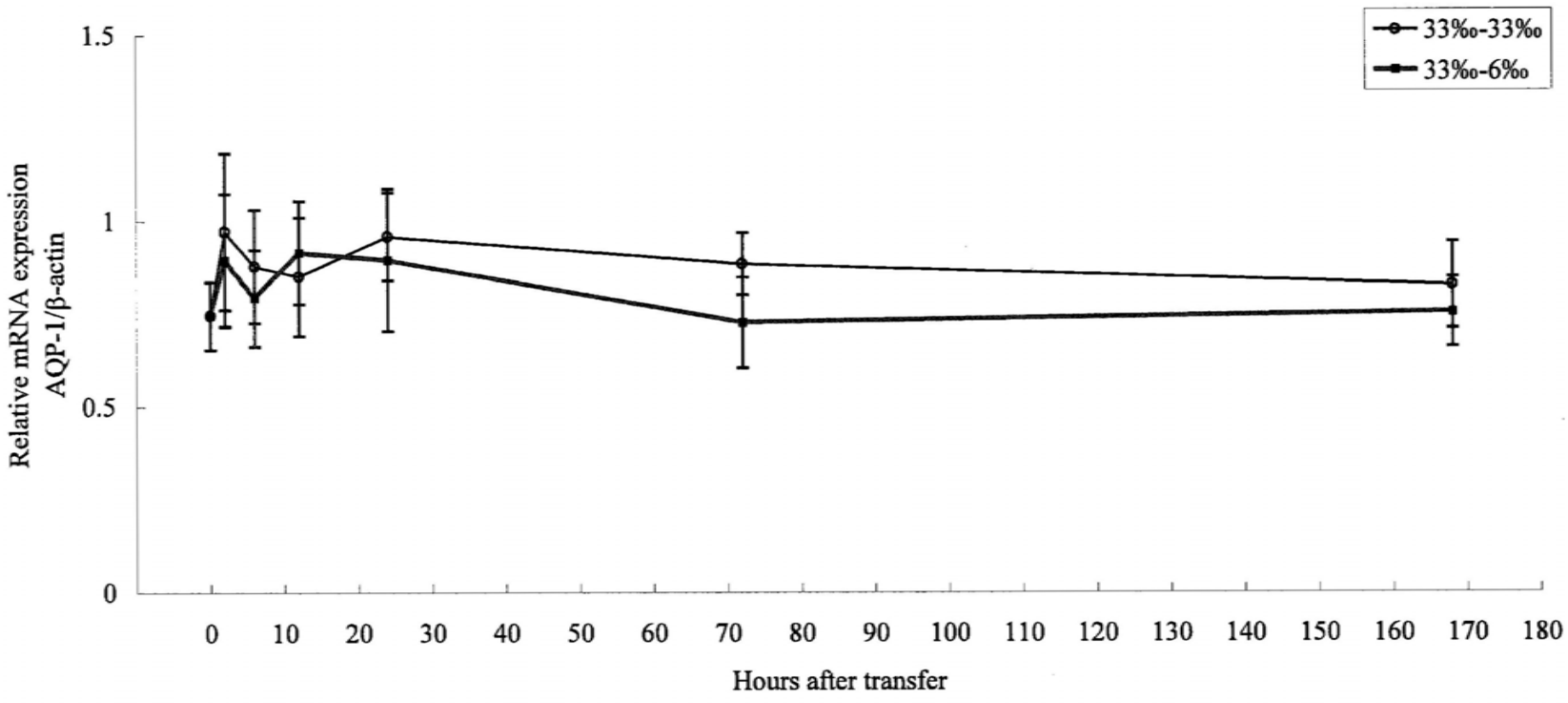

Figure 5.15. Expression of AQP-1 in kidney of sea bream after hypo-osmotic transfer. Expression was expressed as a ratio of AQP-1 to β-actin expression (n=4). Values are means ± S.E.M. No significant differences was found (One way ANOVA, Tukey's test, $p>0.05$; unpaired t-test, $p>0.05$).

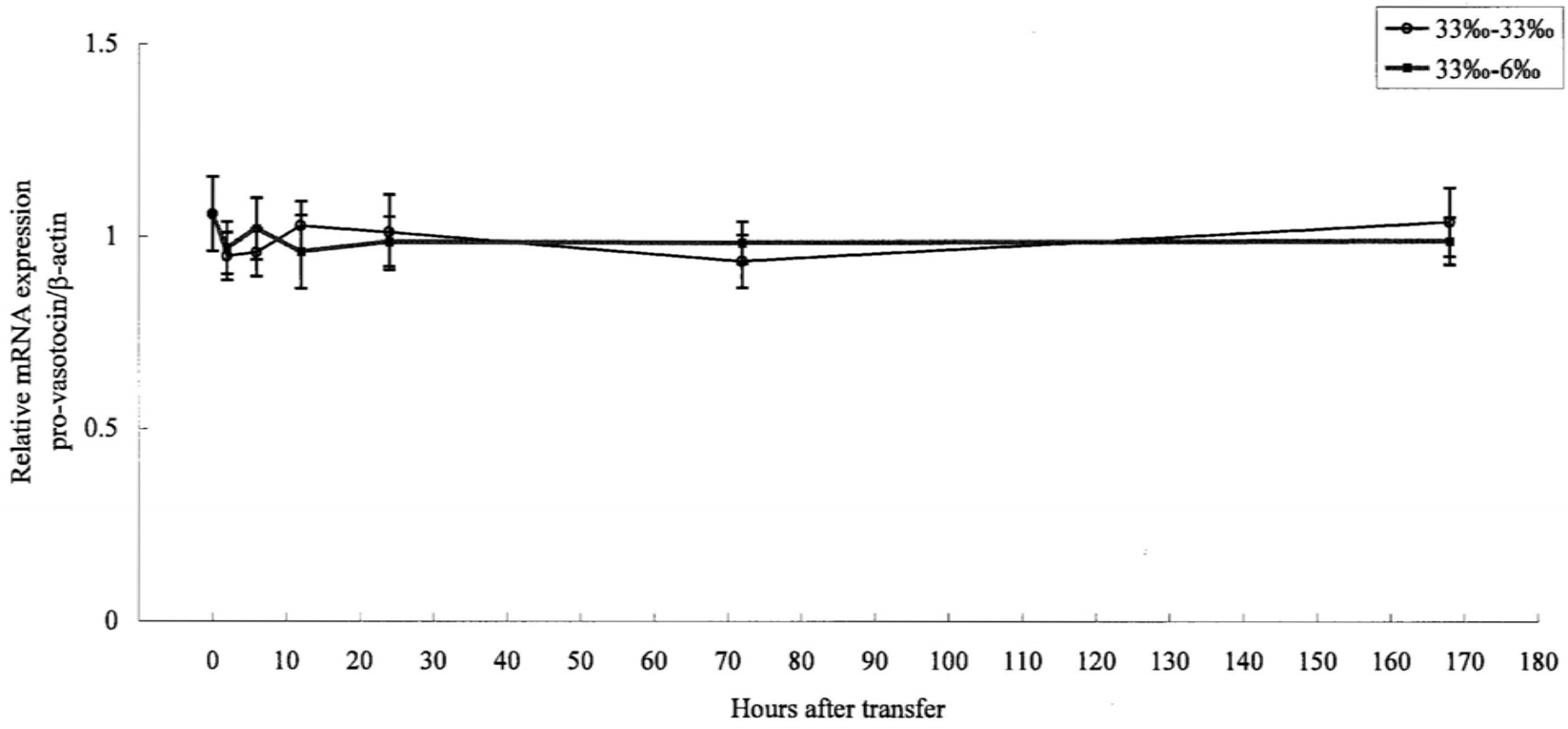

Figure 5.16. Expression of hypothalamic pro-vasotocin of sea bream after hypo-osmotic transfer. Expression was expressed as a ratio of pro-vasotocin to β-actin expression (n=7). Values are means ± S.E.M. No significant differences was found (One way ANOVA, Tukey's test, $p>0.05$; unpaired t-test, $p>0.05$).

