Effects of Blood Withdrawal and Angiotensin II on Prolactin Release in the Tilapia, *Oreochromis mossambicus*

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI'I IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

ANIMAL SCIENCE

MAY 2005

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I dedicate this thesis to my family for their tireless support, encouragement, and occasional nagging.

ACKNOWLEDGEMENTS

I thank faculty staff and students of the fish endocrinology laboratory at the Hawaii Institute of Marine Biology, especially Larry Riley and Andre Seale for their contributions and assistance throughout the production of this thesis.

I would like to thank Dr. Hal Richman and Professor Milt Stetson for their valuable advice and encouragement which led to the maturation of this thesis work as well as my personal professional development.

Finally, special gratitude goes out to Professor Tetsuya Hirano and my advisor, Professor E. Gordon Grau for teaching me the art of science, for their encouragement, and for their assistance in guiding me through the countless hurdles of my thesis research. I further thank Gordon and Tetsuya for allowing me to realize my potential and for the opportunity to have worked and learned under their guidance.

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Chapter I

General Introduction

The Tilapia

The tilapia, *Oreochromis mossambicus*, is a cichlid teleost of East-African origin. Through evolutionary adaptation to its native coastal lagoon and estuarine habitat, the tilapia has developed unique characteristics that allow for survival in poor water conditions and fluctuating salinities (Philippart and Ruwet, 1982; Trewas *et al.*, 1983; Payne *et al.*, 1988). Tilapia are capable of inhabiting environments with fluctuating salinities from 0 –120 ppt and can reproduce in salinities up to 49 ppt (Popper and Licharowich, 1975; Philippart and Ruwet, 1982). The ability of tilapia to adapt readily to a variety of conditions, combined with a steadily growing popularity as a food source and an aquaculture species, has lead to its rapidly expanding worldwide distribution. In fact, tilapia represent the fastest growing component of aquaculture in the United States and are a major source of protein worldwide (USDA/ERS, 2001).

Growth and development are governed in fish, as in all vertebrates, through the orderly release of hormones from the neuroendocrine system, which integrates environmental, physiological, and genetic information. Knowledge of the hormones to regulate growth and development is currently used to enhance growth and the efficiency of feed utilization in livestock industry (Scanes and Bailey, 1993; Stenholm and Waggoner 1992). However, their use to enhance aquaculture yields has not occurred due to a lack of basic understanding on their mode of action in fish. Of particular note is the overlapping roles of hormones that regulate growth, metabolism, and osmoregulation.

Fish Osmoregulation

In all vertebrates, salt and water balance is governed to an important extent by the neuroendocrine array, which maintains precise control of blood osmolality and blood volume. Teleost fishes in particular face osmotic challenges that are not faced by terrestrial vertebrates. Due primarily to the direct contact of osmotic surfaces with the aquatic environment, a direct route for water and ions to move into or from the body is provided. In fact, blood is separated from environmental water by as little as a single cell layer in the gill epithilium (Maetz *et al.*, 1971). Thus, osmotic gradients between blood and the external environment promote passive movement of water and ions across permeable membranes to and from the environment.

Compensating for the osmotic dilution or gain of water and ions is a complex process as are the systems responsible for maintaining them. In fish, the kidney, gill, and intestine are the principal osmoregulatory surfaces and hence play critical roles in the accumulation or excretion of water and ions during both fresh water (FW)-and seawater (SW)-adaptation. The continuous maintenance of body fluid homeostasis may be the most fundamental physiological task performed by fish. In fact, between 30 - 50% of non-swimming metabolic output is consumed for osmoregulation in fish (Rao *et al.*, 1968; Nordlie *et al.*, 1975, 1991; Toepfer *et al.*, 1992; Bushnell *et al.*, 1992).

Most vertebrates maintain blood osmolality between 300 and 350 mOsmol/Kg. In contrast, osmolality of the marine environment is around 1000 mOsmol/Kg. Thus, teleosts in SW are faced with constant dehydration due largely to osmotic processes that passively draw water to the environment. This chronic loss of body water acts to concentrate ions and to reduce blood volume and pressure. To compensate, marine

teleosts replace lost body water by drinking environmental water (Hirano *et al.*, 1974; Takei, 2000). The embibed seawater is first diluted and subsequently absorbed, together with Na^+ and Cl^-, in the posterior intestine thus, compensating for the loss in blood volume while contributing to hypernatremia (Hirano *et al.*, 1976).

The majority of excess ions gained through drinking and subsequent water and ion absorption in the intestine are excreted from the body via well-developed chloride cells in the gill. These chloride cells, characterized by a high concentration of the sodium-potassium adenosinetriphosphatase (Na^+, K^+-ATPase), are essential for monovalent ion secretion in SW and likely for ion uptake in FW (Zadunaisky *et al.*, 1984; Marshall *et al.*, 1995). Intracellular Na^+ is excreted from chloride cells into an internal basal-lateral tubular system through the enzymatic action of Na^+, K^+-ATPase. The active extrusion of Na^+ establishes a negative intracellular potential, which is believed to facilitate the migration of Cl^- out of the cell. Consequently, it is believed that the excess Na^+ passively follows the excretion of Cl^- via leaky cellular junctions. Hence, blood osmolality is restored.

Organs important for maintaining osmoregulation in fish such as the gill, intestine, and kidney are characterized by high numbers of chloride cells. While the kidney is a likely route for the elimination of excess ions, the teleost glomeruli lacks the Loop of Henle and subsequently the capacity to produce a hyperosmotic urine. Moreover, since osmoregulation in the marine environment requires water conservation, the production of large volumes of urine for removal of excess ions would be contradictory to SW adaptation. Indeed, renal adaptation to SW transfer in rainbow trout is characterized by a reduction in overall glomerular filtration rate (Brown *et al.*, 1978).

