รายงานวิจัยฉบับสมบูรณ์

โครงการ Involvement of osmoregulatory hormones, prolactin and cortisol and their receptor gene expression to salinity in striped snake-head fish (*Channa striata*)

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ชื่อโครงการ ความเกี่ยวข้องของฮอร์โมนโพรแลคตินและคอร์ติซอลและการแสดงออกของยีนตัวรับฮอร์โมนในการตอบสนองต่อความเค็มในปลาช่อน

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บทคัดย่อ

ปลาช่อนเป็นปลาน้ำจืดที่สามารถอาศัยอยู่ได้ในแหล่งน้ำที่มีความเค็ม ซึ่งกลไกที่ช่วยในการปรับตัวของปลาช่อนนี้ยังไม่เป็นที่ทราบอย่างแน่ชัด ซึ่งการศึกษาในครั้งนี้ ได้ทำการศึกษาผลเฉียบพลันของความเค็มต่อระดับฮอร์โมนโพรแลคติน การแสดงออกของยีนตัวรับฮอร์โมนโพรแลคตินในอวัยวะที่ทำหน้าที่ควบคุมเกลือแร่และน้ำรวมถึงผลทางเคมีของเลือด โดยทำการย้ายปลาช่อนจากน้ำจืดลงไปเลี้ยงในน้ำเค็มที่ระดับ 2 5 10 15 20 และ 35 ส่วนในพันส่วน (‰) เป็นเวลา 1 ชั่วโมง พบว่าปลาช่อนสามารถมีชีวิตรอดได้ ยกเว้นที่ระดับ 35‰ ค่าออสโมลาริตีและค่าไอออนในเลือดเพิ่มขึ้นตามระดับความเค็มที่สูงขึ้น ปลาในกลุ่มที่ความเค็ม 10 15 และ 20‰ พบว่าระดับฮอร์โมนคอร์ติซอลและระดับน้ำตาลในเลือดเพิ่มขึ้น รวมทั้งมีภาวการณ์สูญเสียน้ำออกจากกล้ามเนื้อ ระดับฮอร์โมนโพรแลคตินลดลงในปลากลุ่ม 20‰ การแสดงออกของยีนตัวรับฮอร์โมนโพรแลคตินในเหงือก ไต และหลอดอาหาร พบว่าลดลงทุกกลุ่มความเค็ม นอกจากนี้เรายังทำการศึกษาการตอบสนองของปลาช่อนในน้ำที่มีภาวะไฮเปอร์ออสโมติกต่อสารน้ำในร่างกายของปลาช่อน โดยทำการย้ายปลาช่อนที่มีการปรับตัวในน้ำจืด (FW) ลงไปในน้ำเค็มที่ระดับ 10‰ (SW) กลุ่มควบคุมคือ FW–FW และในทางกลับกันทำการย้ายปลาช่อนที่ได้รับการปรับตัวที่ SW ลงไปใน FW กลุ่มควบคุมคือ SW–SW ในกลุ่ม FW–SW พบว่าระดับฮอร์โมนคอร์ติซอล น้ำตาล และแลคเตท ในเลือดเพิ่มขึ้นอย่างรวดเร็วแล้วลดลง แต่หลังจากนั้นระดับฮอร์โมนคอร์ติซอลเพิ่มขึ้นอีกครั้งซึ่งสัมพันธ์กับการเพิ่มขึ้นของกิจกรรมเอนไซม์ Na^+/K^+-ATPase ส่วนระดับฮอร์โมนโพรแลคตินลดลงในวันสุดท้ายของการทดลอง (วันที่ 15) ระดับการแสดงออกของยีนตัวรับฮอร์โมนโพรแลคตินลดลงในทุกอวัยวะที่เกี่ยวข้องกับการควบคุมเกลือแร่และน้ำ ส่วนปลาช่อนในกลุ่ม SW–FW พบว่าระดับน้ำตาลและคอร์ติซอลเพิ่มขึ้นในช่วงแรก ส่วนระดับฮอร์โมนโพรแลคตินไม่เปลี่ยนแปลง แต่ระดับการแสดงออกของยีนตัวรับฮอร์โมนโพรแลคตินเพิ่มขึ้นจากกลุ่มควบคุม ค่าออสโมลาริตีของเลือดลดลงแต่ค่ากิจกรรมของเอนไซม์ Na^+/K^+-ATPase ไม่เปลี่ยนแปลงเมื่อเปรียบเทียบกับกลุ่มควบคุม ดังนั้นสามารถสรุปได้ว่า การเปลี่ยนแปลงความเค็มของน้ำโดยเฉพาะในกลุ่ม FW–SW จะกระตุ้นต่อการตอบสนองต่อความเครียดโดยจะไปกระตุ้นการหลั่งของฮอร์โมนคอร์ติซอล ซึ่งนำไปสู่การเพิ่มระดับน้ำตาลและแลคเตทในเลือด เพื่อใช้เป็นแหล่งพลังงานให้กับเอนไซม์ Na^+/K^+-ATPase เพื่อช่วยรักษาระดับไอออนของน้ำในร่างกาย

คำหลัก: คอร์ติซอล; กิจกรรมเอนไซม์ Na^+/K^+-ATPase; ความเค็ม;

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Abstract

Snakehead (*Channa striata*), a freshwater stenohaline fish, is capable of living in natural water sources with salinity fluctuation and osmotic stress despite unclear osmoregulatory mechanism. Herein, the acute effect of salinity changes on prolactin level and its receptor expression in osmoregulatory organs including blood chemistry were investigated by transferring snakehead fish into water with various salinities of 2, 5, 10, 15, 20 and 35 per mill (‰) for 1 h. It was found that fish could survive for 1 h in ≤ 20‰, but not 35‰ salinity. The plasma osmolality and ionic concentrations increased positively with environment salinities. Exposure to hyperosmotic condition (10, 15, 20‰ salinities) was found to elevate plasma cortisol and glucose levels, while inducing muscle dehydration. Plasma prolactin level decreased in 20‰. The relative expressions of prolactin receptor of saltwater-treated group to freshwater group in gill, kidney and esophagus were <1. We also demonstrated time-dependent responses in fish after being transferred from fresh water (0‰) to salt water (10‰), FW—SW and vice versa. In FW—SW, the plasma cortisol level, which was indicative of stress response, was rapidly increased together with increases plasma glucose and lactate levels. Interestingly, the plasma cortisol returned to the baseline after prolonged exposure, followed by the second peak that was postulated to enhance the Na^+/K^+-ATPase activity. Prolong exposure fish caused prolactin level dropped at the end of the experiment when compare to FW—FW. The expression of prolactin receptor was also down in all osmoregulatory organs. Moreover, SW—FW also led to transient increases in glucose and cortisol levels. The plasma prolactin unchanged, however its receptor was up regulated in all osmoregulatory organs. Although the plasma osmolality was decreased in SW—FW, Na^+/K^+-ATPase activity was not altered compared to SW-adapted fish. It could be concluded that salinity change, especially FW—SW, induced a stress response and hence cortisol release, which might, in turn, help increase plasma glucose and lactate to energize Na^+/K^+-ATPase to maintain ion concentrations in the body fluid.

Keywords: cortisol; Na^+/K^+-ATPase activity; salinity

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La-iad Nakkrasae

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Part I

Introduction and Objectives

Many million years ago, Northeastern Thailand was submerged under the sea. The present landscape is dominated by an extensive outcrop of Mesozoic rock, the Maha Sarakham formation, which covers about one third of Khorat, plateau is divided into two depositional basins by the mountain range of Phu Phan: the Sakon Nakhon basin in the north and the Khorat basin in the south. Due to its geological history, the region contains underground rock salt (NaCl), which plays a major role in the distribution of saline soil scattered throughout the region. The level of salt surface soil is related to the dispersal of dissolved rock salt underground, resulting in variable levels of salinity in surface water bodies and in underground water (Rimwanich and Suebsiri, 1984). There are altogether about 2.8 million ha of saline soils in the Khorat basin, which makes up 17% of Northeast Thailand (Arunin, 1992). Salinity has a broad ecological impact on water dwelling flora and fauna, including fish. Salt water can cause reduce establishment, vigour and yield in fisheries. As salinity increases, traditional fish do not grow or have very low productivity.

Snakehead fish *Channa striata* is the most widely distributed economically important members of the genus. In Thailand it is found throughout the country, except in the mountain region. The common snakehead or mudfish is known in Thai language as *Pla Chon* (Thai: ปลาช่อน). Culture of snakehead has expanded rapidly and mostly takes place in the central and eastern parts Thailand. Snakehead fish is tolerant of water lacking in dissolved oxygen and can survive without water for a number of months as long as the skin and breathing apparatus are kept moist. The fish can live in water having pH values of 4–5, with desirable range of 6.5–8.5. Positive growth occurs between water temperatures 28–35 °C, whereas optimum temperature is 30–32 °C (Smith, 1945) But no report in how much salinity of water environment which the snakehead fish can stand. In Northeastern parts Thailand, culture of snakehead fish was spared and mostly the fish were found in natural water sources, rivers, lakes, swamps, marshes, canals, ponds and rice farms.

As the primary link between environmental change and physiological response, the neuroendocrine system is a critical part of osmoregulatory adaptation in teleost. Prolactin is

able to promote survival in freshwater of certain hypophysectomized teleosts, mostly cyprinodonts, which require the pituitary gland for freshwater tolerance (Pickford and Phillips, 1959). In *Fundulus heteroclitus, Anguilla anguilla,* and *Poecilia latipinna* prolactin has been shown to ameliorate the fall in blood electrolyte level that follows hypophysectomy in freshwater, and in the first two species it acts to limit the sodium out flux from the body in freshwater, mainly at the gills, although in other fishes (Gasterosteus, *Fundulus kansae*) a renal effect has been demonstrated (Pickford and Phillips, 1959; Manzon, 2002). Prolactin is probably one of several hormones that regulate electrolyte metabolism in freshwater. It has not been found to exert an effect on electrolyte regulation in seawater. Cortisol has been viewed as the seawater-adapting hormone in seawater and mostly interacts with other hormone like growth hormone and prolactin (Evans, 2002). Cortisol under some conditions may promote ion uptake and interacts with prolactin during acclimation to fresh water (Zhou et al., 2003). Current researches indicate that growth hormone promotes acclimation to seawater, prolactin promotes acclimation to fresh water, and cortisol interacts with both of these hormones thus having a dual osmoregulatory function.

In most animals, the majority of cells are not in direct contact with the external environment but are bathed by an internal body fluid. Homeostatic mechanisms hamper changes in an animal's body fluid, which both gives protection from harmful external environments and impedes quick exchange between intracellular compartments. The cells of the animal cannot survive much additional water gain or loss. Water continuously enters and leaves an animal cell across the plasma membrane; however, uptake and loss must balance. Animal cells swell and burst if there is a net uptake of water or shrivel and die if there is a net loss of water. No information on the effect of rock salt on the physiological responses in snakehead fish. Rock salt dissolve in the water will make hyperosmotic environment to the internal body of the fish. So, we would like to demonstrate that the prolactin and cortisol involve ion and water balance in snakehead fish when the fish expose to salt water from rock salt contamination. We also clone receptor genes of both hormones to use as the marker for demonstrating that which tissues of fish affect from both hormones. We also study in long term adaptation of the fish to salt water. The outcomes from this study will know the prolactin and cortisol responses and relative expression of both hormone receptor genes in

various tissues cause of saltwater in snakehead fish. We also get the physiological adaptation data that expose fish in salt water for long period of time.

Objectives

1. To demonstrate the involvement of prolactin and cortisol responses to saltwater exposure in snakehead fish.

2. To clone the prolactin receptor gene in snakehead fish

3. To examine the expression profiles of prolactin receptor in snakehead fish's tissues.

4. To study physiological responses of snakehead fish that raise in salt water.

Part II

Literature review

1 Osmoregulation of fish

1.1 Osmoregulation of freshwater fish

The freshwater teleosts or marine teleosts in freshwater were hyperosmotic to their environment, with Na^+ and Cl^- which are the dominant ions in the plasma (Evans et al., 2005). Because of these osmotic and ionic gradients, freshwater teleosts were over hydrated and salt depleted. They eliminate excess of water by producing large volumes of dilute urine and uptake salt for replacement salt depletion across the gill. The Cl^- uptake not depend on Na^+ uptake by measuring Cl^- uptake from a variety of Cl salt solution (e.g., NaCl, KCl, NH_4Cl and $CaCl_2$) showed no difference (Krogh, 1938). Likewise, the uptake of Na^+ was similar from solutions of NaCl, BaBr, $NaHCO_3$ and $NaNO_3$ (Perry, 1997), demonstrating that Na^+ uptake was independent of Cl^- uptake. The study in frog skin showed that Na^+ uptake is actually via an apical channel, driven by an electrochemical gradient produced by active proton secretion and other works found that Na^+ uptake by the perfuse trout gill was correlated with proton (H^+) (Pickford, 1982). Like the frog skin, the gill epithelium of freshwater fish extrudes H^+ actively which draws in Na^+ via a channel down the electrochemical gradient across the apical membrane (Perry, 1997).

1.2 Osmoregulation of seawater fish

Plasma of marine teleost was hyposmotic to water, with ~60% less NaCl per liter than the marine environment (Evans et al., 2005). Because of these osmotic and ionic gradients, marine teleosts were constantly dehydrated and salt loaded. They excrete a small volume of urine that is approximately isosmotic to the plasma. Marine teleost must ingest the seawater medium to replace the water loss osmotically and excrete the excess salts taken in by diffusion from the hyperosmotic seawater, as well as by drinking. The ingestion rates and then gut fluid and urine contents in eels, anglerfishes and longhorn sculpin were demonstrated (Smith, 1930). The information got from the experiments showed that total ionic content and volume of gut fluids declined in the intestine (~90% and ~70%,

respectively), with a significant reduction of Na^+ and Cl^-, but an increase in Mg^{2+} and SO^{2-}_4 (Butler, 1999). The urine was at most isoosmotic to the plasma, containing nearly the same plasma levels of Na^+ and Cl^-, but higher Mg^{2+} and SO^{2-}_4 concentrations than the plasma, and even higher divalent concentrations than surrounding seawater.

Because the NaCl concentration in the urine was slightly below the plasma concentrations and certainly below seawater concentration, there must be an external pathway for net extrusion of both Na^+ and Cl^-. Gill was proposed as organ secreted of hyperosmotic solution. Later works demonstrated that Cl^- was secretes across the gill in marine teleost by active process. The mechanism for gill extrusion came from the newly discovered Na^+/K^+-ATPase. The enzyme had high activity in fish gill tissue and that the activity was higher in marine species (sculpin, killifish, sea raven, flounder, angler fish) than freshwater (minnow, bass, eel) and increased in the eel gill when that euryhaline species was acclimated to seawater (Jampol and Epstein, 1970). The concentration of Na^+/K^+-ATPase activity with salinity was generally corroborated in subsequent studies, but the euryhaline Asian milkfish and stingray display an inverse pattern (Evans, 2002).