Chapter 6

Influence of cortisol and vasotocin on the expression of hypothalamic provasotocin and AQP-l in gills, intestine and kidney of the silver sea bream

6.1 Introduction

To date, knowledge on hormonal regulation of fish aquaporins was solely based on several studies in European eels (Martinez et al., 2005a, 2005b, 2005c). Cortisol stimulated expression of AQP-1, AQP-1a (AQP-1 dup) and the aquaglycerporin AQP-1e in intestine of freshwater eels and reduced the renal expressions of these aquaporins in freshwater yellow eels. Eels are catadromous euryhaline species and migrate to and from waters with large salinity differences, so the applicability of these data to marine teleosts in general is questionable.

Cortisol is regarded as a seawater adapting hormone and it promotes salt secretion and water conservation in fish (McCormick, 2001). Cortisol has been shown to increase branchial water permeability in Japanese eels (Ogawa, 1975). Some recent studies had also demonstrated its dual effect in adaptation to lower salinities (Perry et al., 1992; Eckert et al., 2001; Mancera et al., 2002).

Substantial evidence has pointed to the involvement of vasotocin in rapidly affecting gill ion and water transport (Maetz et al., 1964; Marshall, 2003). In a trout isolated nephron preparation, elevated cAMP level was observed after administration

of vasotocin, indicating possible existence of the V2 receptor which is responsible for stimulation of AQP-2 in mammalian kidney (Perrott et al., 1993).

In general, cortisol is a versatile hormone that is capable of influencing osmoregulatory responses in freshwater and seawater fish; while vasotocin regulates many rapid responses to osmotic changes. In the present study, the effect of cortisol and vasotocin on AQP-1 was studied by a single-dose administration of these hormones to the marine teleost *Sparus sarba*. In order to study the role of these hormones during the early phrase of salinity adaptation, seawater-adapted fish were abruptly transferred to 6‰ or 33‰ after injection with cortisol or vasotocin. After these hormone administration and salinity acclimation processes, various osmoregulatory organs (gills, intestine, and kidney) were removed for the extraction of RNA and used for the subsequent determination of gene expression profiles. Prior to organ removal, the osmoregulatory organs were perfused to remove the blood (containing high AQP-1 expression) so as to obtain precise expression levels of the organs examined.

6.2 Materials and methods

6.2.1 Fish

Silver sea bream weighing 50-100 g, were purchased from a local fish farm and kept in a recirculating seawater (33‰) system in Simon F. S. Li Marine Science Laboratory, The Chinese University of Hong Kong for not less than three weeks to allow recovery from transportation stress. Fish were fed *ad libitum* with fish meal pellets according to Woo & Kelly (1995) throughout the stabilizing period.

In order to minimize stress, administration of cortisol and vasotocin followed the procedures previously described for hormone administration (Sangiao-Alvarellos et al., 2006; Pelis & McCormick, 2001). The fish were initially divided into three groups and each group of fish were lightly anaesthetized with MS-222 (Sigma), weighed, and either intraperitoneally injected with 10 μl/g body weight of slow releasing vegetable oil implant alone (mixture 1 : 1 coconut oil and olive oil), containing cortisol (50 μg/g body weight; hydrocortisone, Sigma) or containing vasotocin (1 μg/g body weight; [Arg^8]-vasotocin acetate salt, Sigma). Three days after implantation, each treatment group was further divided into two sub-groups and the subgroups were either abruptly transferred to 6‰ or 33‰ (transfer control) with individual recirculating filter systems.

The fish were acclimated to respective salinity regimes for further three days prior to sampling.

6.2.2 Tissue sampling

Silver sea bream were removed from the tanks and were deeply anesthetized by MS-222 (Sigma, U.S.A.). Blood was withdrawn into a syringe from the caudal vein and the blood samples were centrifuged to obtain serum.

In order to remove the blood from gills and kidney, perfusion was performed through the first branchial afferent artery as described in the Materials and Methods section in Chapter 4.2.4. Perfusion was also performed via the dorsal aorta on several occasions when blood inside the kidney had not been removed properly by the branchial afferent artery perfusion. The operation was deemed complete when the red color of the organs disappeared Gills, kidneys and intestine were dissected and put into 1 ml Tri-reagent (Molecular Research Center, USA). Muscle tissues were removed for determination of muscle moisture content. All samples were then stored at -70 °C.

6.2.3 Serum cortisol, serum electrolytes, and muscle moisture content

Serum cortisol were measured using commercial enzyme immunoassay (ELISA) and the ELISA kit was obtained from Cayman Chemical Company (Michigan, USA). Analysis of serum Na^+ and Cl^-, muscle moisture content followed exactly the same as previously described in the Materials and Methods section in Chapter 5.2.1.3.

6.2.4 RNA extraction, first strand cDNA synthesis and semi-quantitative RT-PCR

RNA extraction, first strand cDNA synthesis and semi-quantitative RT-PCR followed exactly the same procedures as previously described in the Materials and Methods section in Chapter 4.2.2 and Chapter 5.2.1.5.

6.2.5 Statistical analysis

All data were expressed as mean values ± standard error of the mean (S.E.M.). The groups were then subjected to a one-way analysis of variance (ANOVA), followed by a Tukey's test (SPSS) with $p<0.05$ to delineate significance.

6.3 Results

6.3.1 Hormonal effect on serum cortisol electrolytes, muscle moisture and hematocrit

Serum cortisol levels were significantly elevated after cortisol injection, with 8- and 20-fold higher than the oil-injected fish acclimated to 33‰ and 6‰ respectively (Fig. 6.1). Vasotocin administration did not significantly modify cortisol levels of silver sea bream, although the cortisol levels after vasotocin injection was apparently lower than the control levels in fish exposed to 6‰.

Serum electrolytes levels were significantly reduced in sea bream abruptly exposed to 6‰ when compared to the levels of corresponding seawater treatment groups (Fig. 6.2, Fig. 6.3). Among the groups exposure to 6‰, administration of cortisol further lowered the serum Na^+ and Cl^-, with marked decline from oil-injected fish by 15% and 18% respectively. The hormone treatment did not significantly alter serum electrolyte levels among the 33‰ control groups.

Cortisol and vasotocin injection did not significantly change the muscle moisture content in seawater-adapted sea bream (Fig. 6.4). Among the 6‰-acclimated fish,

muscle water content of fish injected with cortisol was significantly higher than those injected with oil. In comparison to seawater treatment groups, muscles were more hydrated in 6‰-adapted fish injected with either cortisol or vasotocin. Hematocrit was not significantly changed after low salinity exposure or hormone treatments, although there was an apparent decrease in cortisol-injected fish acclimated to 6‰ (Fig. 6.5).

6.3.2 Relative expression of AQP-1 in intestine, gills and kidney, and hypothalamic pro-vasotocin

Administration of cortisol significantly upregualated the mRNA expression of AQP-1 in the intestine of fish acclimated to 33‰ and 6‰, with the upregulation being nearly two-fold higher than the oil control group (Fig. 6.6). There was no significant difference in abundance of AQP-1 transcript between vasotocin treatment and control treatment in both 33‰- and 6‰-acclimated fish. In addition, the AQP-1 expression levels were comparable between 33‰- and 6‰-acclimated fish with the same hormone treatment.

The branchial expression levels of AQP-1 transcript were not significantly different among different treatment groups adapted to the same salinity (Fig. 6.7). In

the cortisol-injected groups, transcript abundance of AQP-1 was higher in gills of 33‰-adapted fish than those of 6‰-adapated fish.

Hormone treatment did not significantly change the expression of AQP-1 in kidney of silver sea bream (Fig. 6.8). Similarly, the expression levels of hypothalamic provasotocin were not significantly different in various hormone treatment groups, although higher levels were found in the 33‰-acclimated fish (Fig. 6.9).