Therefore, marine teleosts produce small amounts of isotonic urine composed largely of divalent ions (Beyenbach *et al.*, 1996). Active extrusion of excess monovalent ions is by chloride cells in the gill epithilium is therefore the key to osmoregulation in SW teleosts (Evans, 1993).

In contrast with SW adaptation, FW teleosts must maintain their internal osmolality above that of the external environment. Maintaining homeostasis in FW centers on counteracting a chronic loss of ions and the elevation of blood volume resulting from water influx from the environment. Thus, drinking is not necessary or even appropriate in FW. As in SW-adapted teleosts, FW fishes rely on chloride cells located on the gill lamellae to assist in maintaining ion balance. However, chloride cells in FW teleosts direct ions into the body, the opposite of what occurs in SW. The kidney plays a similarly important role as it is the sole means for the elimination of excess water while conserving ions. Thus, FW teleosts produce large amounts of hypotonic urine. Ions are further sequestered in the gut, which is relatively impermeable to water (Hirano, 1986).

The Role of Prolactin in Osmoregulation

As previously described, osmoregulatory processes in vertebrates are regulated largely by the neuroendocrine array. Among the most important elements controlling osmotic regulation are the pituitary hormones prolactin (PRL) and growth hormone (GH). As in all vertebrates, fish PRL plays a critical role in many different physiological processes (reviewed by Bole-Feysot, 1998). However, in fish the most important role of PRL is likely in maintaining ion homeostasis in hypoosmotic environments (Clarke and Bern, 1980; Hirano, 1986; Lorentz and Bern, 1982). The critical nature of PRL in FW

adaptation was first elucidated in the killifish (*Fundulus heteroclitus*) when Pickford and Phillips (1959) demonstrated that hypophysectomized killifish were incapable of survival in FW without supplementation with PRL. PRL has since been established as a central hormone to FW hydromineral regulation not only in killifish but in many other euryhaline teleost species such as salmonids and tilapias (Dharmamba *et al.*, 1967; Dharmamba *et al.*, 1972; Auperin *et al.*, 1995). These studies as well as many others have lead researchers to consider PRL as the most important FW-adapting hormone (Ball *et al.*, 1969; Bern, 1975, 1983; Brown *et al.*, 1987; Clarke *et al.*, 1980; Hirano, 1986; Hirano *et al.*, 1987; Lorenz and Bern, 1982)

As with other peptide hormones, PRL exerts its regulatory action through membrane bound receptors in target tissues. To date, a single class of high affinity PRL receptors (PRL-R) has been identified in the tilapia (Sandra *et al.*, 2001). The fish PRL-R is a single pass transmembrane receptor in cytokine family similar in primary structure to the long form mammalian PRL-R (Sandra *et al.*, 1995). Regardless of environmental salinity the PRL-R gene expression is highest in gill, kidney, and posterior intestine (Dauder *et al.*, 1990; Auperin *et al.*, 1994a, 1995). Moreover, *in situ* hybridization studies have revealed that PRL-R expression is localized on chloride cells in the gill, kidney and intestine (Sandra *et al.*, 2000), underscoring the importance of PRL in the osmoregulatory process.

Given the role of chloride cells and Na^+, K^+-ATPase activity in FW-and SW-adaptation, an understanding of the modulation of Na^+, K^+-ATPase by PRL is clearly important. Consistent with its role in ion retention, the inhibitory effects of PRL on gill Na^+, K^+-ATPase have been established in several euryhaline fish (see review by

McCormick, 1995). PRL reduces gill Na^+, K^+-ATPase in killifish (Mayer-Gostan, 1978), rainbow trout, and tilapia (Fosket *et al.*, 1981, 1982). The reduction of Na^+, K^+-ATPase activity and sodium extruding capacity may be due to a decrease in chloride cell size and number, a characteristic of chloride cell morphology in FW-adapted teleosts. (Fosket *et al.*, 1982; Herndon *et al.*, 1991; Pisam *et al.*, 1993).

In the kidney, the role of PRL seems to be related primarily to water balance as assessed by measurement of Na^+, K^+-ATPase, urine composition, and urine flow rate indicating changes in water reabsorbtion and salt excretion (Lam *et al.*, 1969). Prolactin appears to act directly on the glomerulus morphology as evidenced by increased surface area (Braun and Dantzler, 1987). Moreover, PRL promotes ion reabsorbtion through stimulation of Na^+, K^+-ATPase in FW fishes (Gona, 1979; 1981; Madsen *et al.*, 1992; Pickford *et al.*, 1970). In addition, glomerular filtration rates and urine flow are increased by PRL, indicating an inhibitory effect of PRL on tubular water reabsorbtion (Clarke and Bern, 1980). This role for PRL in stimulating urine output suggests of a role in maintaining water balance in addition to its well-established role in ion conservation.

The intestine of the SW-adapted teleosts represents an important site of water and ion transport from the lumen to the blood. Conversely, it is necessary for the intestine in FW teleosts to be relatively impermeable to water while actively absorbing ions. Indeed, consistent with FW adaptation, PRL reduces water absorption in the Japanese eel and trout (Hirano, 1986; 1976; Morley *et al.*, 1981; Utida *et al.*, 1972) by reducing gut permeability. Furthermore, PRL promotes ion uptake through modulating of Na^+, K^+-ATPase in the intestine (Kelly *et al.*, 1999).