2 Osmoregulatory hormones

2.1 Prolactin

The importance of prolactin in osmoregulation of freshwater fishes was first demonstrated by Pickford and Phillips (Pickford and Phillips, 1959) who showed that hypophysectomized *Fundulus heteroclitus* (killifish) could only survive in freshwater if injected with prolactin. Two subsequent studies found that this prolactin therapy was associated with a reduction in ionic efflux rather than an increase of ionic uptake, suggesting that prolactin reduces branchial permeability rather than stimulates active transport (Potts and Evans, 1967) but hemodynamic changes could have produced the same effects. However, Pickford's group subsequently showed that prolactin injections inhibited branchial Na^+/K^+-ATPase in hypophysectomized killifish in freshwater (Pickford and Griffith, 1970). Prolactin also reduced Na^+/K^+-ATPase activities in the gill of the euryhaline *Chelon labrosus* (Gallis et al., 1979) as well as *Oncorhynchus mykiss* (rainbow trout; (Madsen and Bern, 1992) but had no effect on either the activity or molecular expression of Na^+/K^+-ATPase in the gill of *Salmo trutta* (brown

trout; (Madsen, 1995). Prolactin also did not affect the activity of the enzyme in the gills (and opercular skin) of *Oreochromis mossambicus* (tilapia) acclimated to seawater (Herndon et al., 1991), or chloride cell (cell that role on salt secretion or salt uptake in seawater or freshwater, respective) density, but it did reduce the size of the individual cells significantly. Similar, prolactin-induced reductions in chloride cell size were also demonstrated in *Oreochromis niloticus* (Nile tilapia; (Pisam et al., 1993), and a recent study found that prolactin reduced the chloride cell density in *Sparus sarba* (sea bream; (Kelly et al., 1999). Foskett et al. (’82) had suggested earlier that (in addition to direct inhibition of ionic extrusion) branchial remodeling may be one mode of action of prolactin. This conclusion was based upon parallel changes in active transport and conductance across the killifish opercular skin. The suggestion that prolactin is involved with freshwater, rather than seawater, osmoregulation is supported by a few studies that have demonstrated that plasma levels of prolactin decreased when fish were transferred to seawater (Morgan et al., 1997) or increased when fish were transferred to freshwater (Yamauchi et al., 1991). The complete cDNA sequence of the gene for the fish prolactin receptor has been determined for three species: *O. mossambicus* (Sandra et al., 1995), *Sparus aurata* (sea bream; (Santos et al., 1999), and *Carassius auratus* (goldfish; Tse et al., 2000). Expression of a prolactin receptor has been demonstrated in the gills of goldfish, using RT-PCR/Southern and Northern analyses (Tse et al., 2000) and, more specifically, in the chloride cells of both *S. aurata* (Santos et al., 1999) and *O. mossambicus* (Weng et al., 1997), using homologous and heterologous (mouse) antibodies, respectively. Paradoxically, the prolactin receptors were most abundant (Western Blot) in the gills of *O. mossambicus* acclimated to seawater, corroborating earlier data (radioreceptor binding analyses) in *O. niloticus* after acclimation to brackish water (Auperin et al., 1995). Clearly, more studies are necessary before to make certain of this apparent up-regulation of the prolactin receptor in high salinities in direct contrast to the plasma hormone levels. It does highlight, however, the caution that the role of a specific signaling agent in a physiological response cannot be ascribed unless levels of expression of the receptor(s) are also known.

2.2 Cortisol

This steroid's role in acclimation to seawater is well established, but more recent evidence suggests that it also plays a role in fish osmoregulation in freshwater. Since the initial finding that injection of cortisol reversed the reduction of gill Na^+/K^+-ATPase activity produced by hypophysectomy (Pickford and Griffith, 1970), it has been shown that cortisol therapy stimulates Na^+/K^+-ATPase activity in a variety of fish species, and also increases tolerance to high salinities (Madsen and Bern, 1995). Moreover, cortisol increased expression of the Na-K-2Cl co-transporter in the gills of *Salmo salar* (Atlantic salmon) acclimated to freshwater (Pelis et al., 2001). Cortisol treatment also increases branchial chloride cell size and density in salmonids (Madsen, 1990). The biochemical and morphological effects are at least partially direct, because they occurred in *O. mossambicus* opercular skin stimulated with cortisol in vitro (McCormick, 1990). Interestingly, cortisol does not stimulate active transport across the tilapia opercular skin (Foskett et al., 1981), suggesting that other factors (e.g., salinity, photoperiod, developmental stage, other hormones) must be involved in facilitating salt extrusion in addition to cortisol-stimulated cell differentiation and upregulation of Na^+/K^+-ATPase. Cortisol receptors have been identified in gill tissue from *Anguilla rostrata* (American eel), *O. mykiss*, *Salvelinus fontinalis* (brook trout), and *Oncorhynchus kisutch* (coho salmon), using radioligand protocols (Sandor et al., 1984) and localized in gill chloride (and pavement) cells from chum salmon fry (*Oncorhynchu keta*), using molecular and immunological techniques (Uchida et al., 1998). In chum salmon, acclimation to seawater was associated with an increase in the expression of cortisol receptors in the gills however, in the coho salmon, cortisol treatment lowered both the affinity and number of gill receptors, just as chronic stress did, presumably via the secretion of cortisol (Maule and Schreck, 1991). This suggests, once again, that factors in addition to cortisol must be involved in tolerance of seawater.

The cortisol receptor has been cloned from *O. mykiss*, and its amino acid sequence shares much closer homology with the mammalian glucocorticoid receptor than the mineralocorticoid receptor (Ducouret et al., 1995). However, spironolactone, a mineralocorticoid receptor antagonist, inhibited the cortisol-induced proliferation of chloride cells in the gill of rainbow trout, but RU486 (which antagonizes glucocorticoid receptors) did

not (Sloman et al., 2001). On the basis of these physiological studies, one might expect that plasma cortisol levels would generally increase during acclimation to seawater. This was found in early studies on the eel and tilapia (Assem and Hanke, 1981), but more recent studies have demonstrated that plasma cortisol levels increase significantly when a variety of fish species (e.g., eel, tilapia, killifish, mullet, flounder, and sea bass) are transferred to low salinities or freshwater. This finding suggests that cortisol also may play an important role in osmoregulation in freshwater. Early work had shown that cortisol treatment stimulated sodium uptake by intact or inter-renalectomized eels and intact goldfish in freshwater and also increased the plasma osmolality of hypophysectomized eels or goldfish in freshwater (McCormick, 2001). Treatment with ACTH (presumed to stimulated cortisol production) increased survival of hypophysectomized *Gambusia sp.* (mosquito fish) and *Amia calva* (bowfin) and increased plasma sodium levels in intact *F. kansae* (killifish) in freshwater.

It appears that both cortisol and prolactin may be necessary for survival in low salinities, because hypophysectomized *Ictalurus melas* (black bullhead) and *Heteropneustes fossilis* (catfish) need both prolactin and cortisol to maintain normal ion homeostasis in isosmotic or freshwater salinities, respectively (Fortner and Pickford, 1982). Cortisol also may synergize with another hormonal axis in seawater; namely, growth hormone and insulin-like growth factor.

3. The snakehead fish

The snakehead fish are members of the freshwater perciform fish family *Channidae*, native to Africa and Asia. They are distinguished by a long dorsal fin, large mouth and shiny teeth. Adults are dark brown in color with faint black bands visible across its entire body. They breathe air with a suprabranchial organ, a primitive form of a labyrinth organ. There are two extant genera, *Channa* in Asia, and *Parachanna* in Africa, consisting of 5-6 species. In nature snakehead is a voracious carnivore feeding mainly on live animals. Small fry feed mainly on zooplankton and insect larvae, while larger fry and fingerlings commonly feed on invertebrates, frogs and smaller fish. Snakehead is a nest-breeding species. The nest is prepared by the parent fish by clearing an area at the water surface of aquatic and emergent vegetation. This is done by biting off the weeds in shallow water near the edge of the water

body. The fertilized eggs from a thin film at the water surface and are guarded closely by the male parent until they hatch and school break. The eggs are yellowish in color, 1.2 – 1.5 mm in diameter, having a large oil globule, floating to the surface and hatch in 24–35 hours at 28–30°C. On the day of hatching the larvae are 3.0–4.5 mm long, dark brown in color. Two days after hatching, fry start to swim vertically with right side up. Orange pigment develops on the fourth day and becomes a red-orange band along the body by day ten. At the end of the fourth week, the fry reach 2.4 – 2.6 cm in length and are dark gray in color and thereafter assume the habits of the adult (Lin et al., 1994).

Under culture in the aquarium snakehead matures in one year, measuring about 21 cm in total length. Breeding season of the fish in Thailand usually commences in April at the onset of the rainy season and extends to October, with a peak in June and August. In nature snakehead can attain a length of one meter. The fish commonly found in natural waters are below 30 cm in length. The snakehead shows a much better growth rate under culture. With proper feed and stocking density, the fish commonly attain 300–500 gm in 9 months and 500–800 gm in 11 months. In Thailand snakehead culture is practiced in Suphanburi, Nakorn Patom, Samut sakorn, Samut Songkhram, Nakorn Nayok, Samut Prakan, Chantaburi and Trad Provinces. Suphanburi is the largest farming area.

Part III

Methodology

1. Animals

Snakehead (*C. striata*) weighing 120 ± 1.24 g were obtained from natural water sources in Khon Kaen Province, Thailand, and maintained in aerated 4-well tanks (150 L/well, dechlorinated tap water) equipped with charcoal filters and air stones. Water temperature was 22–26 °C. Fifty percent of water was renewed daily. They were fed brine shrimp (*Artemia salina*) twice a day (~2% of body weight per day). When fish looked healthy and ate well, they were gradually transferred to salt water. Salinity in the tank was reduced at a rate of 2‰ per day and fish were allowed to acclimate to new salinity for 24 h until the endpoint salinity of 10‰. A half of water in each tank was replaced with salt water of the same salinity every 4 days. Another group of fish was acclimated in dechlorinated tap water. Two animal groups were acclimated to these conditions for at least 4 weeks prior to the experiment. During the experiment, fish were maintained under natural photoperiod. Stressors (e.g., noise, crowding, and vibration) were avoided in order to minimize variation of cortisol levels. This study has been approved by the animal ethics committee of Khon Kaen University, Thailand (no. 0514.1.12.2/26), and all animals were cared for in accordance with the Ethic of Animal Experimentation of National council of Thailand.

2. Chemicals

All chemicals were analytical grade purchased from various commercial companies: Merck, Sigma, Qiagen, Roche, Promega, Fermentas or USBiological. *Escherichia coli,* DH5α [*sup*E44 Δ*lac*U169 (φ80 *lac*Z ΔM15) *hsd*R17 *rec*A1 *end*A *gyr*A96 *thi*-1 *rel*A1] was used as a host for plasmid propagation. The pGEM-T easy vector (Promega) was used in all cloning steps.

3. cDNA cloning of snakehead PRLR

3.1 RNA preparation

3.1.1 RNA isolation

Total RNAs were extracted from tissues, gill, liver, hepatopancrease, kidney, gastrointestinal tract, urinary bladder and brain using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Frozen samples were ground by mortar and pestle and then 1 ml TRIzol reagent was added per 50-100 mg of sample weight. The homogenates were incubated at room temperature for 5 min, then 0.2 ml chloroform per 1 ml of TRIzol reagent was added and the homogenates were vigorously shaken for 15 sec. They were incubated at room temperature for 2-5 min. The mixtures were centrifuged at 14,000 rpm, 4 ºC for 15 min. The upper phase was transferred to the new tubes. The aqueous phase was mixed with 0.6 volume isopropanol. The mixtures were stored at -20 ºC for 1 h for precipitating RNA. The precipitated RNA was collected by centrifugation at 14,000 rpm, 4 ºC for 20 min. The pellet was washed with 1 volume of 70% and 100% ethanol respectively. After the pellet was dried, it was dissolved in DEPC-H_2O (sterile water treated with DEPC reagent) and incubated at room temperature for 30 min. The completely dissolved RNA samples were stored at -80 ºC.

3.1.2 Determination of RNA concentration

The amount of RNA samples was measured by a spectrophotometer at 260 nm and 280 nm. The RNA concentration was calculated from the absorbance value measured at 260 nm using the following formula:

$$\text{RNA concentration } (\mu g/\mu l) = \frac{A_{260} \times (\text{dilution factor}) \times 40}{1000}$$

The purity of RNA samples was determined by the absorbance ratio of A_{260}/A_{280}. A ratio between 1.8-2.0 represents a high RNA purity.

3.2 cDNA amplification of a putative *vasa* gene

3.2.1 Degenerate primer design

Two degenerate primers were designed from conserved regions of PRLR homologue genes from fugu (accession number: XP_003965258.1), sea bream (accession number: ABO61870.1), rainbow trout (accession number: NP_001118071.1), zebrafish (accession

number: NP_001122149), goldfish (accession number: AF1044012), tilapia (accession number: AF080247), chicken (accession number: ADD69774), pig (accession number: ABA41035), human (accession number: XP_001150064.2), rat (accession number: AAA61784) and bullfrog (accession number: XP_002940001). They were aligned using Clustal X program. Two conserved regions were chosen to design the forward and the reverse degenerate primers are shown in Table 3-1.

Table 3-1 Primers and the degenerate oligonucleotide sequences used in the studies. Abbreviations of mixed nucleotides are: N= A, T, G, C; K= T, G; R= A, G; Y= C, T.

Primer name	Sequences (5'–3')	Size (bp)
PRT oligo(dT)	CCGGAATTCAAGCTTCTAGAGGATCC TTTTTTTTTTTTTTT	42
PRT oligo(dG)	CCGGAATTCAAGCTTCTAGAGGATCC GGGGGGGGGGGGGGG	42
Adaptor primer (PM-1)	CCGGAATTCAAGCTTCTAGAGGATCC	26
shPRLR-F1	CGNTCNCCNGARAARGARACNTT	23
shPRLR-R1	CCNCCNGTNCCNGGNCCNAARAT	23
shPRLR-WF	CCATGCGGCAAGGATCGCGTTTCT	24
shPRLR-WR	GGGAGGGGGACGGCGGGATGTGCT	24
3’-RAEC-SF	TGGCTACTCCACATGATGAAACGT	24
5’-RACE-SR	CGTGAGAGCTGGTTTGCCCGGGGGG	25
PRLR-SF2	TGTCCCGCATACCGACGGGG	20
PRLR-SR2	ATTCAGATAGGGATTGACGC	20
β-actinF	AGGCACGACATTGAATGGGC	20
β-actinR	ACATGGCTCCACTAATCTTG	20

3.2.2 First stranded cDNA synthesis by reverse transcription

To clone the putative PRLR gene in the snakehaed, the first stranded cDNA was synthesized from gill by reverse transcription. The reverse transcription reaction was performed according to the manufacturer's instruction of and iScript kit (Biorad, Hercules, CA, USA). One μg of total RNA was mixed with 800 nM of PRT oligo(dT) primer (Table 3-1) in a final volume of 11 μl. The mixture was incubated at 80 ºC for 5 min, chilled on ice and added the following in the order indicated, 4 μl of 5X reaction buffer, 2 μl of 10 mM dNTPs Mix and dH_2O to a final volume of 19 μl. The mixture was incubated at 37 ºC for 5 min and then 200 units of Reverse Transcriptase were added. The reaction mixture was incubated at 42 ºC for 60 min. The reaction was inactivated by heating at 70 ºC for 10 min followed by chilling on ice. After the first stranded cDNA was successfully synthesized, it was stored at -20 ºC until used

3.2.3 RT-PCR

After the reverse transcription, the first stranded cDNA was used as a template for PCR amplification using 2 degenerate primers, shPRLR-F1 and shPRLR-R1. In this study, 2 rounds of PCR amplifications were carried out. To eliminate a problem of DNA amplification from genomic DNA, first round PCR was amplified with shPRLR-F1 and PM-1, an adaptor primer complementary to adaptor sequence in the PRT oligo(dT) primer used in the first stranded cDNA synthesis. The PCR reaction was amplified in the presence of 1 μl of the first stranded cDNA, 2.5 μl of 10X *Taq* DNA polymerase buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl and 0.8% Nonidet P40), 2 μl of 25 mM $MgCl_2$, 2 μl of 10 mM dNTPs, 2 μM of each primer, 0.25 μl of 5 units/μl *Taq* DNA polymerase and dH_2O to a final volume of 25 μl. To increase specificity of primer binding, hot start PCR was performed by adding *Taq* DNA polymerase into the reaction mixture after denaturation at 94ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 45 sec, annealing at 55ºC for 45 sec and extension at 74ºC for 2 min. Final extension was run at 74ºC for 7 min. Then, the first PCR product was diluted to 1:100 dilutions for the semi-nested PCR. The second PCR was performed using two degenerate primers, shPRLR-F1 and shPRLR-R1, with the same reaction conditions. The second PCR products were separated by electrophoresis on a 1.2% agarose gel.