6.4 Discussion

6.4.1 Effect of hormones on serum cortisol and serum electrolyes, muscle moisture and hematocrit

6.4.1.1 Serum cortisol

The concentration of serum cortisol of oil-injected control and vasotocin-injected fish were comparable to the control groups of a cortisol experiment on silver sea bream reported by Deane et al. (2000). The serum cortisol concentrations of saline-injected and untreated sea bream were relatively low (less than 20 ng/ ml), but the concentration was 11-fold higher after cortisol injection (Deane et al. 2000). Administration of cortisol in the present study caused the silver sea bream to develop a

similar state of hypercortisolemia and the present results had shown a single dose of slow-releasing cortisol implant could maintain high concentrations of serum cortisol throughout the experiment period of six days.

There is no significant modification of serum cortisol concentrations of silver sea bream after vasotocin administration. Previous study on gilthead sea bream indicated that vasostocin implant did not increase plasma cortisol levels in seawater fish but the levels were elevated after transfer to higher or lower salinities (Sangiao-Alvarellos et al., 2006). The authors suggested vasotocin and osmotic stimulation may exert additive or synergic effect on plasma cortisol levels. This action of vasotocin on circulatory cortisol levels was not observed in the silver sea bream within the six-day experimental period, further investigation with a shorter time interval is necessary to elucidate this transient action of the hormone.

6.4.1.2 Serum electrolytes and muscle moisture

Although cortisol has been regarded as a seawater acclimating hormone for its action on salt secretion in hyperosmotic salinities, many studies also suggested a dual role for cortisol in the uptake of salt when interacting with prolactin during low salinity

acclimation (McCormick, 2001; Mancera et al., 2002). In fact, cortisol was maintained at elevated levels for days to weeks after the transfer of several marine teleosts from high to low salinity (Mancera et al., 1994). Eckert et al. (2001) reported that prolactin and cortisol interact to restore ion balance of hypophysectomized channel catfish (*Ictalurus punctatus*) acclimated to fresh water and isosmotic salinity.

Curiously, injection of chronic level of cortisol in the present study produced an effect that resembled its classical hypo-osmoregulatory role in that a deepened drop of serum Na^+ and Cl^- occurred after silver sea bream were abruptly acclimated to hypo-osmotic salinity. Indeed, significant drop in plasma osmolality had been reported in freshwater adapted brook trout after cortisol injection (Weisbart et al., 1987). Administration of cortisol did not further modify the concentrations of serum electrolytes in seawater acclimated silver sea bream, which were similarly observed in seawater acclimated fish (Deane et al., 2000; Singer et al., 2003). The results of muscle moisture content followed the trend of serum electrolytes levels with highest muscle water content in 6‰-transferrred fish injected with cortisol. These results indicated that elevation of cortisol alone may not be sufficient to activate the reverse mechanism to operate hyperosmoregulation in fish and a coupled magnitude of increase in circulatory prolactin is likely responsible to mediate the dual response of cortisol

during low salinity acclimation.

Vasotocin did not significantly modify the serum electrolyte concentration or muscle moisture content of silver sea bream transferred to 33‰ or 6‰. These results concur with the unaltered levels of plasma Na^+ and osmolality reported in gilthead sea bream exposed to either low salinity or seawater after vasotocin administration (Sangiao-Alvarellos et al., 2006). In fact, vasotocin plays an important role in fish osmoregulation by increasing drinking rates, lowering urine production during seawater adaptation (Kulczykowska, 2007) as well as alterations in branchial and renal Na^+-K^+-ATPase activity during salinity acclimations (Sangiao-Alvarellos et al., 2006). More work is necessary to elucidate the precise action of vasotocin on electrolyte and water balance in fish living in different osmotic conditions.

6.4.2 Effect of cortisol on AQP-1 expression

Our results showed that cortisol significantly stimulated AQP-1 transcript abundance in intestine of silver sea bream. The cortisol-mediated increase in AQP-1 expression in 6‰-transferred fish was comparable to the extent of increase in 33‰ control groups though high intestinal water absorption was unnecessary during

hypo-osmotic adaptation. Similarly, Martinez et al. (2005b) reported a three-fold increase of AQP-1 expression in epithelial scrapes of intestine of freshwater-acclimated yellow eels (*Anguilla anguilla*) after cortisol implantation. Results of the present study suggested that cortisol may act as a regulator for AQP-1 transcription process in intestine.

In fact, a number of studies revealed the effect of cortisol on intestinal ion and water transport in fish at the physiological level (Cornell et al., 1994; Epstein et al., 1971; Gaitskell & Jones, 1970; Hirano & Utida, 1968, 1971; Hirano et al., 1976; Hirano et al., 1976). The fluid uptake rate across the posterior intestine of Atlantic salmon *Salmo salar* was roughly doubled by cortisol treatment during post-smolt stage (Cornell et al., 1994). Injection of cortisol into freshwater-adapted eels mimicked changes in intestinal water movement during seawater transfer with elevation in net absorption of monovalent ions and water through the intestine (Epstein et al., 1971; Gaitskell & Chester Jones, 1970; Hirano & Utida, 1968, 1971; Hirano et al., 1976). Cortisol-mediated upregulation of AQP-1 transcript in the present study may substantially relate to the cortisol-enhanced intestinal water transport observed in these fishes.

The present study was the first attempt to study effect of cortisol on branchial

AQP-1 expression levels in teleosts acclimated to 6‰ and 33‰. Contrary to its significant effect on intestine, cortisol administration did not induce significant changes in AQP-1 expression levels in gills of silver sea bream. Physiological study had shown injection of cortisol alone could increase water influx and water permeability in isolated gills of Japanese eel, the end result is counteractive to the effect of prolactin observed in the same experiment (Ogawa, 1975). However, cortisol had been shown to cooperate with prolactin to maintain stable osmotic conditions (Eckert et al., 2001; Mancera et al., 2002) to promoting ions uptake and surface area of chloride cells in gills of several teleost species during low-salinities acclimation (Perry et al., 1992). Lack of clear influence in cortisol observed in the present study may be overshadowed by the putative interaction between cortisol and prolactin.

Interestingly, AQP-1 mRNA expression level in kidney was not stimulated by cortisol, in contrast to the cortisol-mediated renal AQP-1 upregulation observed in European eels (Martinez et al., 2005a). It is premature to give solid explanation to this disparity as the function of cortisol may rely on abundance of its receptors or interaction with other osmoregulatory hormones, such as growth hormone and prolactin.

6.4.3 Effect of vasotocin on AQP-1 expression

The present study failed to detect significant changes in AQP-1 transcript abundance in all osmoregulatory organs tested. In addition, levels of hypothalamic pro-vasotocin expression were not sensitive to administration of cortisol and vasotocin. Osmoregulatory actions of vasotocin are generally considered to be more rapid in fish (McCormick, 2002), the six-day hormone acclimation period would be too long to detect the transient action of injected vasotocin. Upon hypo-osmotic exposure, immediate down-regulation of AQP-1 transcript in gills of silver sea bream was observed and this could be a consequence of rapid hormonal regulation. Sampling within short time intervals after vasotocin injections would be necessary to observe the possible immediate action of vasotocin in the osmoregulatory organs of silver sea bream. Besides, although administration of vasotocin in slow-releasing oil vehicle had successfully elevated the plasma levels of vasotocin in gilthead sea bream (Sangiao-Alvarellos et al., 2006), the efficacy of this vehicle for vasotocin in the present study is largely unknown as the plasma levels of the hormone was not monitored. In future experiment, the plasma vasotocin levels in the treated fish should be checked by quantitative radioimmunoassay and the experiment can be repeated with daily injections of vasotocin in saline as an alternative method of hormone

administration.