Regulation of Prolactin Synthesis and Release

The effects of environmental salinity and neuroendocrine factors on pituitary PRL secretion *in vitro* and *in vivo* have been well studied (Auperin *et al.*, 1994; Ayson *et al.*, 1993; Borski *et al.*, 1992; Dharmamba *et al.*, 1967; Grau *et al.*, 1987; Grau *et al.*, 1982; Helms *et al.*, 1991; Kelley *et al.*, 1988; Nagahama *et al.*, 1975; Nicoll *et al.*, 1981; Nishioka *et al.*, 1988; Shepherd *et al.*, 1997; Wigham *et al.*, 1977; Yada *et al.*, 1994), as have the specific stimuli responsible for PRL release and the first and second messenger systems involved (Borski *et al.*, 1991; Grau *et al.*, 1987, 1981, 1994; Nishioka *et al.*, 1994; Richman *et al.*, 1991, 1990; Wendelaar Bonga *et al.*, 1980, 1981, 1985, 1988). As in other vertebrates, tilapia PRL release is under strong inhibitory control by the hypothalamus (Grau *et al.*, 1982; Grau *et al.*, 1985; Nishioka e*t al.*, 1988; Helms *et al.*, 1991). Principal among hypothalamic inhibitors include somatostatin and dopamine (Grau *et al.*, 1982, 1985; Helms *et al.*, 1991; Kelley *et al.*, 1988; Nishioka *et al.*, 1988; Olivereaau *et al.*, 1984; Peter *et al.*, 1990; Wigham *et al.*, 1977). On the other hand, several PRL stimulators have recently been identified including three native forms of gonadotropin-releasing hormone (Weber *et al.*, 1997), thyrotropin-releasing hormone (Barry and Grau, 1985) and Carassius Rfamide, a homologue of mammalian prolactin-releasing peptide (Seale *et al.*, 2002).

A clear inverse relationship between plasma PRL levels and plasma osmolality has been established in many teleost species (Ball *et al.*, 1969; Olivereau *et al.*, 1968). Changes in plasma levels of PRL in during FW or SW adaptation are mediated largely through the direct effects of changes in extracellular osmolality on PRL cell function (Shepherd *et al.*, 1999). For example, PRL release from the tilapia pituitary is

remarkably sensitive to changes in extracellular osmolality as PRL release increases by four-fold in response to as little as 5% reduction in external osmolality (Grau, *et al.*, 1980; Nishioka *et al.*, 1988; Bern 1980; Grau *et al.*, 1994). In addition, medium osmolality well within the range observed in plasma during FW and SW adaptation is highly influential in pituitary PRL release *in vitro* (Grau, *et al.*, 1980; Ingleton *et al.*, 1973, Nagahama *et al.*, 1974, Sage *et al.*, 1965; Nishioka *et al.*, 1988; Grau and Helms, 1990; Grau *et al.*, 1994 In the tilapia, pituitary PRL release is proportional to changes in medium osmolality indicating direct action of extracellular osmolality on PRL release (Nagahama *et al.*, 1975; Grau *et al.*, 1987; Wigham *et al.*, 1977; Grau *et al.*, 1961).

The tilapia pituitary produces two distinct forms of PRL possessing 69% homology at the amino acid level. Although both PRLs are encoded by separate genes, they appear to be produced in the same cell (Specker *et al.*, 1993; Yamaguchi *et al.*, 1988; Rentier-Delrue *et al.*, 1989; Nishioka *et al.*, 1993). Prolactin$_{188}$ (PRL$_{188}$) containing 188 amino acid residues is similar in primary structure to other fish PRLs than prolactin$_{177}$ (PRL$_{177}$), which possesses 177 amino acid residues. In general, both forms possess similar bioactivity with respect to their role in hypoosmoregulation. Although both PRLs respond in a similar manner to changes in extracellular osmolality *in vivo* and *in vitro*, the release of both PRL's appears to be differentially regulated. For example, when tilapia are transferred from FW to SW, plasma levels of both PRLs are reduced. However, the ratio between the circulating levels of both PRLs often shifts in favor of PRL$_{177}$, suggesting a potential role for PRL$_{177}$ in SW adaptation. The underlying mechanisms for such a differential regulation remain largely unknown (Borski *et al.*, 1992; Ayson *et al.*, 1993; Yada *et al.*, 1994).

Both PRLs exhibit strong ion-retaining actions *in vivo*. Prolactin$_{188}$ injection results in a dose-related increase in plasma ion content in SW-adapted tilapia, a condition contradictory to SW adaptation. Prolactin$_{177}$ similarly increased plasma ion content in FW-and SW-adapted tilapia, although a larger dose was needed and the effect was not found to be dose-related (Sakamoto *et al.*, 1997; Auperin *et al.*, 1994). Thus, PRL$_{188}$ seems to be more potent in ion-retention than PRL$_{177}$. A change in the ratio between plasma levels of both PRLs has been used to suggest a differential regulation upon transfer to SW from FW. While PRL$_{188}$ decreases to below detection, plasma PRL$_{177}$ levels, although significantly reduced, plateau suggesting a possible role in SW adaptation (Auperin *et al.*, 1994; Auperin *et al.*, 1995; Ayson *et al.*, 1993; Borski *et al.*, 1992; Yada *et al.*, 1994; Yoshikawa-Ebesu *et al.*, 1995; Specker *et al.*, 1985). Furthermore, PRL$_{177}$ but not PRL$_{188}$, is capable of binding GH receptors, suggesting a somatotropic action for PRL$_{177}$ (Shepherd *et al.*, 1997; Leedom *et al.*, 2002). Interestingly, the tilapia PRL receptor seems to have a higher affinity for PRL$_{188}$ than PRL$_{177}$ (Auperin *et al.*, 1994), indicating a potential for differential signal transduction at the receptor level.