3.2.4 DNA purification by QIAquick gel purification kit (Qiagen)

After cDNA was amplified by RT-PCR, it was purified before sequencing. DNA products were purified using QIAquick gel purification kit. The DNA fragments were separated by electrophoresis on a 1.2% agarose gel. After the expected DNA bands were positioned, the DNA fragments were excised from the agarose gel with a clean blade. The purification reaction was performed according to the manufacturer's instruction. The sliced gel was weighed and 3 volume of QC buffer was added to 1 volume of the gel. The solution was incubated at 50 ºC for 10 min (or until the gel slice was completely dissolved). The tube was vortexed every 2-3 min during the incubation to help dissolving the gel. The gel dissolved tube was added with 1 gel volume of isopropanol and was then transferred to the QIAquick column and centrifuged at 10,000 rpm for 1 min. The flow-through in the collection tube was discarded and the QIAquick column was placed back to the same collection tube. QA buffer was added into the QIAquick column and then it was centrifuged at 10,000 rpm for 1 min. For washing step, 0.75 ml of PE buffer was added to QIAquick column; the mixture was centrifuged for 1 min. Flow-through was discarded and centrifuged additional for 1 min. The DNA sample was collected by placing the QIAquick column on a clean 1.5 ml microtube. EB buffer was added into the QIAquick column which was centrifuged at 10,000 rpm for 2 min. The amount of purified DNA in 1.5 ml microtube was estimated by electrophoresis on a 1.2% agarose gel.

3.2.5 DNA sequencing

The DNA were sequenced using ABI PRISM® 3100 Version 3.7 automated DNA sequencer. The DNA sequences were edited and translated to a deduced amino acid sequences using Expacy Translate Tool in Expacy website (http://ca.expacy.org). All of the cloned DNA and amino acid sequences were aligned using the Clustal X program. The nucleotide and amino acid sequences were blasted using Genbank databases in National Center for Biotechnology Information (NCBI) (http://www .ncbi.nlm.nih.gov).

3.3 RACE-PCR

3.1.1 The 3' end amplification

The 3' end cDNA of putative snakehead PRLR gene was performed using 3'-RAEC-SF (Table 3-1). They were specific primers which were designed from previously identified

sequence. Two rounds of PCR were carried out using 3'-RAEC-SF and PM-1 primers. The Before the second PCR was set, the first PCR product was diluted to 1:50 dilutions. Both PCR reactions contained 1 μl of DNA template, 2.5 μl of 10X *Taq* DNA polymerase buffer, 2 μl of 25 mM $MgCl_2$, 2 μl of 10 mM dNTPs, 2 μl of 10 μM of each primer, 0.25 μl of 5 units/μl of *Taq* DNA polymerase and dH_2O to a final volume of 25 μl. The PCR reaction profiles were performed in the same condition of section 3.2.3. The PCR products were separated by electrophoresis on 1.2% agarose gel.

3.4 The 5' end amplification

The 5' end cDNA of a putative snakehead PRLR gene was performed by 5' RACE method. The method was similar to that of 3' RACE but the first stranded cDNA was poly-C tailed at the 3' end before the PCR amplification. Five μg of total RNA from the gill was reverse transcribed using 0.5 μM of 5'-RAEC-SF, the specific primer (Table 3-1) in order to construct the first stranded cDNA and to obtain only the first stranded cDNA of a putative snakehead PRLR gene. The first stranded cDNA synthesis was carried out using iScript kit and the protocol was described as in section 3.2.2. After reverse reaction, 2.5 μl of 5 N NaOH was added and incubated for 30 min to eliminate RNA; the mixture was neutralized by 72 μl of 1% acetic acid.

Before poly-C tailing reaction (adding cytosine bases at the 3' end of cDNA), the first stranded cDNA was purified using QIAquick PCR purification kit (Qiagen). The kit was used to separate the first stranded cDNA from dNTPs, salts and primers. The reaction was performed according to manufacturer's instructions. The cDNA sample was added with 5 volumes of PB buffer. It was then applied to a QIAquick spin column placed on a 2 ml collection tube and was centrifuged at 12,000 rpm at room temperature for 1 min. After the flow-through was discarded, 0.75 ml of PE buffer was added following with centrifugation at 12,000 rpm for 1 min. The flow-through was discarded and the column was centrifuged at 12,000 rpm for 1 min. The column was then placed in a new microtube and the EB buffer was added. After incubation at room temperature for 1 min, the column was centrifuged at 12,000 rpm for 1 min. The eluted cDNA was collected in a microtube.

In order to allow PRT oligo(dG) primer (Table 3-1) bind to the first stranded cDNA, poly-C tail was added to the 3' end of the first stranded cDNA. A 21 μl of purified first stranded cDNA was added into the reaction mixture containing 2 μl of 25 mM dCTP, 4 μl 5X reaction buffer (1 M potassium cacodylate, 0.125 M Tris, 0.05% triton X-100, 5 mM $CoCl_2$, pH 7.2) and 2 μl of 20 units/μl Terminal Deoxynucletidyl Transferase (TdT). The mixture was then incubated at 37 ºC for 5 min. The TdT was inactivated by incubating at 70 ºC for 10 min. The poly-C tailed first stranded cDNA was stored at -20ºC until used.

To amplify the 5' end cDNA of the putative snakehead PRLR gene, two rounds of PCR amplification were performed (Figure 4-5). The first PCR was performed using 5'-RAEC-SF specific primer and PRT oligo(dG) primer and the first stranded poly-C tailing as a template. Then the first PCR product was diluted to 1:100 dilutions and subjected to the second PCR reaction with 5'-RAEC-SF and PM-1. Both PCR reactions were performed in the presence of 1 μl of cDNA template, 1X *Taq* DNA polymerase buffer, 2 μl of 25 mM $MgCl_2$, 2 μl of 10 mM dNTPs, 2 μl of each 10 μM primer, 0.25 μl of 5 unit/μl of *Taq* DNA polymerase and dH_2O to a final volume of 25 μl. To increase specificity of the PCR reaction, combination of hot start and touch down PCR were performed. At first, hot start PCR was carried out by adding *Taq* DNA polymerase after incubation the PCR reaction at 94 ºC for 5 min, followed by touch down PCR in which annealing temperature was decreased 1 ºC per cycle from 60-55 ºC at the first 6 cycles. Then PCR amplification for 30 cycles of denaturation at 94 ºC for 30 sec, annealing at 55 ºC for 30 sec and extension at 72 ºC for 2 min was carried out, followed by a final extension at 72 ºC for 10 min. The semi-nested PCR was performed with the same conditions. The PCR products were separated by electrophoresis on a 1.2% agarose gel, and then they was purified, cloned and sequenced.

3.5. Full-length cDNA amplification and cloning

The full-length cDNA of the putative snakehead PRLR gene was amplified using 2 specific primers, shPRLR-WF and shPRLR-WR (Table 3-1), corresponding to the 5' and 3' ends respectively. These primers were designed from combined full-length sequences, sequence of 5' end, middle sequence motifs and sequence of 3' end. The first stranded cDNA was generated from a total RNA of the gillas described in section 3.2.2. To avoid

mutation from error amplification by *Taq* DNA polymerase, *Pfu* DNA polymerase containing proofreading activity was used to amplify the full-length cDNA. Two rounds of PCR were carried out (Figure 4-6). The shPRLR-WF and PM-1 primers were used in the first round of PCR reaction. shPRLR-WF and shPRLR-WR primers were used in the second of PCR reaction. The PCR condition contained 1 μl of cDNA template, 1X *Pfu* DNA polymerase buffer, 2 mM $MgCl_2$, 0.4 mM dNTPs and 0.4 μM of each primers and 0.1 unit of *Pfu* DNA polymerase. The second round of PCR reaction was performed with the same condition except for the template. The template was diluted before amplifying (1:50). The hot start and touch down PCR were set. At first, hot start PCR was carried out by adding *Pfu* DNA polymerase after incubation at 94 ºC for 5 min, followed by touch down PCR in which annealing temperature was decreased 1 ºC per cycle from 62-57 ºC at the first 6 cycles. After that the PCR reaction was incubated for 30 cycles of denauration at 94 ºC for 30 sec, annealed at 57 ºC for 30 sec and extended at 68 ºC for 5 min, followed by a final extension at 72ºC for 10 min. The PCR products generated from *Pfu* DNA polymerase were blunt-ended DNA. To ligate with the pGEM-T easy vector, an A-tailing at the 3' ends of the full-length DNA was required. Therefore, the PCR products were added an oligo A at the 3' end by *Taq* DNA polymerase and the PCR reaction was then incubated further at 72 ºC for 10 min. The PCR products were separated by electrophoresis on a 1.0 % agarose gel. It was then purified using the QIAquick gel purification kit as described in section 3.2.4.

3.6. RT-PCR analysis

Total RNAs from several tissues (gill, liver, hepatopancrease, kidney, gastrointestinal tract, urinary bladder and brain) of snakehead were separately extracted and RT-PCR was carried out using specific primers, PRLR-SF2 and PRLR-SR2 (Table 3-1). Primers for β-*actin* gene (accession number: GQ214252), β-actinF and β-actinR, were used for the internal control. Reverse transcription was carried out as follows: 5 μg total RNAs were denatured at 80 °C for 5 min, the PvasR PRLR-SR2 or β-actinR primers were added and annealed at 25 °C for 5 min. The reaction mixtures were chilled on ice and then 100 μM of each dNTPs and 400 units of the reverse transcriptase were added. The reactions were performed at 42 °C for 2 h, and were stopped at 70 °C for 15 min. The synthesized cDNAs

were used as templates in PCR reactions. The reaction conditions were: 94 °C for 5 min, followed by 36 cycles of 94 °C for 30 sec, 57 °C for 45 sec, 74 °C for 1 min, and finally, 74 °C for 7 min. The PCR products were separated by electrophoresis on a 1.2% agarose gel.

4. Experimental design

4.1. Experiment 1: Acute effect of the various salinities on the blood chemistry of C. striata

Salt waters (SW; 2, 5, 10, 15, 20 and 35‰) were prepared by dissolving NaCl (Anivar, Australia) in 100-L tanks of dechlorinated tap water, and salinity was measured by a light refractometer. Chemical and ion compositions of fresh water and salt water are shown in Table 1. Animals were fasted for 24 h before performing the experiment. The freshwater-acclimated fish were randomly placed into each concentration of SW for 1 h. The control group [0‰ salinity, fresh water (FW)] was exposed to dechlorinated tap water. Finally, they were subjected to the measurement of plasma osmolality, muscle water content, and plasma concentrations of cortisol, glucose, Na^+, Cl^-, K^+, and HCO_3^-. Measurement of muscle water content and plasma HCO_3^- level after an acute salinity exposure reflected the ability of fish to restrict water loss and to maintain blood pH, respectively.

4.2 Experiment 2: Time-dependent adaptation to hyperosmotic environment

SW (10‰ salinity) was prepared by dissolving NaCl in 150-L tanks of dechlorinated tap water. The experimental group (FW—SW) was fish exposed to abrupt salinity change from FW (0‰ salinity) to SW (10‰ salinity), whereas the control group (FW—FW) was fish transferred from FW to FW. Plasma osmolality, blood chemistry (plasma concentrations of cortisol, glucose, lactate, Na^+, and Cl^-) and branchial Na^+/K^+-ATPase activity were measured at 0 h (FW), and then at 1, 3, 4, 6, 12, 24, 72, 168, 240 and 360 h after SW exposure (n= 8 per each time point). Normally, an increase in lactate concentration indirectly indicates stress in fish (Arends et al., 1999).

4.3. Experiment 3: Response of SW-acclimated fish to hypoosmotic environment

In the experimental group (SW—FW), SW-acclimated fish were transferred to FW (0‰ salinity), whereas the control group (SW—SW) was fish transferred from SW to SW. Plasma

osmolality, blood chemistry (plasma concentrations of cortisol, glucose, lactate, Na^+, and Cl^-) and branchial Na^+/K^+-ATPase activity were measured at 0 h (SW), and then at 1, 3, 4, 6, 12, 24, 72, 168, 240 and 360 h after FW exposure (n= 8 per each time point).

5. Sample collections

Prior to blood collection by cardiac puncture, fish were anesthetized by 0.5 g/L tricaine methanesulfonate (catalog no. MS222, Sigma, St. Louis, MO, USA). The water used for anesthesia had the same temperature and electrolyte composition as that fish has been exposed previously. Under anesthesia, the heart was exposed immediately, and a blood sample was drawn from the bulbus arteriosus using a syringe coated with 200 U/mL ammonium heparin (Sigma). Plasma was stored at –80 °C before analysis.

The muscle tissue sample (~0.5 g) were taken from epaxial side in duplicate and weighed immediately for a wet weight. Then they were dried at 60 °C for 48 h and weighed. The muscle water content was calculated as: (wet weight–dry weight)/wet weight × 100.

6. Blood and water analyses

Plasma and water osmolality was measured by a freezing point osmometer (Advanced Instruments, Norwood, MA, USA). Concentrations of Na^+, Cl^- and K^+ in plasma and water were determined by ion-selective electrode system of Cobas 6000 analyzer module c501 (Roche Diagnostics, Rotkreuz, Switzerland). Concentrations of HCO_3^-, glucose and lactate were also measured by Cobas 6000 analyzer. Plasma samples were analyzed in triplicate or duplicate, depending on available plasma volume. Hematocrit measurement was performed in duplicate using microhematocrit capillary tubes centrifuged at 11,000 rpm for 5 min in a microhematocrit centrifuge.