6.5 Conclusions

Administration of cortisol exerted pronounced effect only on intestinal AQP-1 expression in the sea bream, regardless of ambient salinities. Nonetheless, intestinal water absorption is tightly coupled with active ion transport and the cortisol-stimulated water absorption could be facilitated independently or synergistically by cortisol-modulated ion transport and/or aquaporin abundance, precise physiological study on these pathways is necessary to elucidate the regulatory process of water transport in intestine of fish. Study of synergic effect of cortisol with other osmoregulatory hormones at the transcriptional and physiological levels is recommended to obtain the complete picture of the dual roles of cortisol in hyper- and hypo-osmoregulatory processes. AQP-1 mRNA expression in major osmoregulatory organs of silver sea bream was not influenced by administration of vasotocin for six day in either 6‰ or 33‰ regimes. Re-examination of impact of vasotocin injection with a shorter exposure time is essential to reveal its immediate role on osmoregulatory functions, in particularly its effect on modulation of aquaporin availability.

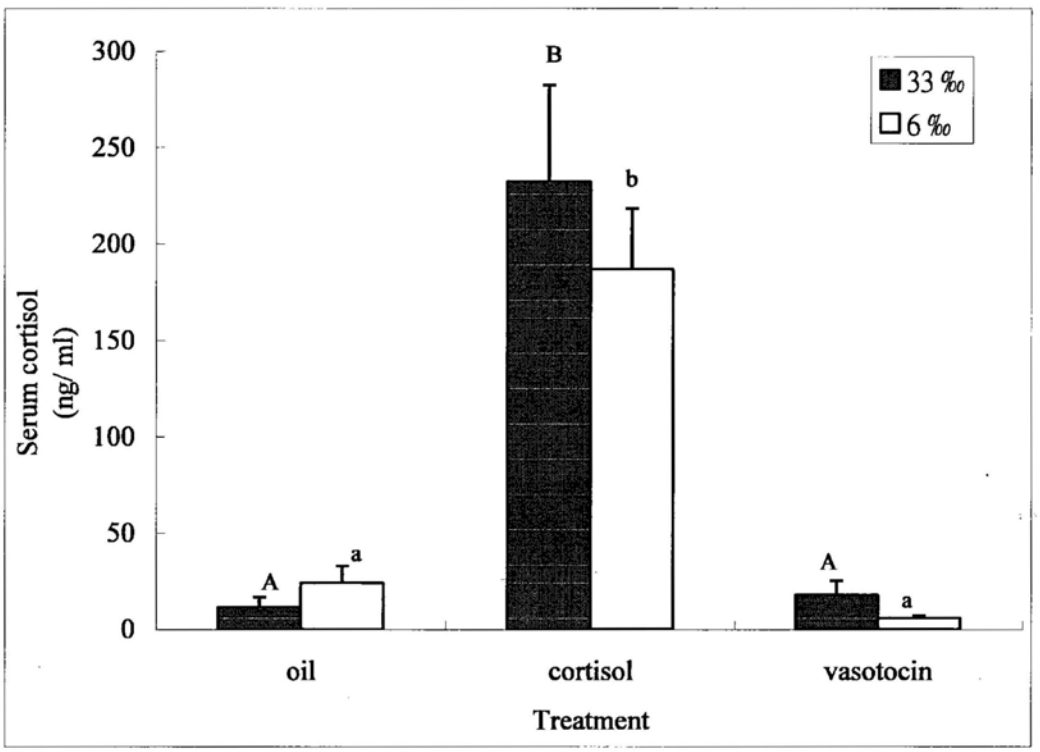

Figure 6.1. Changes in serum cortisol of 33 ‰- and 6 ‰-adapted sea bream after administration of oil alone, cortisol (50 μg/ g) or vasotocin (1 μg/ g) mixed with oil (n=7). Capital letters denote significant differences among the 33 ‰ groups. Small letters denote significant differences among different treatments in the 6 ‰ groups (One way ANOVA, Tukey's test, $p<0.05$). Values are means ± S.E.M.

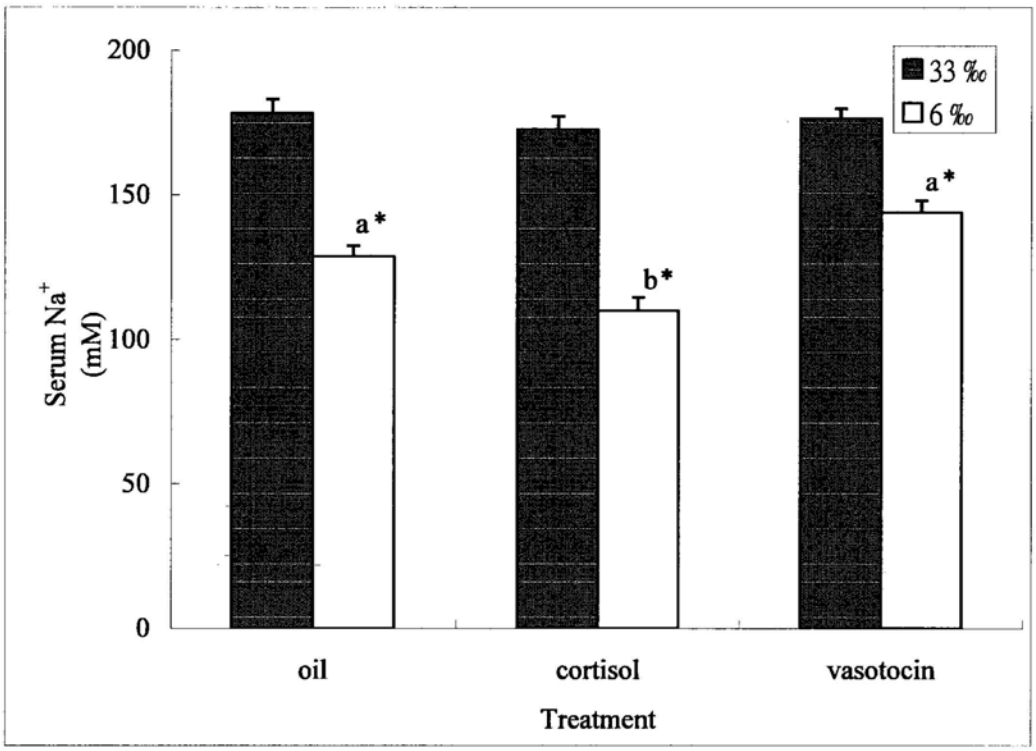

Figure 6.2. Changes in serum Na^+ of 33 ‰- and 6 ‰-adapted sea bream after administration of oil alone, cortisol (50 μg/ g) or vasotocin (1 μg/ g) mixed with oil (n=7). No significant difference was found among the 33 ‰ groups. Small letters denote significant differences among different treatments in the 6 ‰ groups. * Denote significant differences between 33‰- and 6‰-adapted sea bream with all treatments (One way ANOVA, Tukey's test, $p<0.05$). Values are means ± S.E.M.