Reflecting an important role in FW adaptation, plasma levels of both PRLs in tilapia are inversely correlated to plasma osmolality. Plasma levels are higher in FW-adapted fish compared with SW-adapted fish. Increases in plasma levels of both PRLs have been observed in several species following transfer of tilapia from SW or brackish water to FW and decrease upon transfer from FW to SW or brackish water (Ayson *et al.*, 1993; Yada *et al.*, 1994; Morgan *et al.*, 1997; Auperin *et al.*, 1994, 1995; Hasegawa *et al.*, 1987; Yada *et al.*, 1991). In tilapia, plasma levels of PRL$_{188}$ increased from a nearly

undetectable level (<0.2 ng/ml) and peaked at nearly 25 ng/ml, 3 days following transfer from SW to FW. The peak levels were subsequently reduced, but remained elevated over initial levels (Yada *et al.*, 1994). Conversely, plasma PRL levels were rapidly reduced following transfer from FW to SW.

Consistent with plasma levels, thymadine uptake by the PRL cell is higher in tilapia adapted to FW than that of tilapia adapted to SW indicating greater cell activity in FW (Nishioka *et al.*, 1993; Auperin *et al.*, 1994; Yada *et al.*, 1994; Shepherd *et al.*, 1997). Moreover, PRL cell activity is correlated to environmental salinity and plasma osmolality *in vivo* (Yada *et al.*, 1994). Changes in plasma osmolality appear to be the primary mediator of PRL release and gene expression in tilapia (Grau *et al.*, 1994; Bern *et al.*, 1982). Not only does this imply that the primary mediator for pituitary PRL release is independent of hypothalamic control, but that mechanisms mediating PRL cell activity *in vitro* may also be active *in vivo*. In support of this, Shepherd *et al.* (1997) found a direct effect of osmolality on PRL release and gene expression, independent of hypothalamic innervation following autotransplantion of tilapia pituitaries onto the optic nerve.

The Role of Growth Hormone in Osmoregulation

GH and PRL share considerable structural and functional similarities and are considered to have evolved from a common ancestral gene. As in other vertebrates, GH plays a crucial role in the regulation of multiple physiological processes such as growth osmoregulation, metabolism, and reproduction in fish (Donaldson, 1979; Bern and Madson *et al.*, 1992; McLean and Donaldson, 1993; Shepherd *et al.*, 1997). Together with its mediator, insulin-like growth factor (IGF-I), GH plays a role in SW adaptation in

several euryhaline species such as salmonids (McCormick *et al.*, 1991; Fruchtman *et al.*, 2000), tilapia (Sakamoto *et al.*, 1997; Fuchtman *et al*, 2000) and killifish (Bern *et al.*, 1993; Sakamoto *et al.*, 1993). Initial investigations in salmonids demonstrated increased survival during SW transfer with GH treatment (Clarke *et al.*, 1977). Similarly, GH therapy was shown to increase hyposmoregulatory ability by reducing plasma osmolality in salmonids (Sakamoto *et al.*, 1997), possibly through increases in size and density of chloride cells or by upregulating Na^+, K^+-ATPase activity (Richman *et al.*, 1987; Sakamoto *et al.*, 1993; Borski *et al.*, 1992; Degani *et al.*, 1985).

More recently, studies in tilapia have confirmed a role for GH in SW adaptation. In tilapia, pituitary GH cell content and gene expression are higher in SW than in FW (Borski *et al.*, 1994). Similarly, plasma GH levels increase in association with SW transfer and increased plasma osmolality *in vivo* (Sakamoto *et al.*, 1994; Yada *et al.*, 1994; Ayson *et al.*, 1993; Borski *et al.*, 1994; Tang *et al.*, 1993; Yada *et al.*, 1994). Helms *et al.*, (1987) observed a correlation *in vitro* between increased GH secretion from the tilapia pituitary and increases in medium osmolality. Moreover, GH replacement therapy reduced plasma osmolality and increased gill Na^+, K^+-ATPase in hypophysecomized tilapia. Thus, GH clearly plays an important role in osmoregulation, particularly for adaptation to hyperosmotic environments.

The Role of Cortisol in Osmoregulation

Cortisol has been identified as a SW-adapting hormone acting primarily to stimulate of Na^+, K^+-ATPase and ion excretion by the gill chloride cell (Madsen *et al.*, 1990; McCormick *et al.*, 2002; Balment *et al.*, 1987; Evans *et al.*, 1990; Henderson *et al.*, 1987). As with PRL and GH, osmoregulatory surfaces such as kidney and intestine are

also the target organs of cortisol (Maule and Shreck, 1990; Shrimpton and Randall, 1994; Shrimpton *et al.*, 1995). An important role for cortisol in SW adaptation is supported by increased circulating levels and metabolic clearance after SW transfer (Bern and Madson, 1992; McCormick e*t al.*, 1995, 2001). In tilapia, cortisol enhances the ability to maintain plasma osmolality upon SW challenge (Lin *et al.*, 1999) and stimulates the development of yolk-sac chloride cells (Ayson *et al.*, 1994). Cortisol was also recently shown to enhance drinking in response to SW transfer in presmolt salmon, juvenile rainbow trout, and tilapia (Fuentes and Eddy, 1996).

The Regulation of Blood Volume and Blood Pressure

The majority of previous studies have focused on the regulation of PRL release by changes in plasma osmolality following FW or SW transfer or by the direct actions of medium osmolality on pituitary PRL release *in vitro*. FW transfer is a hypertensive event, characterized by ion loss and passive influx of water. Compensating for the reduction in osmolality, PRL stimulates Na^+ uptake and promotes ion conservation. However, elevated plasma Na^+ has the potential to accentuate hypertension. In contrast, SW transfer results in a loss of water and a concentration of ions resulting in hypotension. Adaptations to SW subsequently involve the excretion of excess Na^+ ions and active drinking to restore lost fluids. In the eel, transfer from FW to SW results in transient elevations of plasma osmolality and blood pressure (Jones *et al.*, 1969). Due to the differential presser effects that occur between FW and SW adaptation it is conceivable that blood pressure, or factors involved in its regulation, may control PRL release in addition to the direct effects of plasma osmolality. However, the effects of blood volume on PRL release remain unknown.