7. Measurement of cortisol level

Plasma cortisol concentrations were determined by radioimmunoassay using a commercial kit (ImmuChemCoated Tube Cortisol ^{125}I RIA kit, MP Biomedicals, NY, USA). Plasma samples and standards were pipetted into anti-cortisol coated tubes. Cortisol-3-carboxymethyloxime-BSA was used as an immunogen to produce anti-cortisol antibody in rabbits. The anti-serum

was coated and bound to the inner surface of a polypropylene tube. Cortisol ^{125}I was added to all tubes followed by 45-min incubation. The coated tubes were then analyzed in a gamma counter to determine the amount of antibody-bound ^{125}I-cortisol. Levels of cortisol in the plasma samples were obtained from the standard curve. The anti-serum (primary antibody) reacted 100% with cortisol, 12.3% with 11-desoxycortisol, 5.5% with corticosterone, 2.1% with cortisone, 0.25% with progesterone, and < 0.1% with testosterone.

8. Measurement of prolactin level

Plasma prolactin concentration was determined by Enzyme-linked immunosorbent assay using a commercial kit (Fish Prolactin/Luteotropic Hormone, Casabio, Japan). Plasma samples and standards were pipetted in duplicate into 96-well plate. A Blank well without any solution was also set up. HRP (horse radish peroxidase)-conjugate was added to each well (not to Blank well) then antibody was to each well. The reactions were mixed well and then incubate for 60 minutes at 37 °C. Each well was aspirated and washed with Wash Buffer, repeating the process two times for a total of three washes. Each well was washed by filling Wash Buffer using a multi-channel pipette and let it stand for 10 seconds. After the last wash, Wash Buffer was removed any remaining by aspirating. Then Substrate A and Substrate B were added to each well, mixed well and incubated for 15 minutes at 37 °C in the dark. Stop Solution was added to each well, gently tap the plate to ensure thorough mixing. Each well was determined the optical density of within 10 minutes, using a microplate reader set to 450 nm.

9. Na^+/K^+-ATPase activity assay

The measurement of Na^+/K^+-ATPase activity was modified from the method of McCormick (1993). Gill tissues taken from the fish were homogenized in 200 µL of SEID buffer (150 mM sucrose, 10 mM imidazole, 0.1% deoxycholic acid), and then centrifuged at 5,000 g for 1 min. Homogenate samples (10 µL) were added into 200 µL of solution A [4 U/ml lactate dehydrogenase, 5 U/ml pyruvate kinase, 2.8 mM phosphoenolpyruvate (PEP), 0.7 mM ATP, 0.22 mM NADH, and 50 mM imidazole, pH 7.5] or solution B [solution A plus 0.5 mM ouabain (Na^+/K^+-ATPase inhibitor)] in 96-well microplates at 25 °C, followed by reading at

340 nm for 10 min by a microplate reader (Biorad, QC, Canada) to determine a decrease in NADH level, which was used to calculate ATP hydrolysis (McCormick, 1993). Each pair of wells (with and without ouabain) was determined, and the Na^+/K^+-ATPase activity (μmol ADP/mg protein/h) was calculated as the difference of ATP hydrolysis in the absence and presence of ouabain. The total protein concentrations were determined by bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA).

10. Expression study of shPRLR in snakehead fish by quantitative real-time PCR

The shPRLR transcript levels in gill, intestine and kidney of each treatment were determined by quantitative real-time PCR. Total RNA was extracted from gill, intestine and kidney by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according the manufacturer's instruction. RNA was treated with DNase (Vivantis, CA, USA), purity and quantities were determined by Nanodrop spectrophotometer. β-actin gene was used as internal control. The primer sequences for shPRLRs and β-actin are listed in Table 1.

First strand cDNA was synthesized from 1 μg total RNA using oligo-dT_{20} and iScript kit (Biorad, Hercules, CA, USA). Real-time PCR were performed on CFX96 Touch system (Biorad). The reactions were performed in duplicate in a 25 μl reaction volume, using SsoFast EvaGreen Supermix (Biorad). The specificity of the reactions was confirmed by melting curve analysis and agarose gel electrophoresis. All amplicons showed a single melting peak on real-time PCR and agarose gel electrophoresis present a single band of the electrophoresis. The PCR products were confirmed by DNA sequencer (ABI PRISM 310, Applied Biosystem, CA, USA). The relative expression of shPRLR between treated group and control group was determined by $2^{-\Delta\Delta C_T}$ (Livak and Schmittgen, 2001).

11. Statistical analysis

All data were analyzed by GraphPad Prism 6.0 (San Diego, CA, USA). Results are expressed as means ± SE. Differences between groups were analyzed by one-way analysis of variance (ANOVA) with Dunnett's posttest. Comparison between the two factors, i.e., salinity and time, were performed by two-way ANOVA. The level of significant for all statistical analysis was $P < 0.05$

Part IV

Results

Results are divided into 4 sections.

Section 1: Cloning and characterization of mRNA prolactin receptor (PRLR) in snakehead fish

The snakehead PRLR cDNA consisted of 2,412 bps, containing an ORF encoding 618 aa (DDBJ/EMBL/GenBank DNA databases with the Accession No. K051404). The 5'-UTR and 3'-UTR consisted of 245 and 313 nucleotides, respectively. The deduced amino acid sequence was composed of an extracellular domain with two pairs of cysteines and a WSXWS motif, a single transmembrane domain and an intracellular domain with highly conserved box 1 and box 2 regions (Fig. 1).

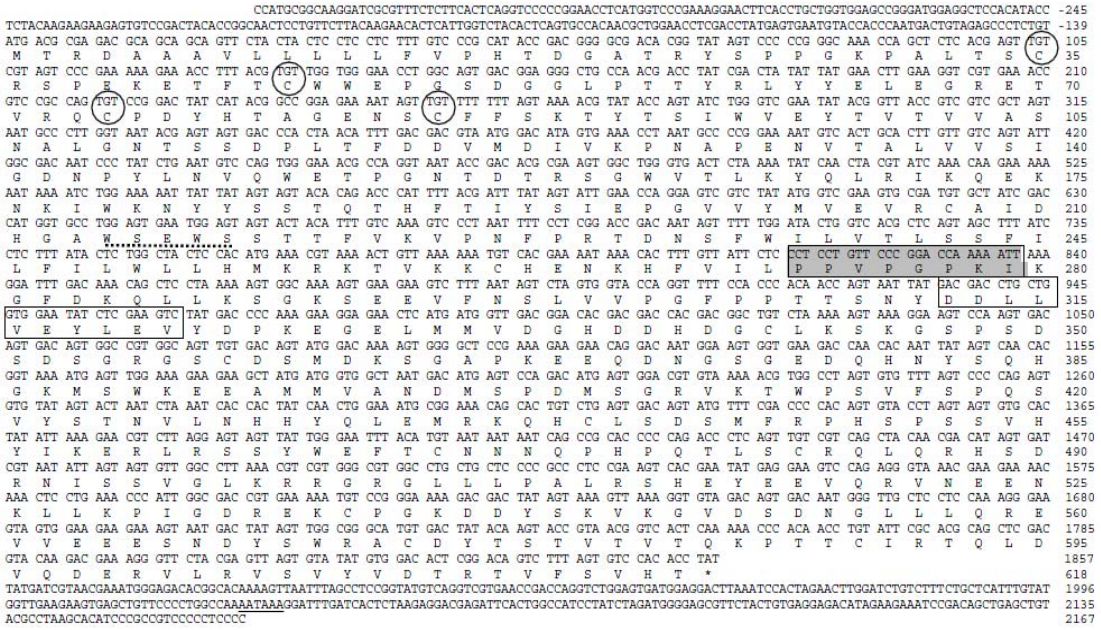

Fig. 4-1. Nucleotide and deduced amino acid sequence of the snakehead PRLR *(shPRLR*). Nucleotide (upper sequence) and aa (lower sequence) are numbered on the right. Conserved cysteine residues (circled), WS motif (underlined with a dot line), Box 1 (boxed in shaded) and Box 2 (boxed), the stop codon (marked with an asterisk), and underline aataaa is the poly-A adding sequence

Determination the tissue expression of PRL receptor, the RT-PCR was performed. A single band of the expected size (400 bp) was observed in the liver, heart, kidney gill, gastrointestine, urinary bladder and brain but the highest expression was found in gill and kidney (Fig. 4-2).

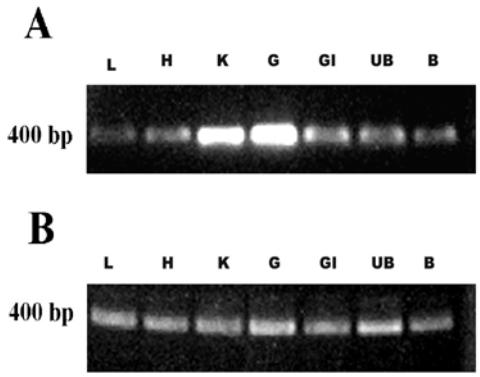

Fig. 4-2. Expression of shPRLR in various tissues of snakehead fish using RT-PCR (A). Expression of β-actin, an internal control (B). L, liver; H, hepatopancrease; K, kidney; G, gill; GI, gastrointestinal tract; UB, urinary bladder and B, brain.

Section 2: Acute effect of the various salinities

Ion concentrations such as Na^+, Cl^-, K^+ and HCO_3^- and osmolality of freshwater and saltwater samples collected from freshwater and NaCl-dissolved water (SW) tanks were measured and the results are shown in Table 4-1. Snakeheads could survive after 1-h exposure to 2, 5, 10, 15 and 20‰ SW, but all fish in 35‰ did not. Nevertheless, fish exposed to 15 and 20‰ SW showed stress behaviors, e.g., decreased movement and increased mucus secretion, and eventually died within 6 h.

Table 4-1. Osmolality and ion concentrations of freshwater and saltwater samples collected from freshwater and NaCl-dissolved water (SW) tanks.

Ion concentrations / osmolality	Salinity (‰)						
	0	2	5	10	15	20	35
Na^+ (mmol/L)	2	30	80	150	226	315	559
Cl^- (mmol/L)	2	30	79	149	227	314	560
K^+ (mmol/L)	0	0	0.05	0.1	0	0.1	0.3
HCO_3^- (mmol/L)	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Osmolality (mmol/kg H_2O)	9	70	166	310	460	640	1126

Their blood chemistry and percent muscle water content are shown in Table 4-2. Percent muscle water content increased significantly at 2‰ salinity and decreased at salinities >5‰. Plasma glucose was elevated in all salinities and reached the highest level at 10‰. Neither plasma levels of K^+ nor HCO_3^- were altered after exposure to SW.

Table 4-2. Blood chemistry and percent muscle water content in the snakehead fish (*C. striata*) after exposure to different salinities for 1 h.

	Salinity (‰)					
	0	2	5	10	15	20
Muscle water content (%)	79.72±1.82	85.69±2.64*	78.16±1.51	74.79±1.48*	74.05±2.01*	75.10±1.47*
Glucose (mmol/L)	6.25±0.87	7.66±1.49*	8.06±0.91*	10.00±1.13*	7.31±0.69*	9.50±1.37*
K^+ (mmol/L)	8.42±1.14	9.17±1.80	9.33±6.54	9.93±0.81	8.23±2.04	6.28±1.97
HCO_3^- (mmol/L)	6.40±1.39	7.07±1.99	6.50±2.35	7.23±3.30	7.88±0.98	8.02±0.86

Values are means ± SE (n = 8/group). * $P < 0.05$ vs. 0‰ salinity (one-way ANOVA).

The plasma concentrations of Na^+ and Cl^- as well as the resultant plasma osmolality were elevated with increasing salinities, especially when plasma osmolality was >310

mmol/kg H_2O (Figs. 4-3A and 4-3B). Plasma cortisol levels remained unchanged after 1-h exposure to hypoosmotic water (9, 70, and 166 mmol/kg H_2O), but were significantly increased in hyperosmotic water (Fig. 4-4). Plasma prolactin was significantly decreased only in fish exposed to at salinity level 640 mmol/kg H_2O (Fig. 4-5)

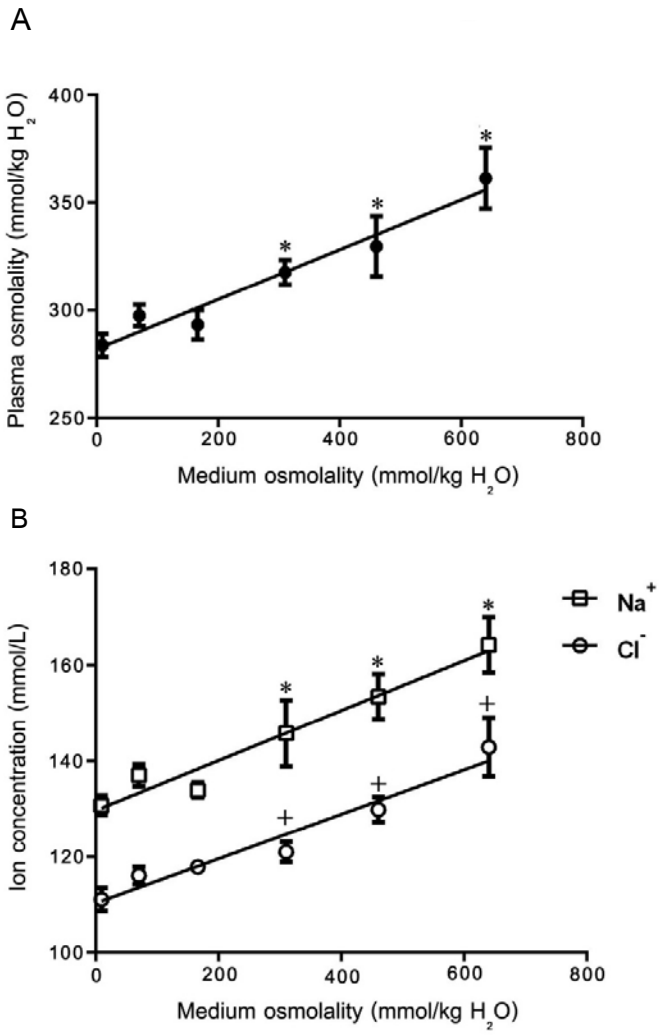

Fig. 4-3. Acute effect of various media with different ion concentrations on (*A*) plasma osmolality, and (B) plasma Na^+ and Cl^- levels in *C. striata*. Media (70, 166, 310, 460 and 640 mmol/kg H_2O) were prepared by dissolving NaCl in tap water. Each value is mean ± SE (n = 8 independent samples per group for each sampling time). * $P < 0.05$ compared with the corresponding control group (one-way ANOVA).

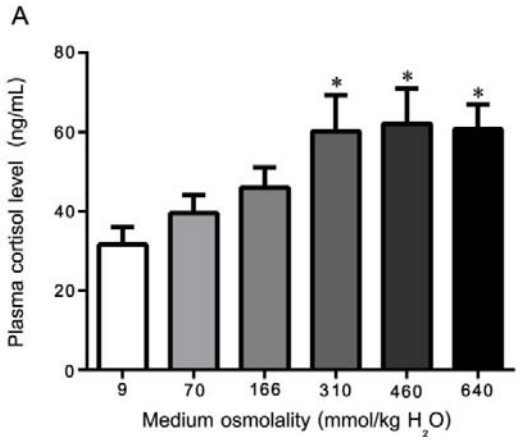

Fig. 4-4. Acute effect of various media with different ion concentrations on plasma cortisol level in *C. striata*. Media (70, 166, 310, 460 and 640 mmol/kg H_2O) were prepared by dissolving NaCl in dechlorinated tap water. The osmolality of dechlorinated tap water (control) was 9 mmol/kg H_2O. Fish were exposed to dechlorinated tap water or different media for 1 h. Each value is mean ± SE (n = 8 independent samples per group for each sampling time). * $P < 0.05$ compared with the corresponding control group (one-way ANOVA).