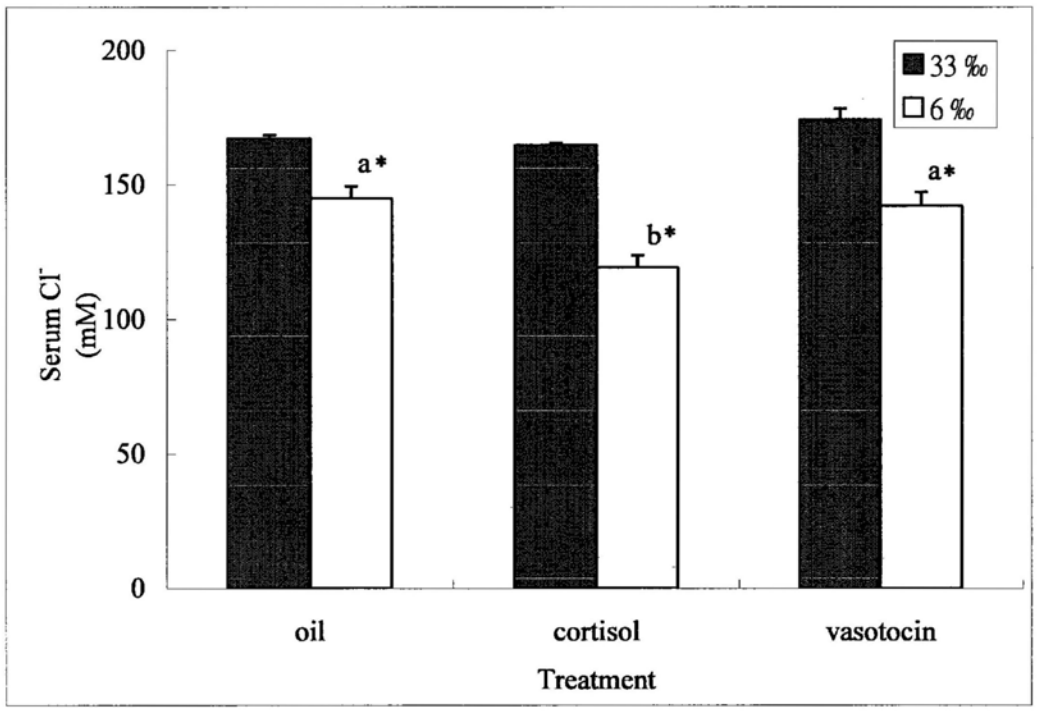

Figure 6.3. Changes in serum Cl^- of 33‰- and 6‰-adapted sea bream after administration of oil alone, cortisol (50 μg/ g) or vasotocin (1 μg/ g) mixed with oil (n=7). No significant difference was found among the 33 ‰ groups. Small letters denote significant differences among different treatments in the 6 ‰ groups.

* Denotes significant differences between 33‰- and 6‰-adapted sea bream with all treatments (One way ANOVA, Tukey's test, $p<0.05$). Values are means ± S.E.M.

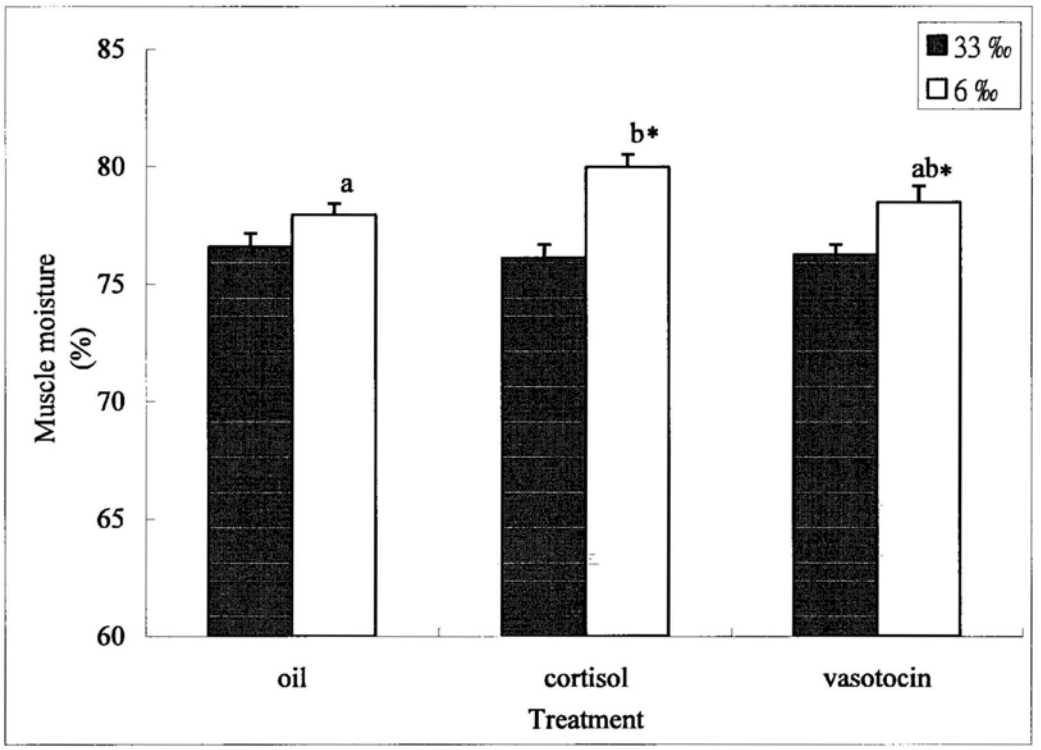

Figure 6.4. Changes in muscle moisture of 33‰- and 6‰-adapted sea bream after administration of oil alone, cortisol (50 μg/ g) or vasotocin (1 μg/ g) mixed with oil (n=7). No significant difference was found among the 33 ‰ groups. Small letters denote significant differences among different treatment in the 6 ‰ groups. * Denotes significant difference between 33‰- and 6‰-adapted sea bream with the same treatment (One way ANOVA, Tukey's test, $p<0.05$). Values are means ± S.E.M.

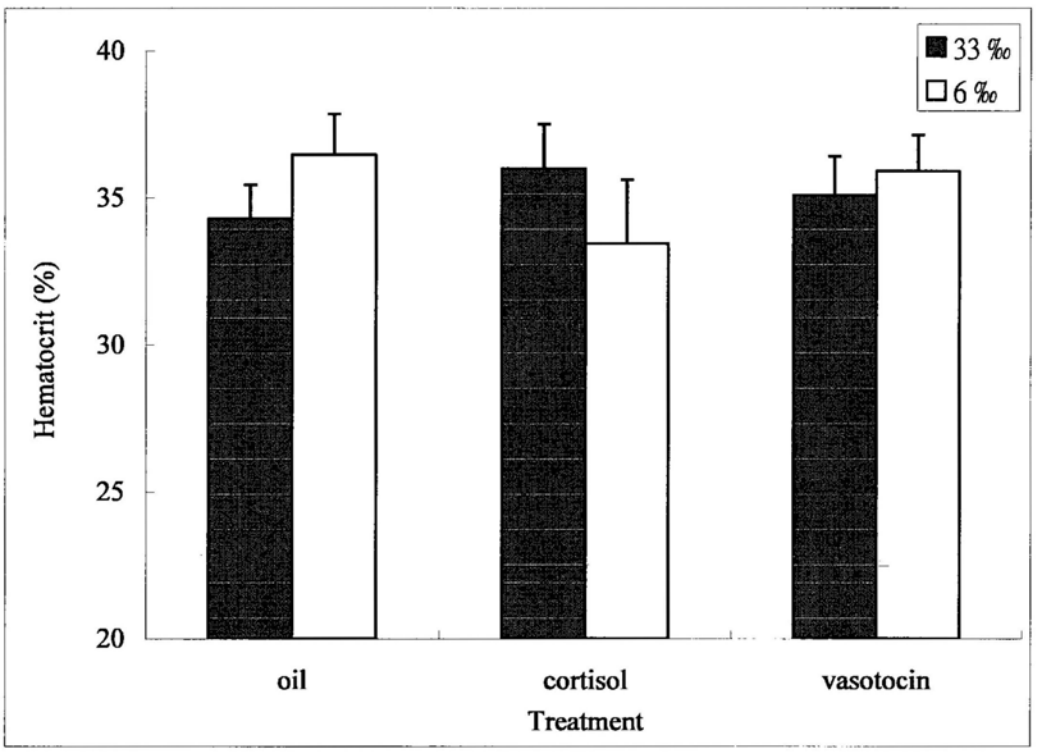

Figure 6.5. Changes in hematocrit of 33‰- and 6‰-adapted sea bream after administration of oil alone, cortisol (50 μg/ g) or vasotocin (1 μg/ g) mixed with oil (n=7). No significant difference was found among different salinity groups and hormone treatments (One way ANOVA, Tukey's test, $p>0.05$). Values are means ± S.E.M.