As described previously, drinking is critical for replacing water lost during adaptation to hypertonic environments (Evans *et al.*, 1993; Takei *et al.*, 2000; Hirano *et al.*, 1974; Fuentes and Eddy, 1996; Takei *et al.*, 1998). In fact, if ingested water is occluded from the gut by ligation of the esophagus, eels die from hypernatremia and hypovolemia after transfer to SW (Takei *et al.*, 1998). The control of drinking during SW adaptation remains poorly understood. Hypotension, induced by the smooth muscle relaxants papaverine or sodium nitroprusside, stimulates the drinking response in FW and SW-adapted flounder, Atlantic salmon, and eels (Balment and Carrick, 1985; Fuentes and Eddy, 1996). Hypotension stimulates drinking even in FW-adapted flounders, in which drinking lacks a physiological role (Balment and Carrick, 1985; Tierney *et al.*, 1995). On the other hand, hypertension reduces drinking in the eel (Hirano and Hasegawa, 1984), suggesting that the stimulus for drinking is directly related to hypotension.

It is feasible that changes in plasma osmolality play a role in the drinking response as well. Changes in plasma osmolality associated with drinking are observed following administration of hypotensive drugs such as papaverine and sodium nitroprusside in the eel and Atlantic salmon (Fuentes and Eddy, 1997), suggesting a role in the drinking stimulus. However, osmolality is not likely to be a major dipsogenic stimulus as observed changes in plasma osmolality occur well after the initiation of the drinking response (Hirano, 1979). Moreover, plasma osmolality changes with respect to acclimation salinity. Thus, it would appear that blood volume is regulated at the temporary expense of plasma osmolality, indicating a more strict conservation of blood volume than osmolality.

The Renin-Angiotensin System

The renin-angiotensin system (RAS) is principally responsible for control over maintaining blood volume and pressure in all vertebrates (Fitzsimmons, 1998). Stimulation of the RAS by a reduction in blood pressure or blood volume has been well established in mammals. Central to the function of the RAS is the enzymatic synthesis of angiotensin II (ANG II), a potent dispogenic and hypertensive hormone (Kobayashi and Takei, 1996; Fitzsimmons, 1998; Takei, 2000). Synthesis of ANG II depends on an enzymatic cascade initiated by the release of renin from juxtaglomerular cells of the kidney in response to a reduction in blood pressure or elevation of extracellular ion concentration. Once in circulation, renin acts on angiotensinogen, produced by the liver, to form ANG I, a decapeptide. Final synthesis of ANG II is completed with the cleavage of 2 amino acids of the carboxy end, in the mammalian lung or fish gill, by angiotensin converting enzyme (ACE) (Wright and Harding, 1994). All the components found in the mammalian RAS have been identified in fish (Tierney *et al.*, 1995; Kobayashi and Takei, 1996; Fuentes and Eddy, 1997; Tsuchida and Takei 1999).

***Regulation of Drinking by the* Renin-Angiotensin System**

While the control of drinking remains poorly understood in fish, the RAS is believed to play an important role. As in mammals, the dipsogenic action of ANG II has been well established in fish (Kobayashi *et al.*, 1983; Takei, 2000). Activation of the RAS or administration of ANG I or ANG II results in increased drinking even in FW (Fuentes and Eddy, 1996). Euryhaline species such as flounders, salmonids, and killifish seem to be more responsive to the actions of ANG II than strictly FW and SW species (Takei *et al.*, 1979; Kobayashi *et al.*, 1983; Malvin *et al.*, 1980; Hirano and Hasegawa, 1984). Plasma renin activity, the rate-limiting step in ANG II synthesis, and ANG II are

higher in SW-adapted fish compared to FW fish (Smith *et al.*, 1991; Tierney *et al.*, 1995). Similarly, plasma levels of renin and ANG II increase during SW adaptation in several teleost species (Kobayashi *et al.*, 1983; Takei, 2000), further suggesting that the RAS plays a critical role during SW adaptation.

Various types of ACE inhibitors and ANG II receptor agonists have been used to evaluate the role of ANG II in drinking. In salmonids, the dipsogenic effects of ANG I or ANG II are inhibited by the receptor antagonist saralasin (Fuentes and Eddy, 1996). ACE inhibitors decrease basal drinking in several SW adapted teleosts, suggesting a role of the RAS in SW adaptation (Tierney *et al.*, 1995; Balment and Carrick, 1985). Additionally, increased drinking associated with the activation of the RAS by elevation of plasma ion concentration, or hypovolemia induced by hemorrhage has been well established in teleost fishes (Hirano *et al.*, 1974; Bath and Eddy, 1979; Evans *et al.*, 1979; Takei *et al.*, 1979; Hirano and Hasegawa, 1984; Balment and Carrick, 1985; Tierney *et al.*, 1995; Kobayashi and Takei, 1996; Fitzsimmons, 1998).

The Renin-Angiotensin System and the Kidney

In the fish kidney, SW transfer results in reduced urine production via regulation of both the number of filtering glomeruli and the single glomerular filtration rate (Hickman *et al.*, 1969). Angiotensin II infusion *in vivo* reduces overall urine production in SW-adapted rainbow trout by reducing singular and overall glomerular filtration rates. However, ANG II had no effect on the singular filtration rate of glomeruli in FW-adapted trout. (Brown *et al.*, 1980). Thus, ANG II appears to control urine production by reducing the total number of actively filtering glomeruli both in FW-and SW-adapted trout (Brown *et al.*, 1980).