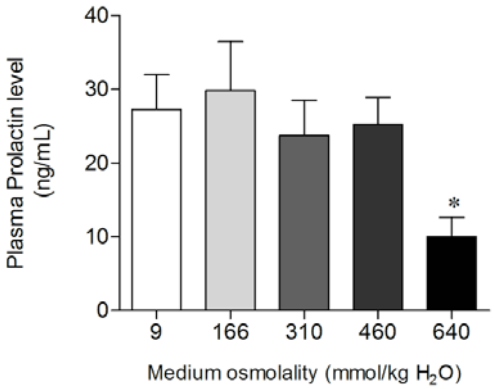

Fig. 4-5. Acute effect of various media with different ion concentrations on plasma prolactin level in *C. striata*. The osmolality of dechlorinated tap water (control) was 9 mmol/kg H_2O. Fish were exposed to dechlorinated tap water or different media for 1 h. Each value is mean ± SE (n = 8 independent samples per group for each sampling time). * $P < 0.05$ compared with the corresponding control group (one-way ANOVA).

The PRLR gene expression in gill decreased in the snakehead fish exposed to salinities, 166, 310, 460 and 640 mmol/kg H_2O for 43.93%, 53.25%, 53.14% and 49.44%, respectively (Fig. 4-6). In kidney and esophagus, the PRLR gene expression increase in the snakehead fish exposed in salinity level at 310 mmol/kg H_2O (Fig. 4-7 and 4-8). The other treated group show lower lever than the control freshwater group (9 mmol/kg H_2O).

The branchial and kidney Na^+/K^+-ATPase activities not altered in the treated group when compared to the control group (Fig. 4-9 and 4-10). However, in the esophagus, the enzyme activity only dropped in salinity 310 mmol/kg H_2O (Fig. 4-11).

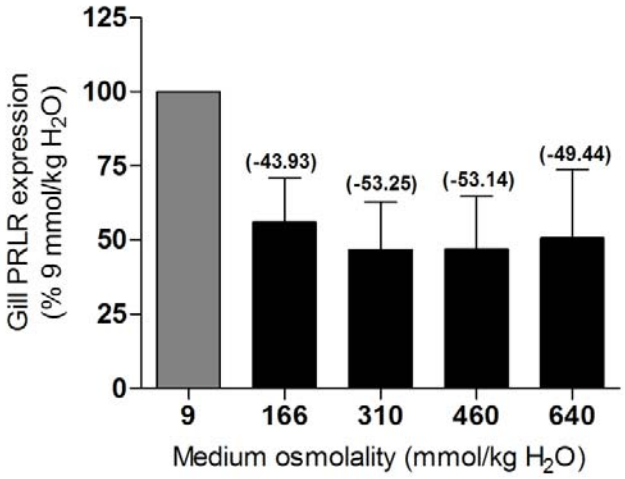

Fig. 4-6. Gill PRLR expression in *C. striata* after transfer from dechlorinated tap water to various media (166, 310, 460 and 640 mmol/kg H_2O) for 1 h. Control data are shown 100% and the treated data are shown as the percentage of increase (+) or decrease (-) from the control

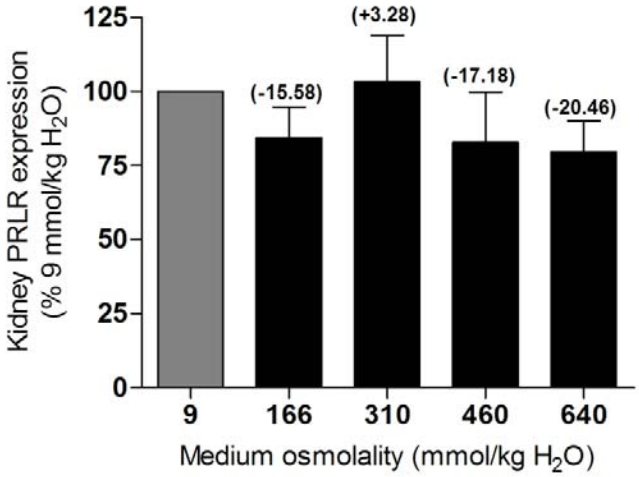

Fig. 4-7. Kidney PRLR expression in *C. striata* after transfer from dechlorinated tap water to various media (166, 310, 460 and 640 mmol/kg H_2O) for 1 h. Control data are shown 100% and the treated data are shown as the percentage of increase (+) or decrease (-) from the control

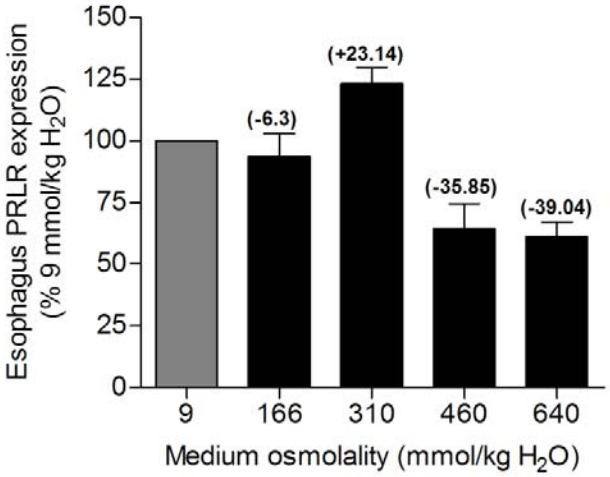

Fig. 4-8. Esophagus PRLR expression in *C. striata* after transfer from dechlorinated tap water to various media (166, 310, 460 and 640 mmol/kg H_2O) for 1 h. Control data are shown 100% and the treated data are shown as the percentage of increase (+) or decrease (-) from the control

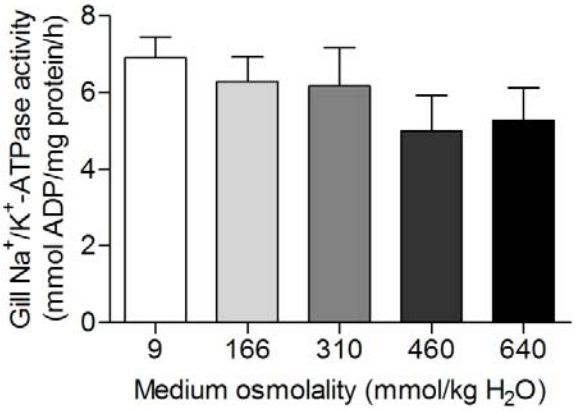

Fig. 4-9. Gill Na^+/K^+-ATPase activity in *C. striata* after transfer from dechlorinated tap water to various media (166, 310, 460 and 640 mmol/kg H_2O) for 1 h.

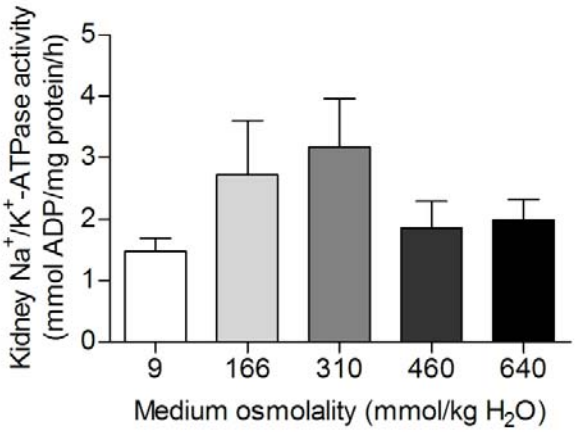

Fig. 4-10. Kidney Na^+/K^+-ATPase activity in *C. striata* after transfer from dechlorinated tap water to various media (166, 310, 460 and 640 mmol/kg H_2O) for 1 h.

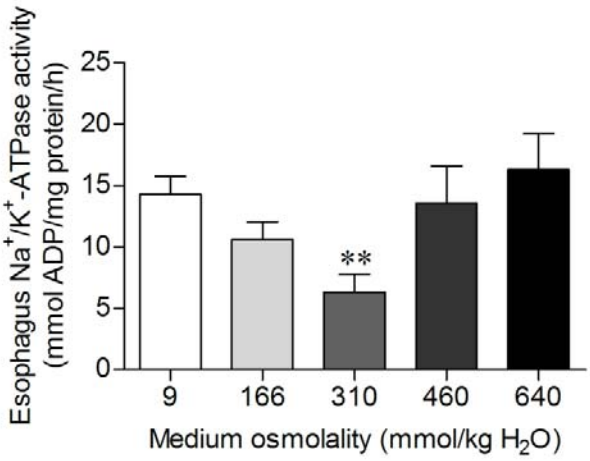

Fig. 4-11. Esophagus Na^+/K^+-ATPase activity in *C. striata* after transfer from dechlorinated tap water to various media (166, 310, 460 and 640 mmol/kg H_2O) for 1 h.

Section 3: Time-dependent responses to hyperosmotic environment

Plasma osmolality of freshwater-adapted fish was elevated at 1 h after being transferred to SW as compared to the value at 0 h (Fig. 4-13). The plasma osmolality after exposure to 0‰ (9 mmol/kg H_2O) was ~280 mmol/kg H_2O, but it could increase to as high as 390 mmol/kg H_2O after exposure to 10‰ SW (310 mmol/kg H_2O) for 168 h, which was greater than that of the baseline in plasma and medium by ~40% and ~25%, respectively. The plasma levels of Na^+ and Cl^- significantly increased at 3 and 1 h, respectively, after being transferred to SW (Fig. 4-12). Both plasma osmolality and ion concentrations remained higher than that of the control levels even after 360 h.

Despite no mortality in both FW—SW and FW—FW groups, there were conspicuous changes in blood ion levels, plasma osmolality, and cortisol levels in the FW—SW group as compared to FW—FW group. Changes in the plasma cortisol levels were complex. Specifically, they were transiently increased during the first hour after being transferred from FW to SW, followed by returning to the baseline level until 72 h, increasing again at 168–240 h, and then returning to the baseline value at 360 h (day 15). There was no change in cortisol level in the FW—FW group (Fig. 4-14).

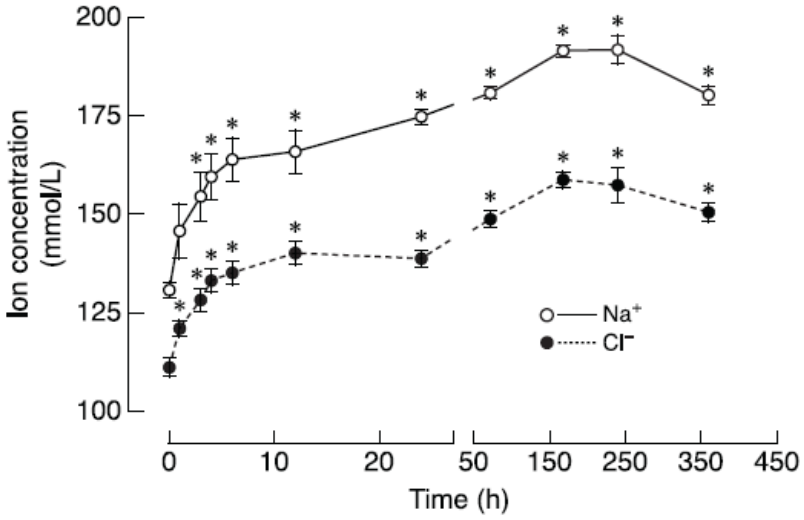

Fig. 4-12. Changes levels in plasma Na^+ and Cl^- *C. striata* after an abrupt transfer from fresh water to salt water (FW—SW), or from fresh water to fresh water (FW—FW). FW (0‰) was dechlorinated tap water. SW (10‰) was prepared by dissolving NaCl in dechlorinated tap water. **P* < 0.05 compared with the value at 0 h (one-way ANOVA). Each value is mean ± SE (n = 8 per group for each sampling time).

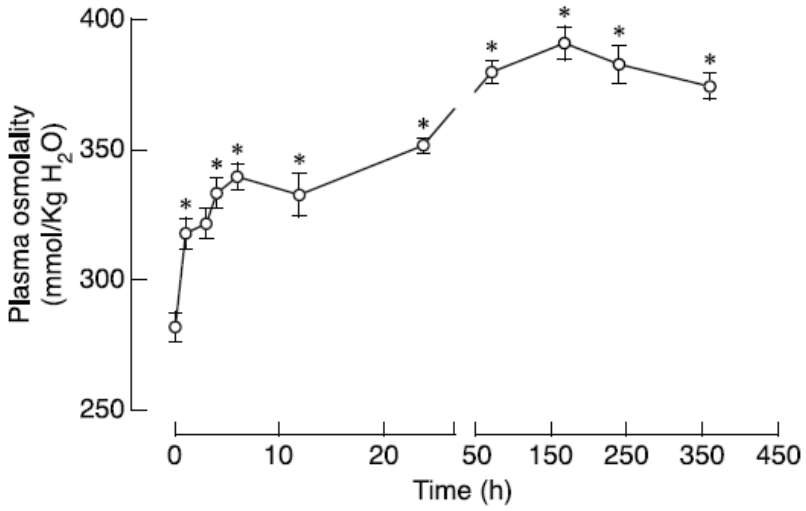

Fig. 4-13. Changes in plasma osmolality in *C. striata* after an abrupt transfer from fresh water to salt water. **P* < 0.05 compared with the value at 0 h (one-way ANOVA). Each value is mean ± SE (n = 8 per group for each sampling time).

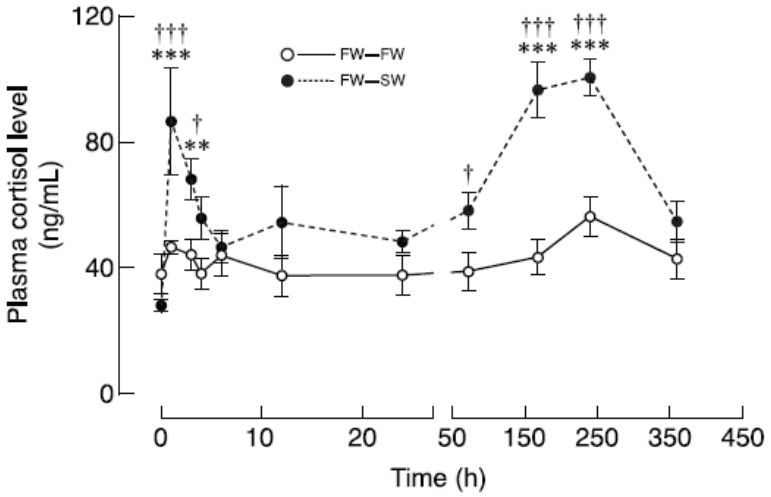

Fig. 4-14. Changes in the plasma cortisol levels in *C. striata* after an abrupt transfer from fresh water to salt water (FW—SW), or from fresh water to fresh water (FW—FW). FW (0‰) was dechlorinated tap water. SW (10‰) was prepared by dissolving NaCl in dechlorinated tap water. ** $P < 0.01$, *** $P < 0.001$ compared with the value at 0 h; $^{\dagger\dagger\dagger}P < 0.001$ compared with the corresponding value in the FW—FW group (two-way ANOVA). Each value is mean ± SE (n = 8 per group for each sampling time).