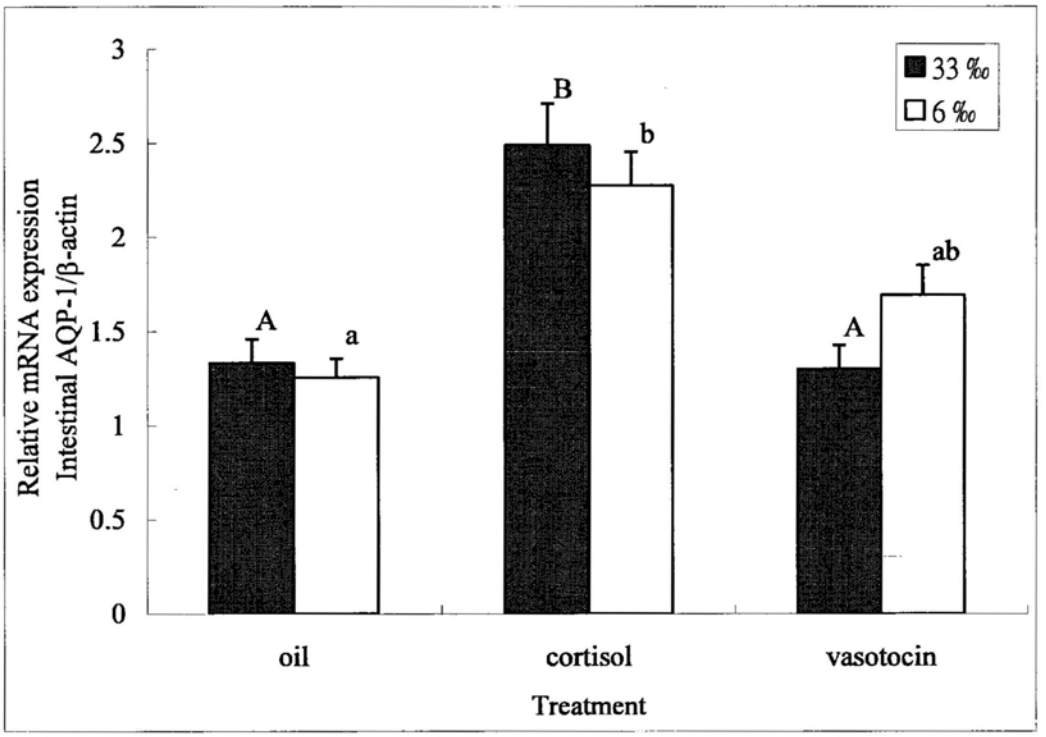

Figure 6.6. Expression of AQP-1 in intestine of 33 ‰- and 6 ‰-adapted sea bream after administration of oil alone, cortisol (50 μg/ g) or vasotocin (1 μg/ g) mixed with oil (n=7). Capital letters denote significant differences among the 33 ‰ groups. Small letters denote significant differences among different treatment in the 6 ‰ groups. No significant difference was found between 6‰- and 33 ‰-acclimated fish with the same treatment (One way ANOVA, Tukey's test, $p<0.05$). Expression was expressed as a ratio of AQP-1 and β-actin expression (n=7). Values are means ± S.E.M.

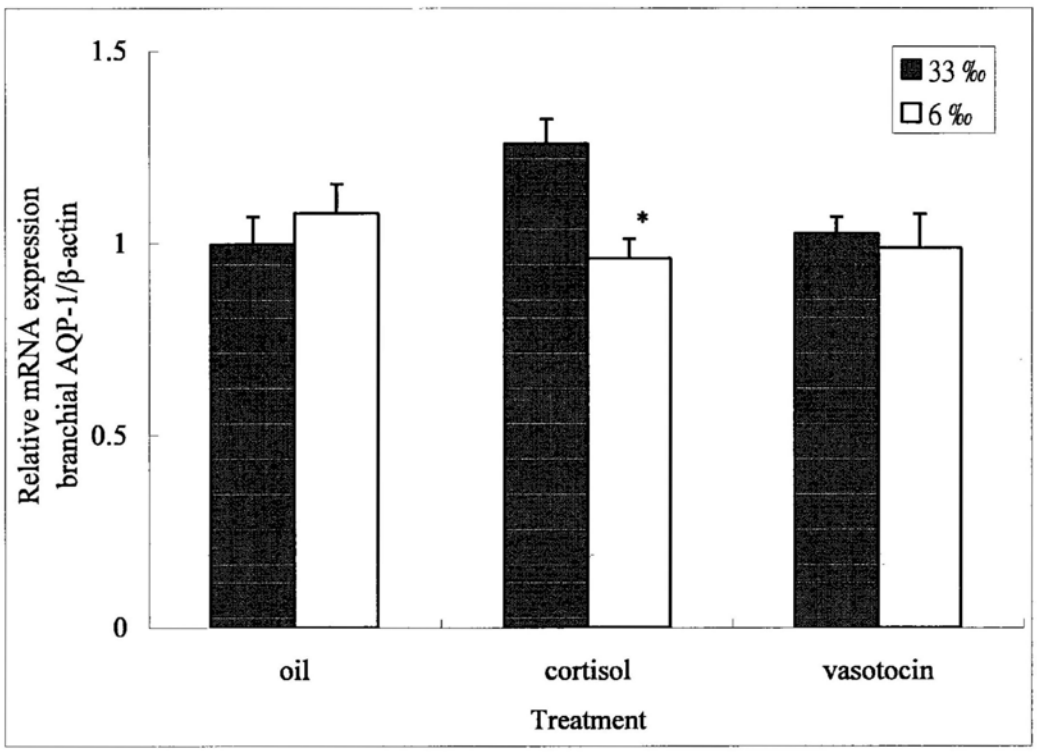

Figure 6.7. Expression of AQP-1 in gills of 33 ‰- and 6 ‰-adapted sea bream after administration of oil alone, cortisol (50 μg/ g) or vasotocin (1 μg/ g) mixed with oil (n=6). No significant difference was found among different hormone treatment groups. * Denotes significance between 6‰- and 33 ‰-acclimated fish in the same treatment groups (One way ANOVA, Tukey's test, $p>0.05$). Expression was expressed as a ratio of AQP-1 and β-actin expression (n=7). Values are means ± S.E.M.