In addition to its stimulation of drinking and of glomerular filtration rate, ANG II plays a role in ion homeostasis through the modulation of Na^+, K^+-ATPase activity in the gill, kidney, and intestine. Physiological levels of ANG II inhibited Na^+, K^+-ATPase in the intestine and increased enzyme activity in gill and kidney *in vitro* (Marsigliante *et al.*, 1997, 2000, 2001). The effects, accompanied by increases in intracellular free Ca^{2+} and activation of protein kinase C, were completely abolished by DuP-703, a specific agonist to the mammalian AT1 receptor subtype. Supporting a suggested role in SW-adaptation, ANG II appears to enhance ion extrusion capability of the chloride cell through an AT-1 like receptor-mediated mechanism involving Ca^{2+} mobilization.

Angiotensin II and Prolactin

Euryhaline teleost species are commonly used to analyze the mechanisms regulating drinking because they possess the mechanisms to control drinking when environmental salinity changes (Evans *et al.*, 1993). Traditionally, drinking has been assessed through the use of dyes such as phenol red (Kobayashi *et al.*, 1983) dissolved in environmental water. However, the technique is relatively insensitive and is ineffective for determining small changes in drinking rates. More recently water-born dyes have been replaced with radioisotopes such as ^{51}Cr-EDTA (Hazon *et al.*, 1989), ^{125}I-polyvinylpyrroridine (Evans *et al.*, 1968), or ^{3}H-polyethylene glycol (Malvin *et al.*, 1980) have been successfully used to increase the sensitivity of drinking estimates.

Tilapia offer an attractive model for the study of interrelationships between regulation of growth and development and hydromineral balance. Not only is the ease with which tilapia adapt to varying salinities attractive for such studies, but the tilapia is rapidly gaining international popularity as a food source. Clearly, with the increasing

global dependence on tilapia as an aquaculture product, efforts must be directed toward the development of technologies to increase the growth of these fish in culture and production increases. Central to these efforts is the neuroendocrine regulation of adaptation and growth in aquatic environments. Of the processes involved, the maintenance of salt and water balance is essential to life. A thorough understanding of the interrelationships between the endocrine regulation of growth and osmoregulation must be developed for the optimal application of neuroendocrine technology toward growth promotion.

While conducting experiments on the uptake and clearance of bovine GH in FW tilapia, we became aware that blood withdrawal elicited a reduction in plasma osmolality which was accompanied by a marked increase in plasma PRL levels (more than 400 ng/ml) (Hirano *et al.*, 2002). PRL levels increased to a greater degree than could be accounted for by the reduction in osmolality alone. Since hypovolemia caused by hemorrhage is known to stimulate the RAS and drinking not only in fish but also in mammals (Kobayashi and Takei, 1976; Fitzsimmons, 1998; Takei, 2000), we hypothesized that the marked increase in plasma PRL after blood withdrawal was due to a synergistic relationship between ANG II and reduced osmolaltiy in mediating PRL release. To clarify the potential synergistic effect of ANG II on the response to the PRL cell to osmotic stimulation, the present study was carried out by examining the changes in plasma PRLs, GH, cortisol, and drinking rate in response to blood withdrawal in tilapia adapted to FW, 30%SW, and SW. In addition, the effects of exogenous ANG II on PRL and GH release and drinking were examined *in vivo* and *in vitro*.

Chapter II:

Effect of Blood Withdrawal and Angiotensin II on Prolactin Release in the Tilapia, *Oreochromis mossambicus*

ABSTRACT

Reflecting their important role in freshwater (FW) osmoregulation, plasma levels of prolactins (PRL_{188} and PRL_{177}) in the euryhaline tilapia, *Oreochromis mossambicus*, are always higher in fish acclimated to FW than in those in seawater (SW). Repeated blood withdrawal (5% of estimated blood volume at 0, 1, 4, 8, 24, 48, 76, and 120 h) from the tilapia acclimated to FW resulted in a marked increase in plasma levels of PRLs. The increase seemed to be correlated with a decrease in plasma osmolality, but the increased PRL levels were more marked than the levels expected from the change in plasma osmolality alone. Repeated blood withdrawal from the fish in SW, on the other hand, did not cause any change in plasma PRL levels, although a significant increase in plasma GH accompanied an increase in plasma osmolality. Blood withdrawal resulted in a significant reduction in hematocrit values regardless of the environmental osmolality, suggesting hemodilution. The reduction in plasma osmolality after blood withdrawal in FW and the increased osmolality in SW suggest that the blood volume is restored at least in part by drinking environmental water. In a separate experiment, a single blood withdrawal (20% of total blood) stimulated drinking in the fish regardless of whether they were held in FW, 30% SW, or SW. Plasma levels of both PRLs were also elevated following a single blood withdrawal in the fish acclimated to FW and 30% SW, but not in the fish in SW. Activation of the renin-angiotensin system after blood withdrawal and dipsogenic action of angiotensin II (ANG II) is well established in fish. Intraperitoneal injection of ANG II (0.1 and 1.0 μg/g) into the fish in FW significantly increased plasma levels of the tilapia PRLs after 1 h. ANG II at concentrations of 10-1000 nM was also

effective in stimulating PRL secretion *in vitro*. There was no effect of ANG II on GH release. These results suggest that the marked increase in PRL concentration after blood withdrawal from the fish in FW is due to a facilitative interaction between ANG II and a reduced plasma osmolality.

INTRODUCTION

Maintenance of hydromineral balance in vertebrates includes the precise regulation of plasma osmolality and blood volume. In teleost fishes, important regulation of plasma osmolality occurs through pituitary hormones, prolactin (PRL) and growth hormone (GH), and through cortisol, a corticosteroid secreted by the interrenal gland (Hirano, 1986; Brown and Brown, 1987; Bern and Madsen, 1992; McCormick, 2001). Blood volume, on the other hand, is regulated primarily by angiotensin II (ANG II), the principal biologically active product of the renin-angiotensin system in all vertebrates (Kobayashi and Takei, 1996; Fitzsimons, 1998; Takei, 2000).