Figs. 4-15 and 4-16 show the plasma glucose and lactate levels, respectively, which were obtained from FW—FW (control) and FW—SW groups. In the FW—SW group, increases in both plasma glucose and lactate levels were observed within the first hour post-transfer as compared to the corresponding FW—FW group, before returning to the control levels at 4 h. The control group exhibited a relatively constant value throughout the experimental period.

The plasma PRL levels in the FW—SW group gradually decreased and significantly different from those the FW—FW group at end of the experiment (15 days after transfer) (Fig. 4-17). The control group exhibited a relatively constant value throughout the experimental period.

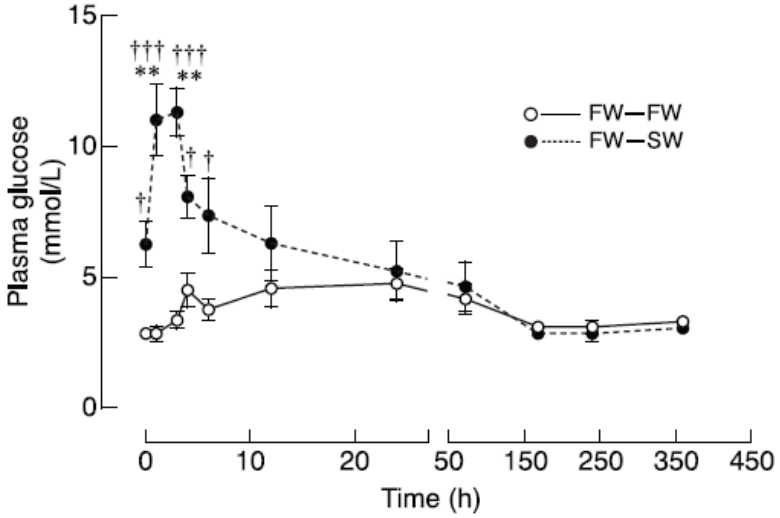

Fig. 4-15. Changes in plasma glucose and in *C. striata* after an abrupt transfer from fresh water to salt water (FW—SW), or from fresh water to fresh water (FW—FW). Each value is mean ± SE (n = 8 per group for each sampling time). ** $P < 0.05$ compared with the value at 0 h; † $P < 0.01$, and ††† $P < 0.001$ compared with the corresponding value in the FW—FW group (two-way ANOVA).

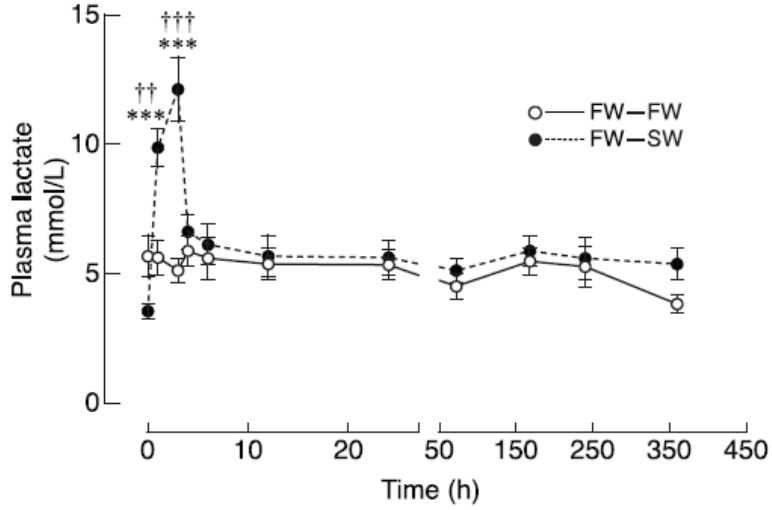

Fig. 4-16. Changes in plasma lactate in *C. striata* after FW—SW), or FW—FW. Each value is mean ± SE (n = 8 per group for each sampling time). *** $P < 0.001$ compared with the value at 0 h; †† $P < 0.05$ and ††† $P < 0.001$ compared with the corresponding value in the FW—FW group (two-way ANOVA).

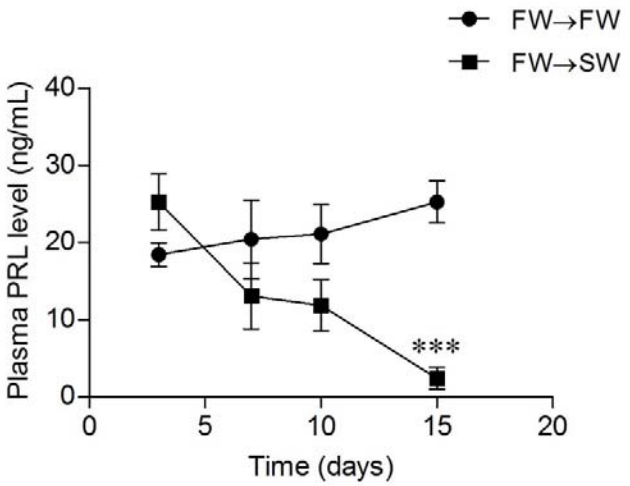

Fig. 4-17. Changes in the plasma PRL level in *C. striata* after an abrupt transfer from fresh water to salt water (FW—SW), or from fresh water to fresh water (FW—FW). Each value is mean ± SE (n = 8 per group for each sampling time). *** $P < 0.001$ compared with the corresponding value in the FW—FW group (one-way ANOVA)

The expression of PRLR gene in the gill of the FW—SW group decreased when compared to the control FW—FW group at days 3, 7 and 10, however, its expression levels showed higher than the control group at the end of the experiment (Fig. 4-18). The expression levels of the gene in the kidney of the FW—SW group were not different from the control FW—FW group (Fig. 4-19). The expression levels of the gene in the esophagus of the FW—SW group were lower than the control FW—FW group at days 3, 7 and 15, but its level was comparable to the control FW—FW group at day 10 (Fig. 4-20).

Moreover, FW—SW group showed a 2-fold increase in the branchial Na^+/K^+-ATPase activity on day 7 (168 h) compared to the baseline level (0 h), which remained at the high level until the end of experiment (Fig. 14-21). The branchial Na^+/K^+-ATPase activity of FW—FW group was not altered.

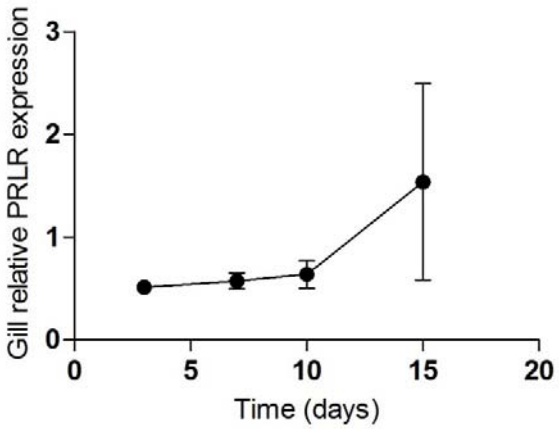

Fig. 4-18. Changes in the relative expression of mRNA PRLR in gill of *C. striata* after an abrupt transfer from fresh water to salt water (FW—SW) to those of from fresh water to fresh water (FW—FW). Each value is mean ± SE (n = 8 per group for each sampling time).

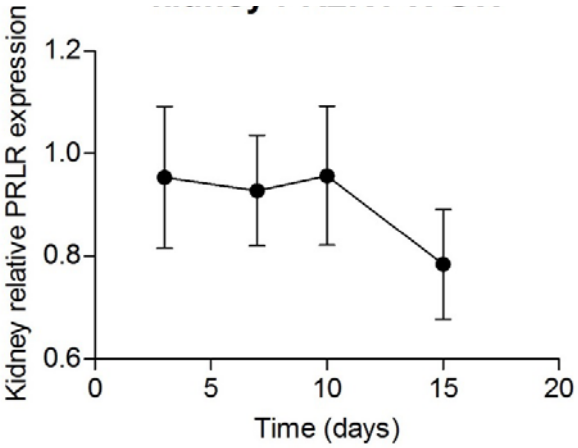

Fig. 4-19. Changes in the relative expression of mRNA PRLR in kidney of *C. striata* after an abrupt transfer from fresh water to salt water (FW—SW) to those of from fresh water to fresh water (FW—FW). Each value is mean ± SE (n = 8 per group for each sampling time).

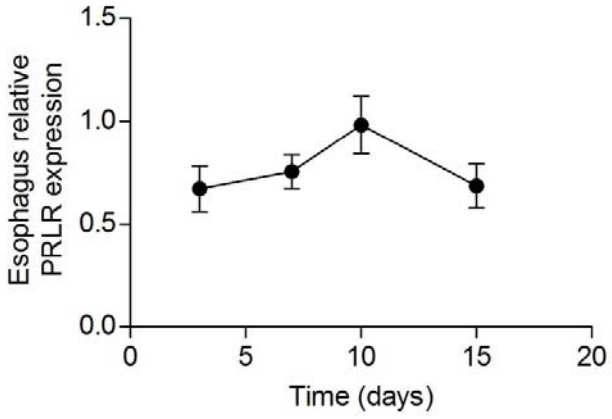

Fig. 4-20. Changes in the relative expression of mRNA PRLR in esophagus of *C. striata* after an abrupt transfer from fresh water to salt water (FW—SW) to those of from fresh water to fresh water (FW—FW). Each value is mean ± SE (n = 8 per group for each sampling time).

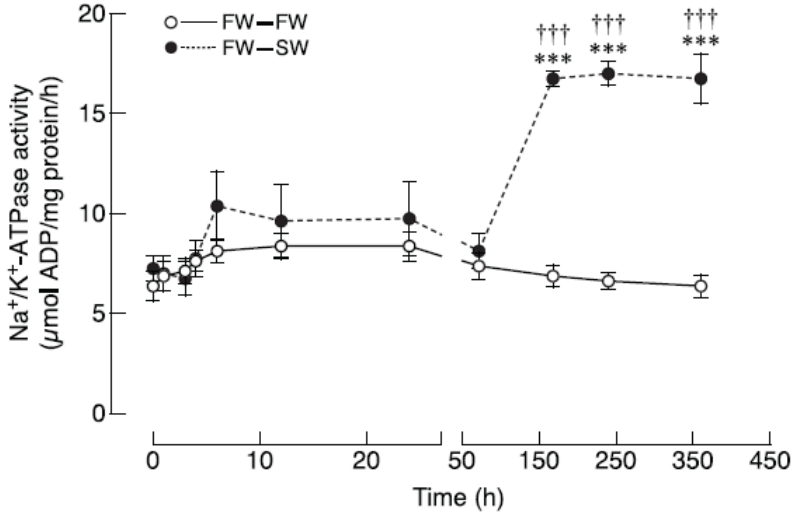

Fig. 4-21. Changes in the branchial Na^+/K^+-ATPase activity in *C. striata* after an abrupt transfer from fresh water to salt water (FW—SW), or from fresh water to fresh water (FW—FW). Each value is mean ± SE (n = 8 per group for each sampling time). *** $P < 0.001$ compared with the value at 0 h; ††† $P < 0.001$ compared with the corresponding value in the FW—FW group (two-way ANOVA).

The kidney Na^+/K^+-ATPase activity of the FW—SW group was significantly increased form the control FW—FW group at day 3, but was not different at day 7 and then increased again at day 10 to the end of the experiment (day 15). The kidney Na^+/K^+-ATPase activity of FW—FW group was not altered (Fig. 4-22). The esophagus Na^+/K^+-ATPase activity of the FW—SW group was comparable to the control FW—FW group and that of the FW—FW group was not altered (Fig. 4-23).

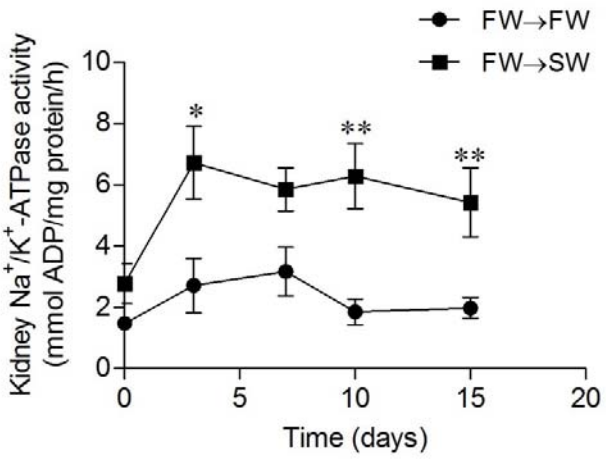

Fig. 4-22. Changes in Na^+/K^+-ATPase activity in kidney of *C. striata* after an abrupt transfer from fresh water to salt water (FW—SW), or from fresh water to fresh water (FW—FW). Each value is mean ± SE (n = 8 per group for each sampling time). ** $P < 0.05$ compared with the corresponding value in the FW—FW group (two-way ANOVA)

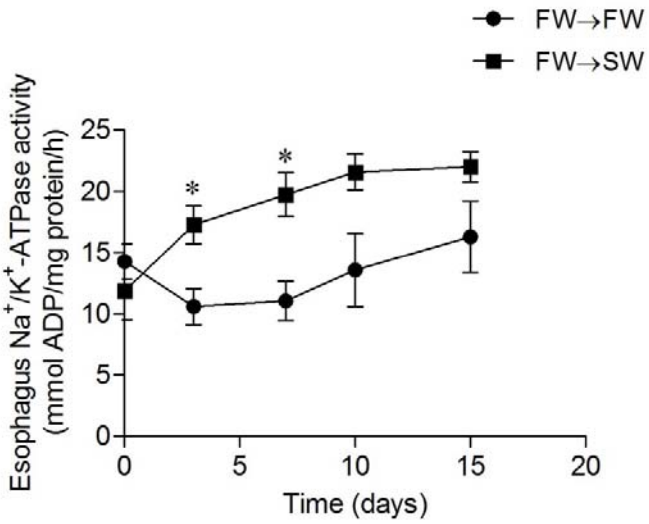

Fig. 4-23. Changes in Na^+/K^+-ATPase activity in esophagus of *C. striata* after an abrupt transfer from fresh water to salt water (FW—SW), or from fresh water to fresh water (FW—FW). Each value is mean ± SE (n = 8 per group for each sampling time).

Experiment 4: Response of SW-acclimated fish to hypoosmotic environment

In this series of experiments, the 10‰ SW-acclimated fish were transferred to either FW (experimental group; SW—FW) or SW (control group; SW—SW). Plasma cortisol levels in both control and experimental groups appeared higher at the first hour post-transfer as compared to the baseline level at 0 h (Fig. 4-24), before returning to the baseline level thereafter. Plasma osmolality and concentrations of Na^+ and Cl^- rapidly decreased during the first hour post-transfer, and remained relatively constant until the end of experiment (Figs. 4-25 and 4-26).