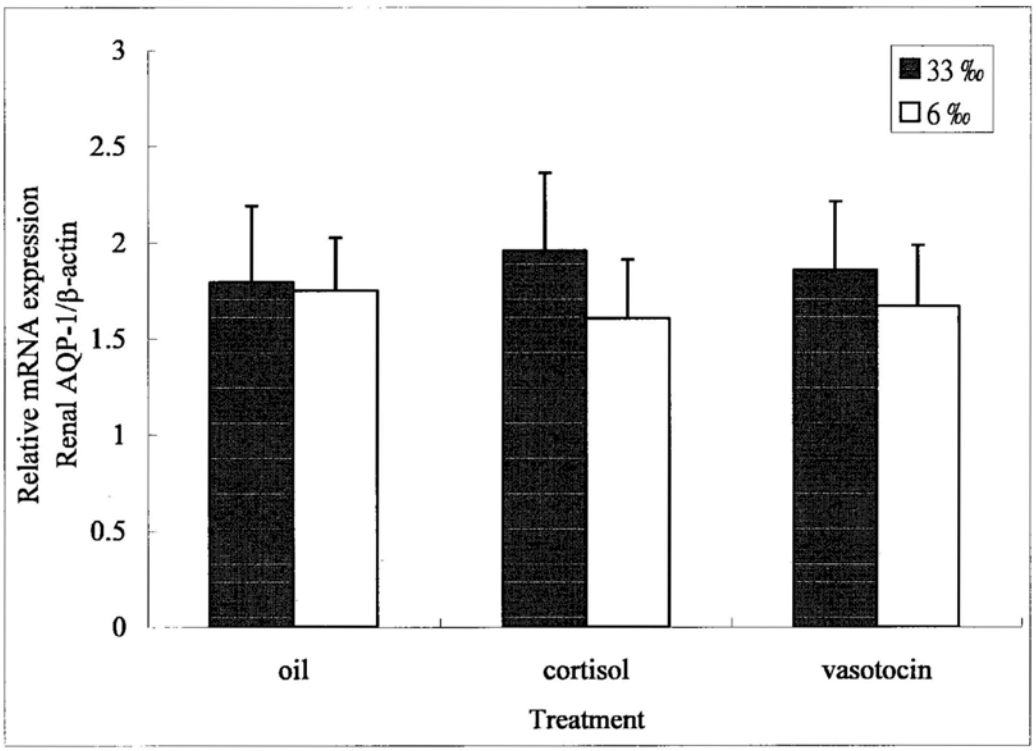

Figure 6.8. Expression of AQP-1 in kidney of 33 ‰- and 6 ‰-adapted sea bream after administration of oil alone, cortisol (50 μg/ g) or vasotocin (1 μg/ g) mixed with oil (n=4). No significant difference was found between 6‰- and 33 ‰-acclimated fish and among different treatment groups (One way ANOVA, Tukey's test, $p>0.05$). Expression was expressed as a ratio of AQP-1 and β-actin expression. Values are means ± S.E.M.

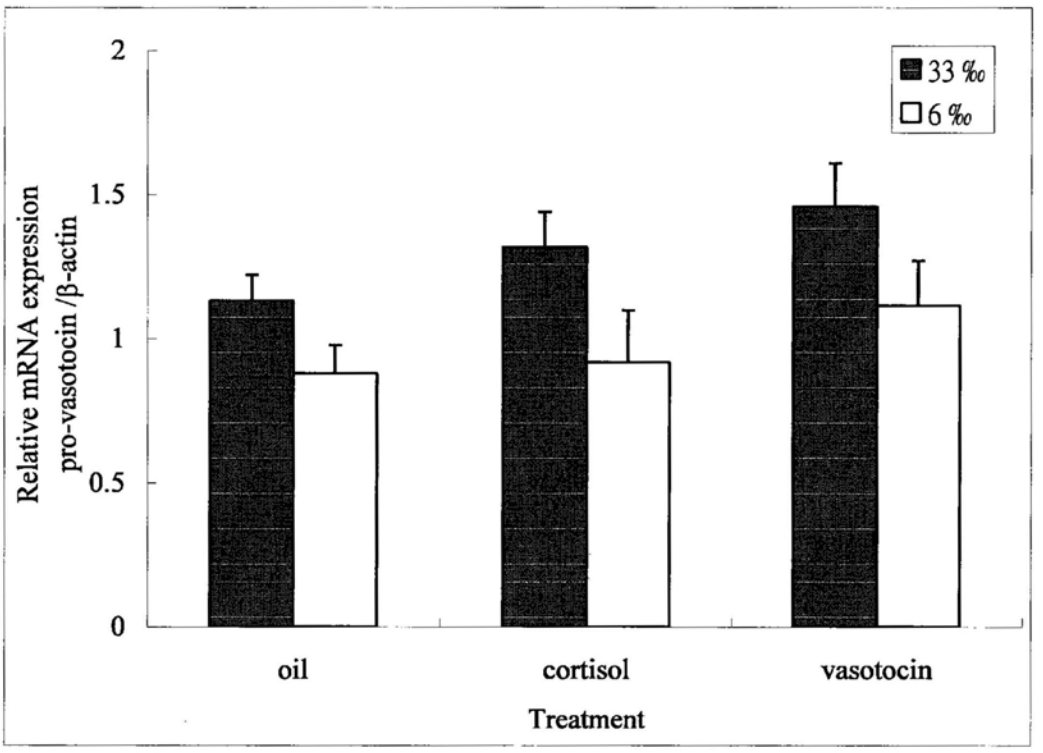

Figure 6.9. Expression of pro-vasotocin in hypothalamus of 33 ‰- and 6 ‰-adapted sea bream after administration of oil alone, cortisol (50 μg/ g) or vasotocin (1 μg/ g) mixed with oil (n=6). No significant difference was found between 6‰- and 33 ‰-acclimated fish and among different treatment groups (One way ANOVA, Tukey's test, $p>0.05$). Expression was expressed as a ratio of pro-vasotocin and β-actin expression. Values are means ± S.E.M.

Chapter 7

General Conclusions

The present study offered insights into the role of aquaporins in osmoregulation of silver sea bream *Sparus sarba*. Influences of environmental salinity perturbations and hormones on AQP-1 expressions and possible correlation between aquaporins and pro-vasotocin synthesis were seriously discussed. A schematic diagram summarizing the main findings of the present study is shown in Fig. 7.1.

By applying mammalian aquaporin anti-sera and immunolocalisation techniques, AQP-1 and AQP-3 were localized in major osmoregulatory organs of the silver sea bream. Both AQP-1 and AQP-3 were predominantly immunolocalised in the chloride cells of the gills. The results concurred with previous studies for these AQPs in gills of other fish (Brunelli et al., 2009; Lignot et al., 2002), however, the hypothesized role for these AQPs in cell volume regulation still requires more functional evidences to justify. In kidney, AQP-1 immunoreactivity was detected in the apical membrane of certain subset of renal tubules. The differential localization of the aquaporin may explain the disparity of water permeability among various regions of the fish nephron. Identification of the type of tubular segments with AQP-1 immunoreactivity would give us better understandings in the correlation between AQPs and water transport across apical membrane in particular tubular segment of marine teleosts. In addition, immunoreactivity of AQP-1 and AQP-3 was located at the basal side of enterocytes of

the sea bream intestines, and the presence of these intestinal aquaporins may contribute to the movement of water molecules between the enterocytes and the blood. The basal localization of AQP-1 immunoreactivity in the intestinal epithelium of sea bream is dissimilar to the apical localization of AQP-1 as observed in other teleosts (Aoki et al., 2003; Raldua et al., 2008). The discrepancy could be due to detection of fish AQP-1 isoforms or other fish AQP homologues that share similar epitopes with human AQP-1, and readily detected by the polyclonal human AQP-1 antiserum. This finding raises considerable interest to for further investigations on this basolaterally located AQP water channels in the enterocytes in the sea bream.

The current experiment also characterized the homologue of AQP-1 and pro-vasotocin in the silver sea bream. By using cloning procedures, a 774-bp cDNA of AQP-1 was isolated from the silver sea bream and the deduced amino acid sequence shared highest identity to that of AQP-1a of gilthead sea bream. Although the silver sea bream AQP-1 appears to possess the major features of a functional aquaporin, functional analysis should be performed to determine the water carrying capacity of this AQP water channel. In addition, a partial cDNA clone of pro-vasotocin was isolated from the hypothalamus of silver sea bream. The nucleotide sequence of the

partial clone is highly similar to those of teleostean pro-vasotocin and they share substantial similarity with pro-isotocin of fishes.

Semi-quantitative RT-PCR analysis was deployed to reveal the influences of salinity variations on AQP-1 and pro-vasotocin expression in the silver sea bream. There is no mortality during the acclimation period and serum electrolytes and muscle water content were only slightly altered in silver sea bream following abrupt hypo-osmotic transfer or when fish were acclimated to six different salinities that ranged from 0‰ (freshwater) to 70‰ (hypersaline). This reflected the extreme euryhalinity of silver sea bream irrespective of its ecologically stenohaline life cycle.

Intestinal AQP-1 transcription was found to be highest in fish acclimated to strongly hyper-osmotic (70‰) salinity, however, an observable elevated level was detected in fish acclimated to isosmotic salinity (12‰). Higher intestinal expression of AQP-1 mRNA in isosmotic salinity contradicts the findings of lower osmoregulatory effort at this salinity reported in our previous studies (Deane & Woo, 2004; Kelly et al., 1999), the inconsistency of which may be due to differential expression responses in various parts of the intestine. Additional works are needed on elucidating the AQP expression profile of different parts of the gut.