Prolactin is essential for freshwater (FW) adaptation in many euryhaline teleosts, including the tilapia, *Oreochromis mossambicus* (Hirano, 1986; Brown and Brown, 1987; Grau *et al.*, 1994). The tilapia pituitary produces two distinct PRL molecules, PRL_{188} and PRL_{177}, which are encoded by separate genes (Specker *et al.*, 1985; Yamaguchi *et al.*, 1988; Rentier-Delrue *et al.*, 1989). Reflecting their important role in FW osmoregulation, plasma levels of PRLs are always higher in the tilapia acclimated to FW than in those in SW (Ayson *et al.*, 1993; Yada *et al.*, 1994; Shepherd *et al.*, 1997a). It is also well established that *in vitro* release of PRLs from the pituitary is inversely correlated with the change in the medium osmolality, increased under hyposmotic conditions and suppressed in hyperosmotic medium (Nishioka *et al.*, 1988; Grau and Helms, 1990; Grau *et al.*, 1994). Recently, Shepherd *et al.* (1999) have shown that changes in plasma osmolality *in vivo* also exert a direct regulatory action on PRL release and gene expression in the tilapia pituitary. Thus, changes in extracellular osmolality are likely to be a major regulator of PRL cell function in the tilapia.

Stimulation of the renin-angiotensin system by changes in plasma osmolality, blood pressure and blood volume is well established in mammals, resulting in enzymatic synthesis of ANG II, a potent dispogenic and hypertensive hormone (Kobayashi and Takei, 1996; Fitzsimons, 1998; Takei, 2000). The dipsogenic action of ANG II has also been well established in fish (Kobayashi *et al.*, 1983; Takei, 2000). In SW, total body water is reduced by passive loss to the hypertonic environment, therefore, drinking is essential to maintain blood volume (Evans, 1993). Plasma renin activity, the rate-limiting factor for ANG II synthesis, and ANG II have been shown to increase during SW adaptation in several teleost species (Kobayashi *et al.*, 1983; Takei, 2000) reflecting an important role in SW adaptation.

We have reported earlier that ANG II stimulates PRL release from the tilapia pituitary *in vitro* (Grau *et al.*, 1984). In mammals, it has also been shown that ANG II stimulates PRL release through a Ca^{2+}-dependent process (Malarkey *et al.*, 1987; Diaz-Torga *et al.*, 1998; Iglesias *et al.*, 2001). While we were conducting experiments on plasma clearance of bovine growth hormone in tilapia, we became aware that repeated blood withdrawal elicited a reduction in plasma osmolality, accompanied by a marked increase in plasma PRL levels (more than 400 ng ml^{-1}) (Hirano *et al.*, 2001). Since hypovolemia caused by hemorrhage is known to stimulate the RAS and drinking not only in mammals but also in fish (Kobayashi and Takei, 1996; Fitzsimons, 1998; Takei, 2000), we hypothesized that the marked increase in plasma PRL after blood withdrawal may be due to a synergistic interaction between ANG II and reduced osmolality. In an attempt to clarify a possible facilitory effect of ANG II on the response of tilapia PRL cells to osmotic stimulation, the present study was carried out by examining the changes in

plasma PRLs, GH, cortisol and drinking rate in response to blood withdrawal in the tilapia adapted to FW, 30% SW and SW. In addition, effects of exogenous ANG II on PRL and GH release were examined *in vivo* and *in vitro*.

MATERIALS AND METHODS

Fish

Euryhaline tilapia (*Oreochromis mossambicus*) were reared from breeding stock at the Hawaii Institute of Marine Biology. Fry were removed from brooding females and reared in 60-liter tanks in FW under a natural photoperiod. They were fed twice daily with ProForm (Agro Pacific, Chilliwaeck, BC, Canada), approximately 5% of body weight per day. Water temperature was maintained at 25 ± 2°C. Large (250-400g) fish were used for repeated blood withdrawal experiments. Juvenile fish (25-50 g) were used for drinking experiments and *in vitro* pituitary incubations. Tilapia used for FW and SW treatments were removed directly from stock populations, previously acclimated to FW or SW for at least one month prior to experimentation. Fish were acclimated to 30% SW by direct transfer of FW-adapted tilapia to 30% SW for two weeks.

Blood withdrawal

Blood was removed serially from fish adapted to FW, 30% SW and SW at 0, 1, 4, 8, 12, 24, 72, and 120 h. Five percent of the total blood volume, estimated as 7% of the body weight (Okimoto *et al.*, 1994), was removed from the caudal vessels via heparinized syringe (200 U/ml ammonium heparin). Prior to blood collections, fish were

anesthetized in tricane methanesulfonate (0.5 g/l) neutralized with $NaHCO_3$ (0.5 mg/l). They were held in outdoor 600-l aquaria without feed throughout the experimental procedure. Immediately following removal, blood samples were kept on ice (< 15 min) until plasma was separated by centrifugation (5 min at 10,000 rpm). Plasma samples were held at –20°C until analyses.

Drinking rate

Drinking rate was estimated following withdrawal of 20% of the estimated blood volume (ca. 500 µl) from young tilapia (30-45 g), adapted to FW, 30% SW or SW. Food was withheld for ten days prior to the experiment to clear the intestine. Blood was withdrawn as described above. Drinking rate was estimated largely following the procedures described by Kobayashi *et al.* (1983). Briefly, after blood withdrawal, fish were transferred to 60 l glass aquaria containing 0.004% phenol red in 20 l water. After 5 h, a final blood sample was taken, the gut was dissected out, and its contents were washed with 3 ml distilled water into a petri dish. Two ml of the wash were transferred to a 2 ml polyethylene centrifuge tube. Following centrifugation (1,000 rpm for 5 min) the supernatant was transferred to a 2 ml polyethylene tube and mixed vigorously with an equal volume of methylene chloride to remove fats and bile. After centrifugation (3,500 rpm for 5 min), 400 µl of the supernatant was decanted into a tube containing an equal volume of trichloroacetic acid and mixed vigorously. Following centrifugation (3,500 rpm for 5 min), 1 ml of the supernatant was removed and added to an equal volume of 1 N NaOH. Phenol red concentration in the mixture was determined by spectrophotometer (Beckman DU 650) at 550 µm.