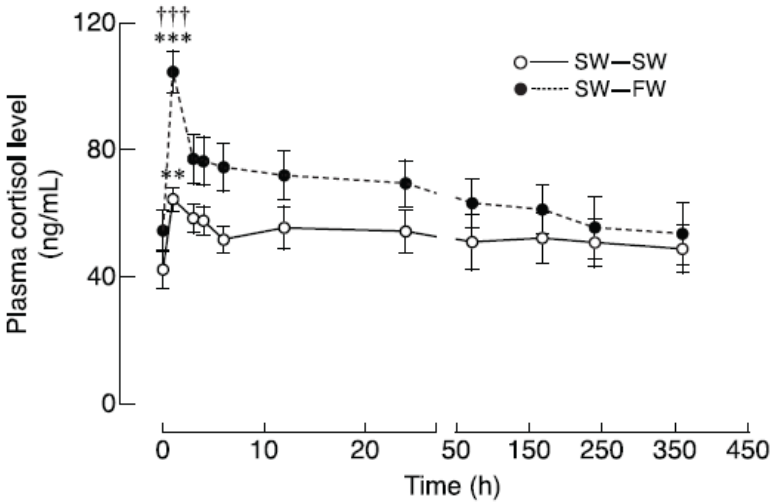

Fig. 4-24. Changes in the plasma cortisol in *C. striata* after being transferred from salt water to fresh water (SW—FW), or from salt water to salt water (SW—SW). ** $P < 0.01$, *** $P < 0.001$ compared with the value at 0 h; ††† $P < 0.001$ compared with the corresponding value in the SW—SW group (two-way ANOVA).

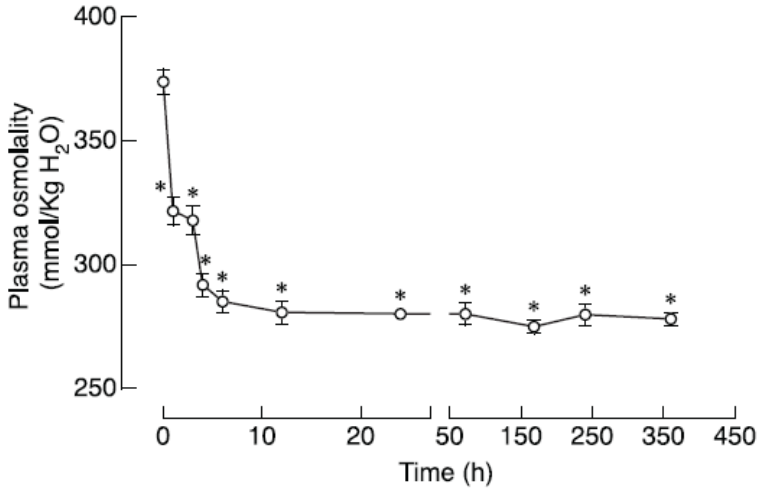

Fig. 4-25. Changes in plasma osmolality in *C. striata* after being transferred from salt water to fresh water. * $P < 0.05$ compared with the value at 0 h (one-way ANOVA). Each value is mean ± SE (n = 8 per group for each sampling time).

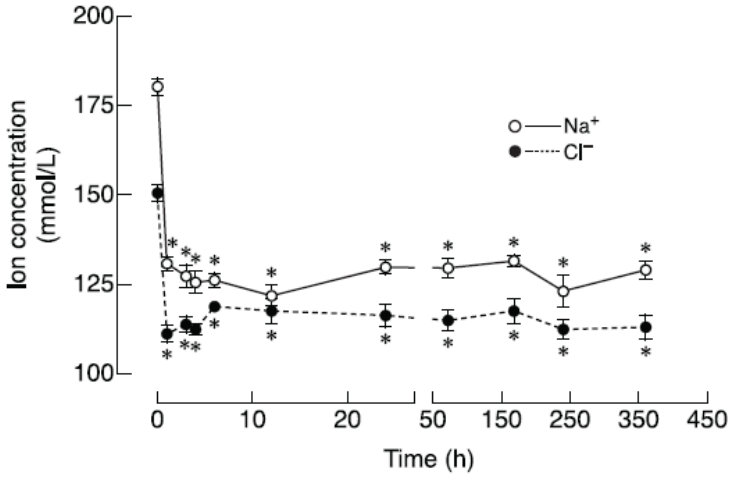

Fig. 4-26. Changes in plasma Na^+ and Cl^- levels in *C. striata* after being transferred from salt water to fresh water. * $P < 0.05$ compared with the value at 0 h (one-way ANOVA). Each value is mean ± SE (n = 8 per group for each sampling time).

Plasma glucose reached the peak at 3 h and then decreased to the baseline level, while that of the control fish (SW—SW) remained unaltered throughout the experiment (Fig. 4-27).

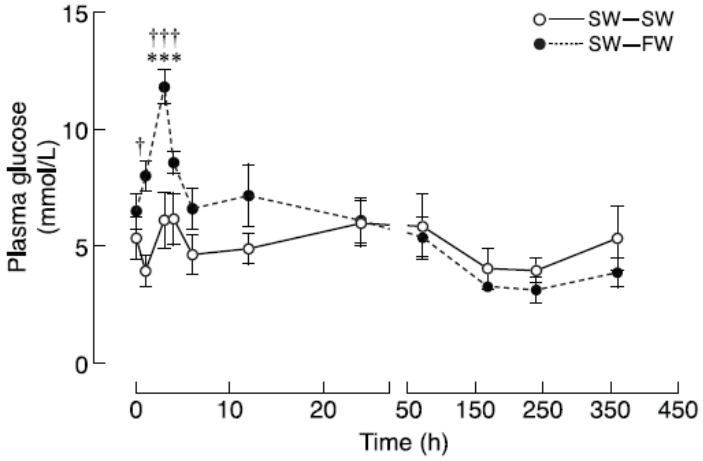

Fig. 4-27. Changes in the plasma glucose in *C. striata* after being transferred SW—FW, or SW—SW. Each value is mean ± SE (n = 8 per group for each sampling time). *** $P < 0.001$ compared with the value at 0 h; † $P < 0.05$, ††† $P < 0.001$ compared with the corresponding value in the SW—SW group (two-way ANOVA).

Both SW—FW and SW—SW groups had comparable levels of plasma lactate (Fig. 4-28) and plasma PRL level (Fig. 14-29).

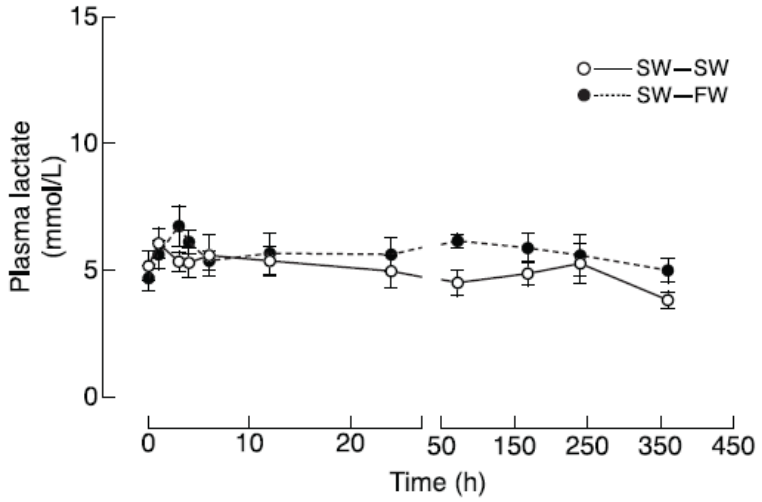

Fig. 4-28. Changes in the plasma lactate in *C. striata* after being transferred from salt water to fresh water (SW—FW), or from salt water to salt water (SW—SW). Each value is mean ± SE (n = 8 per group for each sampling time).

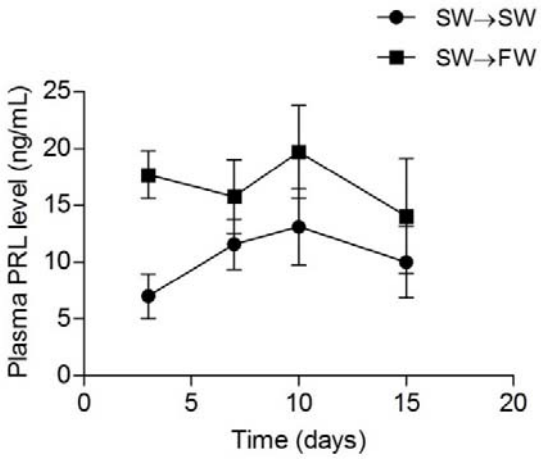

Fig. 4-29. Changes in the plasma prolactin in *C. striata* after being transferred from salt water to fresh water (SW—FW), or from salt water to salt water (SW—SW). Each value is mean ± SE (n = 8 per group for each sampling time).

The expression of PRLR mRNA in gill of snakehead fish that were transferred from SW–FW was elevated higher than the SW–SW group approximated 3 folds in day 3 after transfer (Fig. 4-30). At day 7 and 10, the expression levels still higher than control group (~2 folds) and at the end of the experiment, the expression level decreased to the same level of the control group

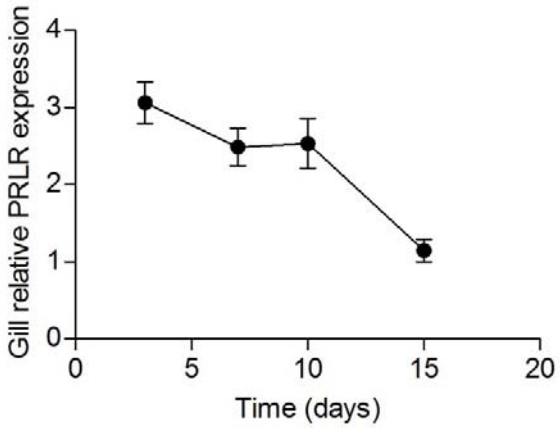

Fig. 4-30 Changes in the relative expression of mRNA PRLR in gill of *C. striata* after an abrupt transfer from salt water to fresh water (SW—FW) to those of from salt water salt water (SW—SW). Each value is mean ± SE (n = 8 per group for each sampling time).

The relative PRLR expression in kidney of snakehead transferred from SW–FW to SW–SW was increased throughout the experimental period (ratio > 1) (Fig. 4-31). In the esophagus, the PRLR expression level was down in group of snakehead transferred from SW–FW at day 3 to day 10 and then the expression level elevated at the end of the experiment (Fig. 4-32).

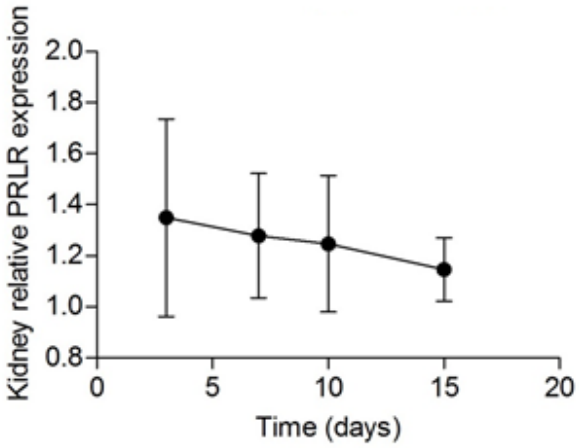

Fig. 4-31 Changes in the relative expression of mRNA PRLR in kidney of *C. striata* after an abrupt transfer from salt water to fresh water (SW—FW) to those of from salt water salt water (SW—SW). Each value is mean ± SE (n = 8 per group for each sampling time).

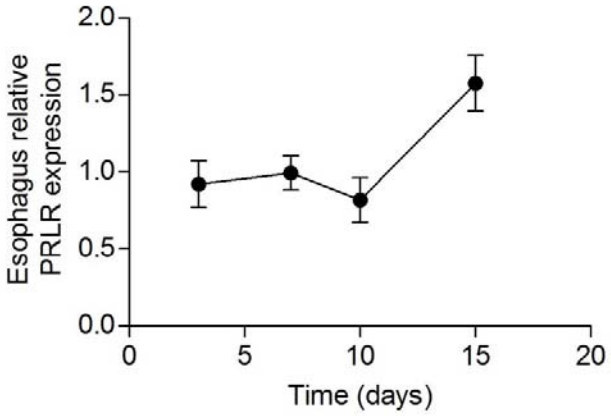

Fig. 4-32. Changes in the relative expression of mRNA PRLR in esophagus of *C. striata* after an abrupt transfer from salt water to fresh water (SW—FW) to those of from salt water salt water (SW—SW). Each value is mean ± SE (n = 8 per group for each sampling time).

Both SW—FW and SW—SW groups had comparable levels of branchial Na^+/K^+-ATPase and kidney activities (Figs. 4-33 and 4-34). The esophageal Na^+/K^+-ATPase activity significantly decreased at day 3 to the end of the experiment when compared to the control SW—SW group (Fig. 4-35). The activity of the control group unchanged throughout the experimental period.

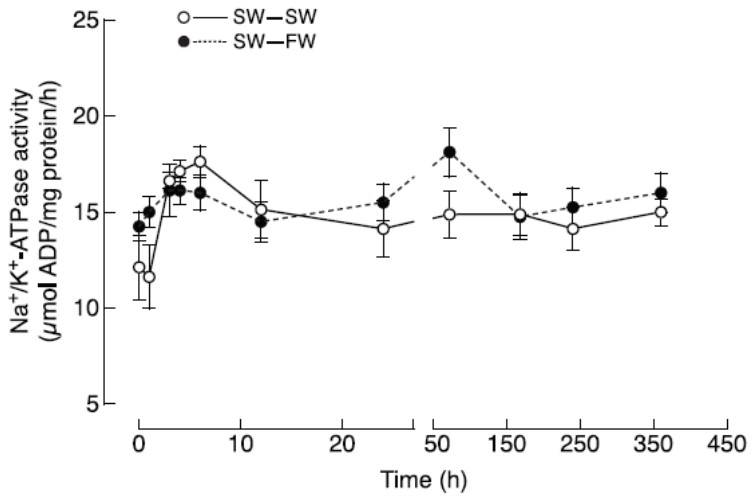

Fig. 4.33. Changes in the branchial Na^+/K^+-ATPase activity in *C. striata* after being transferred from salt water to fresh water (SW—FW), or from salt water to salt water (SW—SW). Each value is mean ± SE (n = 8 per group for each sampling time).

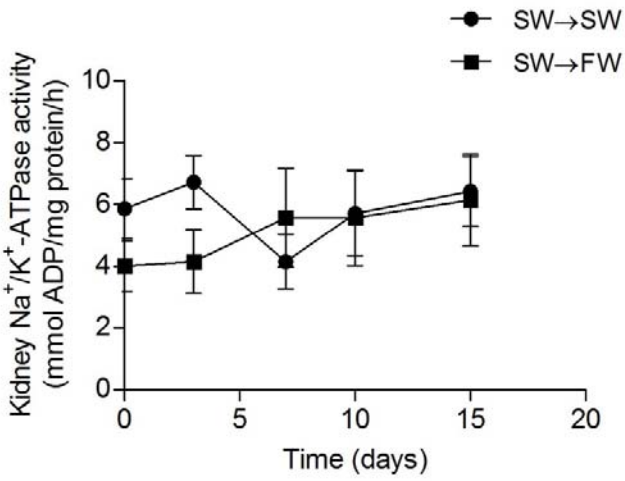

Fig. 14.34. Changes in the kidney Na^+/K^+-ATPase activity in *C. striata* after being transferred from salt water to fresh water (SW—FW), or from salt water to salt water (SW—SW). Each value is mean ± SE (n = 8 per group for each sampling time).