In gills, adaptation to freshwater for four weeks resulted in a statistically significant increase of AQP-1 mRNA expression in the silver sea bream. This elevated AQP-1 expression may facilitate basolateral water transport to the serosal fluid to prevent the chloride cells from swelling during hypo-osmotic adaptation. In contrast to long-term salinity acclimation, the branchial AQP-1 expression was initially decreased after abrupt 6‰ exposure. This downregulation is coherent with some observations in fish with inhibited branchial water transport after similar low salinity transfer (Ogasawara & Hirano, 1984; Gallis et al., 1979). The results suggested that initial downregulation of branchial AQP-1 transcription may serve as a temporary strategy to prevent further water gain during abrupt hypo-osmotic adaptation, and to resume its volume regulatory role in the long run. In order to prove this hypothesis, measurements of gill water permeability should be performed in sea bream after abrupt and long-term hypo-osmotic adaptation. There is no significant difference of AQP-1 expression level detected in kidney of silver sea bream in the two salinity acclimation experiments. These insignificant results should be interpreted cautiously due to relatively high variations of the data obtained from this organ, which may be partly due to perfusion procedures performed on the fish in the present study. More studies have to be done before any firm conclusions can be drawn regarding the role of AQP-1 in the kidney of sea bream.

In the hormone treatment experiment, cortisol injections markedly upregulated intestinal AQP-1 transcription in both hypo-osmotic and hyperosmotic salinities, however, administration of vasotocin did not produce any alterations in the transcription level of AQP-1 in both salinity groups. The data had shown that cortisol is a potent stimulator of AQP-1 transcription in intestine, the result is coherent with the stimulatory effect of cortisol on AQP-1 expression in other teleosts (Aoki et al., 2003; Martinez et al., 2005b). Indeed, cortisol can interact with other hormones to exert its specific action on hyper- or hypo-osmoregulation in fish. The present study and those previous experiments on fish had only focused on the single effect of cortisol on the AQP-1 expression. Further research on the influence of other osmoregulatory hormones and possible interaction between cortisol and these hormones are essential to portrait the complete picture of AQP-1 regulation in fish.

Lastly, abundance of hypothalamic pro-vasotocin transcript was significantly downregulated in 0‰-acclimated sea bream and this decreased expression level was parallel to the downregulation of AQP-1 mRNA expression in the same group of fish. Although An et al. (2008) also reported a correlation between expression of vasotocin receptors and AQP-1 expression in the black porgy, the linkage of the regulatory action of vasotocin hormone axis with AQP-1 function in these organs is still obscure.

Nonetheless, hypothalamic pro-vasotocin expression levels did not significantly change after abrupt 6‰ transfer. Similarly, vasotocin administration did not alter AQP-1 mRNA expressions in intestine, gills and kidney after a similar hypo-osmotic acclimation. Taking these results together, pro-vasotocin synthesis and perturbations in circulating vasotocin levels may not directly influence AQP-1 expression and function, instead, the effect of the hormone on AQP-1 possibly occurs througb the adjustment of the abundance of the vasotocin receptors in the target organs. Hence further experiments are required to find out the possible relationship between these receptors and AQP-1 function.

In conclusion, the data of the present study demonstrate the sea bream AQP-1 is likely to be a functional water channel and performs an osmoregulatory role in the intestine and gills during adaptation to different salinities. Cortisol is a key hormone to stimulate AQP-1 synthesis at least in the intestine, while the actions of other osmoregulatory hormones on AQP-1 expressions, though not having been decisively studied, are also of considerable interest in the overall osmoregulatory strategy of euryhaline fish. Expression of pro-vasotocin showed a substantial relationship with AQP-1 synthesis during freshwater adaptation, however, the influence of vasotocin regulatory axis is yet to be confirmed by further experiments.

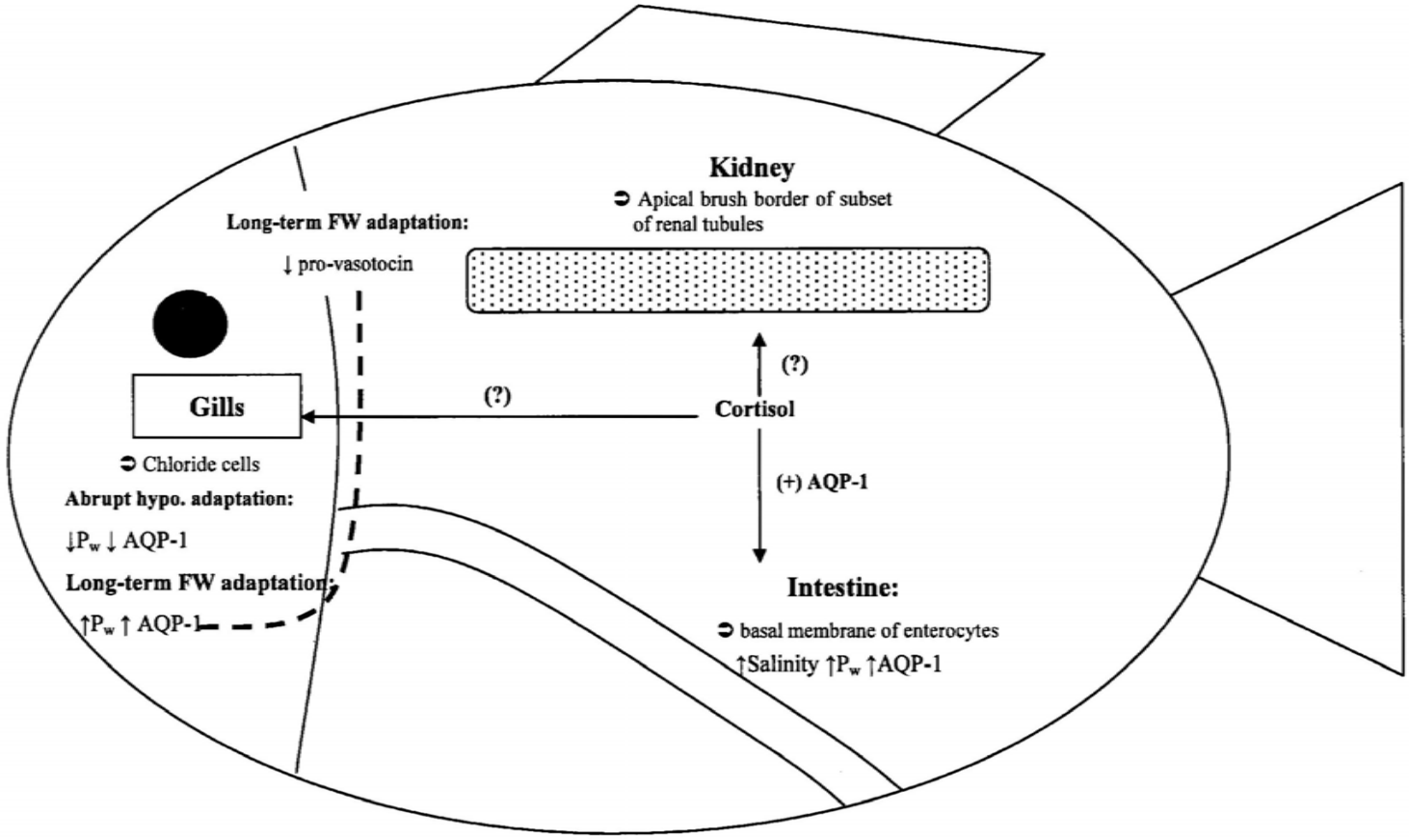

Figure. 7.1 Schematic model of the findings of the present study together with water permeability data from previous studies. Abbreviations and notes: Hypo.: hypo-osmotic; ↑/↓: increase/decrease; Dashed line: possible correlation ; P_w: Water permeability; ➲: localization of AQP-1 in particular tissues.

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