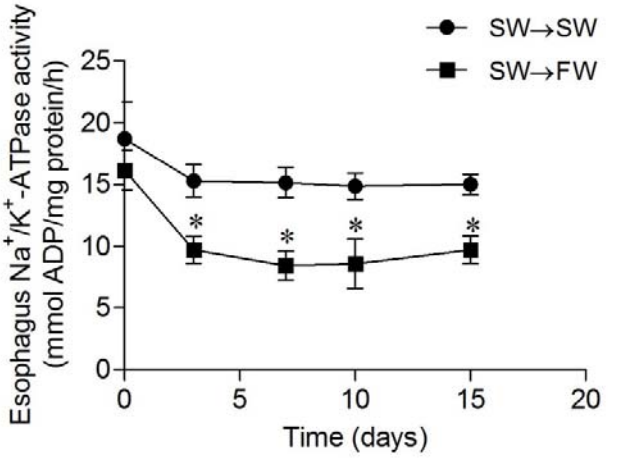

Fig. 4.35. Changes in the esophagus Na^+/K^+-ATPase activity in *C. striata* after being transferred from salt water to fresh water (SW—FW), or from salt water to salt water (SW—SW). Each value is mean ± SE (n = 8 per group for each sampling time).

Part V

Discussion

PRL initiates its actions through binding to a specific cell surface PRL receptor (PRLR), which has been cloned in several mammalian (Boutin et al., 1989; Edery et al., 1989; Scott et al., 1992; Shirota et al., 1990), avian (Chen and Horseman, 1994; Tanaka et al., 1992; Zhou et al., 1996), and amphibian (Huang and Brown, 2000) species. These receptors belong to the class I cytokine receptor superfamily, including receptors for growth hormone and leptin. In teleosts, PRLR cDNAs have been cloned in tilapia (*Oreochromis mossambicus*) (Sandra et al., 1995), goldfish (*Carassius auratus*) (Tse et al., 2000), rainbow trout (*Oncorhynchus mykiss*) (Le Rouzic et al., 2001), and sea bream (*Sparus aurata*) (Santos et al., 2001). The overall structures of these fish PRLRs are similar to those of the long isoform of mammalian PRLRs. The PRLR mRNA was heavily expressed in osmoregulatory organs such as gills, kidney, and intestine in tilapia (Sandra et al., 1995), goldfish (Tse et al., 2000), and rainbow trout (Le Rouzic et al., 2001). The results are in good agreement with the observation that PRL modulates hydromineral balance in those organs of FW and euryhaline teleosts (Auperin et al., 1994; McCormick, 1995).

In vertebrate species, PRLRs conserve structural features common to the members of the class I cytokine receptor superfamily, such as two pairs of cysteine residues, a WSXWS motif, a single transmembrane domain, and the box 1 region to interact with Jak kinases mediating signals of PRL (Bole-Feysot et al., 1998; Hennighausen et al., 1997; Lebrun et al., 1995). Snakehead PRLR also possesses all these characteristic features, sharing amino acid sequence identities with those of other teleosts (58-68%)) and mammalian species (42-48%).

The present RT-PCR analysis demonstrated that PRLR was intensively expressed in the gills and kidney. It should be noted that those PRLR-expressing organs are closely associated with osmoregulation, suggesting the involvement of PRL and PRLR in regulation of hydromineral balance in those organs. The study in puffyfish (*Takifugu rubripes*) showed that hybridization signals were detected in osmoregulatory epithelia in those organs; chloride

cells in the gills, proximal convoluted tubule cells in the kidney, and mucosal epithelial cells in the intestine.

The snakehead is an economic food fish in Southeast Asian countries with high market value. In the Northeastern Thailand, snakehead are mostly captured from natural water sources that are often contaminated by rock salt and had salinity fluctuation; however, they have an ability to tolerate in this threaten condition. To live in ion-rich waters, the snakehead has to develop some osmo- and ionoregulatory mechanisms to prevent stress from salinity change. We showed in the present study that snakehead was capable of adapting to salt water with low-to-moderate salinity, similar to that observed in other freshwater teleosts, e.g., white sucker (*Catostomus commersoni*) (Wilkes and McMahon, 1986), common carp (*Cyprinus carpio*) (Wang et al., 1997) and goldfish (*Carassius auratus*) (Luz et al., 2008). Exposure to hyperosmotic environment was found to induce loss of free water from the plasma and then soft tissues, such as muscle, thereby leading to an increase in hematocrit and a reduction in muscle water content, respectively. However, exposure to > 10‰ salinity resulted in 100% mortality within 24 h, indicating that salinities around 10‰ were the upper limit of tolerance for this species.

The common responses of stenohaline freshwater fish to salinity changes are the increased plasma osmolality and ion concentrations (Benli and Yildiz, 2004; De Boeck et al., 2000; Eckert et al., 2001; Luz et al., 2008; Tam et al., 2003), both of which were observed in snakehead. The plasma hyperosmolality-induced tissue water loss—as indicated by muscle dehydration—was not only found in snakehead but also occurred in common carp (Van der Linden et al., 1999) and goldfish (Luz et al., 2008). In addition, a decrease in plasma free water also increased hematocrit in snakehead (data not shown), similar to that observed in white sucker (*C. commersonii*) (Wilkes and McMahon, 1986). However, hematocrit was not altered in some other species, such as goldfish (*C. auratus*), tilapia (*O. mossambicus*) and gray snapper (*Lutjanus griseus*) after a direct salinity exposure (Kammerer, et al., 2010; Luz et al., 2008; Sardella and Brauner, 2008; Sardella et al., 2004; Serrano et al., 2011). Indeed, the upper limit of salinity tolerance in stenohaline freshwater fish was determined by their ability to deal with soft tissue dehydration and to restrict the increasing plasma osmolality. Unlike the snakehead, several euryhaline species had a robust osmoregulatory capacity, and

a tremendous salinity change may not much affect plasma osmolality. Therefore, some euryhaline teleosts (e.g., *Poecilia latipinna*, *Cyprinodon variegates, O. mossambicus* □ *O. urolepis hornorum,* and *L. griseus*) could be reared in extreme salinities before plasma osmolality variation was observed (Gonzalez et al., 2005; Nordlie, 1985; Nordlie and Walsh, 1989; Sardella et al., 2004; Serrano et al., 2011).

Once being exposed to hyperosmotic environment (i.e., salt water), the body ion homeostasis was markedly disturbed, leading to acute stress and hence elevation of plasma cortisol concentration. Salinity changes could signal to stimulate the hypothalamic-pituitary-interregnal axis, which ultimately resulted in the increased plasma cortisol, as observed in the killifish (*F. heteroclitus*) (Jacob and Taylor, 1983; Marshall et al., 1999) and tilapia (*O. mossambicus*) (Morgan et al., 1997). Our results indicate that, after being transferred into salt water, the snakehead exhibited two peaks of cortisol release, i.e., (*i*) an increase within 1 h presumably due to stress, and (*ii*) an increase from day 3–10 as a result of osmoregulatory adaptation, presumably to help enhance the activity of Na^+/K^+-ATPase.

Since acute stress was an energy-demanding process as evidenced by the stress-induced increases in metabolic rate and oxygen uptake in fish (Barton et al., 2002), the first peak of cortisol owing to acute stress might play an important role in a rapid production of glucose and lactate, both of which could be used as energy sources for cell metabolism. Elevation of plasma glucose and lactate rapidly occurred after hyperosmotic exposure, suggesting that glycogenolysis known to be enhanced by cortisol (Mommsen et al., 1999; Vijayan et al., 1997) had contributed to a rapid provision of glucose and lactate. Such the cortisol action appeared to be non-genomic, involving alteration in the phosphorylation-dephosphorylation status of glycogen phosphorylase (Gomez-Munoz et al., 1989). However, since this hyperglycemia was transient, gluconeogenesis, the activation of which was time-consuming, was not involved in providing glucose during saltwater exposure (van der Boon et al., 1991; Wendelaar Bonga, 1997). Meanwhile, lactate was generated from pyruvate in anaerobic respiration to produce additional energy during saltwater acclimation. The saltwater-acclimated tilapia (*O. mossambicus*) showed an increased expression of lactate dehydrogenases in the branchial mitochondria-rich cells (Tseng et al., 2007). Taken together, to cope with osmotic stress, plasma glucose and lactate were used as additional energy

sources for the body as well as branchial Na^+/K^+-ATPase in snakehead during adaptation in hyperosmotic environment.

The second phase of cortisol release might be directly associated with the long-lasting osmoregulatory mechanism. It was evident that cortisol was also a seawater-adapting hormone by stimulating the branchial chloride cell differentiation and Na^+/K^+-ATPase activity (Madsen and Bern, 1993; Madsen et al., 1995). Gill is the major osmoregulatory organ in fish for both saltwater and freshwater acclimation. In fish acclimated to hyperosmotic environment, Na^+/K^+-ATPase in the gill epithelium is an essential transporter in removing excess ions from the body (Marshall, 2002; McCormick, 2001). Cortisol might also provide glucose and lactate to energize the process of de novo Na^+/K^+-ATPase synthesis and Na^+/K^+-ATPase activity. Indeed, cortisol itself has been reported to directly activate Na^+/K^+-ATPase in several teleost species, such as *Oncorhynchus kisutch* (Björnsson et al., 1987), *Anguilla rostrata* (Butler and Carmichael, 1972), *O. mossambicus* (Dange, 1986) and *F. heteroclitus* (Pickford et al., 1970). In addition, a reduction in the gill Na^+/K^+-ATPase activity in hypophysectomized killifish (*F. heteroclitus)* could be restored by cortisol supplementation (Pickford et al., 1970). In vitro treatment of cortisol to primary gill tissue culture of coho salmon (*O. kisutch*) was also found to increase Na^+/K^+-ATPase activity in a dose-dependent manner (McCormick and Bern, 1989).

Although the second phase of cortisol release on day 3 post-saltwater exposure could explain a high activity of branchial Na^+/K^+-ATPase on day 7, there was a latent period in the saltwater adaptation between the plasma cortisol peak and the increased activity of Na^+/K^+-ATPase. In *Anguilla japonica*, the plasma cortisol increased 2 h after saltwater exposure, and the drinking rate increased after a lag and reached its maximum within 48 h (Hirano and Utida, 1971). On the other hand, in *A. rostrata*, the plasma cortisol increased within 24 h after saltwater exposure, while the branchial Na^+/K^+-ATPase activity gradually increased and reached the maximal rate within 9–14 days (Forrest et al., 1973). The presence of latent period suggested the induction of time-consuming molecular or metabolic processes by cortisol in association with the adaptive mechanisms.

When saltwater-acclimated snakehead fish were transferred to fresh water, they exhibited a rapid loss of ion from the body, leading to acute stress with the elevated plasma

levels of cortisol and glucose. Glucose could be used to energize the branchial Na^+/K^+-ATPase in snakehead, which showed high activity throughout 15 days (360 h) post-transfer. Several investigations suggested that cortisol played a role in ion uptake in freshwater- and brackish-adapted fish by increasing Na^+/K^+-ATPase density in the gill chloride cells (Dang et al., 2000; Eckert et al., 2001; Laurent and Perry, 1990). Many studies have shown the upregulation of Na^+/K^+-ATPase activity in freshwater-acclimated fishes (Ciccotti et al., 1994; Doneen, 1981; Gallis et al., 1979; Kelly et al., 1999; Lasserre, 1971; Lin et al., 2003; Stagg and Shuttleworth, 1982; Woo and Chung, 1995). During hypoosmotic acclimation, the branchial Na^+/K^+-ATPase becomes an important transporter to help restrict salt loss and maintain Na^+- and H^+-gradients, which generate driving force for ion regulation (Lin and Randall 1993; Evans el al. 2005; Hirose et al. 2003). In teleost species, several studies have localized the Na^+/K^+-ATPase to the basolateral membrane of the mitochondria rich cells (Christensen et al., 2011; Uchida et al., 1996; Witters et al., 1996). Therein, the role of Na^+/K^+-ATPase is to generate low intracellular Na^+ concentration by pumping Na^+ across the basolateral membrane into the blood, which leads to the inward movement of the surrounding Na^+ down a steeper Na^+ gradient across the apical membrane into the cell (Avella and Bornancin, 1989; Lin and Randall, 1993). Therefore, it was not surprising to observe an increase in Na^+/K^+-ATPase activity in both FW—SW and SW—FW adaptations. Studies in Atlantic salmon (*Salmo salar*), rainbow trout (*O. mykiss*) and Arctic char (*Salvelinus alpines)* agreed with our observation where the Na^+/K^+-ATPase activity was found to increase during freshwater and saltwater acclimation (Bystriansky et al., 2006; Bystriansky et al., 2007; Bystriansky and Schulte, 2011). Since these three fish species showed the gill Na^+/K^+-ATPase isoform switching between α1a and α1b catalytic subunits during acclimation, the snakehead might also use similar mechanism to modulate the activity of Na^+/K^+-ATPase.

Meanwhile, the plasma K^+ level did not change significantly after exposure to different salinities, similar to that reported in *O. mossambicus* (Morgan et al., 1997). This was probably due to the lower gradient of K^+ concentration between the blood (8.42 ± 1.14 mM in freshwater-acclimated fish) and salt water (ranging 0–0.3 mM) compared to that of Na^+ and Cl^-. Exchanging K^+ between plasma and the intracellular compartment could also prevent an

alteration in the plasma K^+ level. In addition, plasma HCO_3^- concentration could indirectly reflect blood pH, which may be decreased by lactic acidosis. However, despite an increase in lactate production in the first hour of saltwater exposure, there was no change in the plasma HCO_3^- concentration, suggesting that lactic acidosis did not occur.

In conclusion, the snakehead was capable of living in low-to-moderate salinities (< 10‰) for an extended period of time (i.e., up to ~15 days). This stressful condition might induce cortisol secretion from the interrenal cells, which, in turn, enhanced the production of glucose and lactate presumably through glycogenolysis and anaerobic respiration (glycolysis), respectively. Both glucose and lactate could further energize Na^+/K^+-ATPase, which was the important transporter for the branchial ion excretion during FW—SW acclimation or for generation of driving force for sodium uptake during SW—FW acclimation. Cortisol itself also directly increased the Na^+/K^+-ATPase activity to maintain osmoregulation and ionoregulation (Björnsson et al., 1987). When saltwater-acclimated snakehead was placed in fresh water, the plasma osmolality and ion concentrations were normalized, but acute stress was also observed as indicated by transient increases in the plasma cortisol and glucose levels. The present findings, therefore, underline the reason why salinity must be monitored and controlled tightly in commercial fish farming as well as in natural water sources.

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