Na^+, K^+-ATPase activity

Gill Na^+, K^+ ATPase activity was determined as described by (McCormick, 1993). Primary gill filaments were stored at –80°C in 100 µl SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) immediately following removal. Prior to assay, samples were thawed and homogenized in 25 µl SEID buffer (SEI buffer with 0.1% deoxycholic acid) in a 1.5 ml polyethylene microcentrifuge tube. The homogenate was centrifuged at 3,000 rpm for 30 sec. The supernatant was removed and assayed for enzyme activity. Duplicate 10 µl supernatant samples (10µl) were added to 200 µl of assay mixture (50mM imidazole, 1 U/ml lactic dehydrogenase, 2.5 U/ml pyruvate kinase, 2 mM phosphoenololpyruvate, 0-5 mM nicotinamide adenine dinucleotide (NADH), 0.5 mM ATP, 0.4 mM KCN, 45 mM NaCl, 2.5 mM $MgCl_2$, 10 mM KCl, pH 7.5) in 96-well microplates. A duplicate set of wells were run simultaneously with assay buffer containing 0.5 mM ouabain. The rate of NADH oxidation was calculated from the linear rate of the reaction as determined by the change in absorbance (340 µm) measured on a plate reader (SpectraCount, Packard) at 24°C . Protein concentration was determined using a protein assay kit (Bio-Rad, Hurcules, CA), using bovine serum albumin (BSA, Fraction V, Sigma, St. Louis, MO) as a standard. Na^+, K^+-ATPase activity was determined as the difference in ouabain-sensitive ATP hydrolysis and expressed as µmol/ADP/mg protein/h.

Plasma PRLs, GH, Cortisol, and serum Osmolality

Plasma levels of PRL_{188}, PRL_{177} and GH were determined by radioimmunoassays (RIAs) using the procedures of Ayson *et al.* (1993) as modified by Yada *et al.* (1994). PRL_{177} was iodinated as described by Seale *et al.* (2001). Plasma cortisol concentrations were determined using a commercially available kit (ImmunoChem Coated Tube Cortisol ^{125}I RIA Kit; ICN Biochemicals, Costa Mesa, CA) as modified by Eckert *et al.* (2001). Plasma osmolality was determined in replicate 8 µl plasma samples using a vapor pressure osmometer (Wescor 5100 C, Logan, UT).

In vivo *effect of ANG II*

Effects of ANG II on plasma PRL and GH levels and drinking rate were measured following intraperitoneal injection of synthetic fish ANG II (^{1}Asp, ^{5}Val - angiotensin II, Peptide Institute, Osaka, Japan). Thirty FW-adapted tilapia (20-40g) were separated into three replicate groups (n = 5/replicate). Each group received 10 µl/g of ANG II solution corresponding to doses of 0, 1.0, or 0.1 µg/g in 0.9% saline. Drinking rate was measured by placing the fish in phenol red solution for 1 h as described above. Concentrations of PRLs and GH were determined in plasma samples taken 1 h after the injection.

In vitro *effect of ANG II*

Pituitaries were isolated from FW-adapted tilapia (40-60 g) and incubated separately in a 96-well culture plate with 200 µl of Eagle's minimal essential medium (330 or 355 mOsm), containing glucose (500 mg/l), glutamine (290 mg/l) and buffered

with 25 mM HEPES and 18 mM $NaHCO_3$, as described by Yada *et al.* (1995). Pituitaries were pre-incubated for 18-20 h at 27°C in a gyratory platform (50 rpm) under a humidified atmosphere composed of 95% O_2 and 5% CO_2. After pre-incubation (ca. 18h), the pituitaries were incubated for 8 h with 0, 10, 100 or 1000 nM ANG II (^{1}Asp, ^{5}Val - angiotensin II). The medium was replaced at 1, 2, 4, and 8 h.

Statistics

All values represent means ± standard errors of the mean (SEM). Differences were determined by one-way or two-way ANOVA (repeated measures) followed by Duncan's multiple-range test or by Mann-Whitney U-test. Calculations were performed using a computer program, STATISTICA (StatSoft, Tulsa, OK).

RESULTS

Effects of repeated blood withdrawal

(1) Hematocrit and plasma osmolality (Fig. 1)

The effects of repeated blood withdrawal on hematocrit and plasma osmolality was determined in tilapia adapted to FW, 30% SW or SW. Five percent of the estimated blood volume was taken from the caudal vessels 5 times during the first 24 h, and then at 48, 72 and 120 h thereafter, thus resulting in removal of 40% of the total estimated blood volume. The initial hematocrit values were higher in the fish in SW than in those in FW. Significant ($P < 0.01$) reduction was observed after 1 h, and the hematocrit values declined gradually thereafter. At the end of the experiment, the hematocrit of the SW fish was reduced to 12%, approximately 30% of the initial level.

Hematocrit was reduced by a similar proportion in the fish in FW and in 30% SW (Fig. 1A).

Repeated blood withdrawal also caused significant changes in plasma osmolality (Fig. 1B). The initial plasma osmolality in the fish in SW (355 mOsm) was significantly ($P < 0.05$) higher than in those in FW (335 mOsm) and 30% SW (318 mOsm). In the SW fish, osmolality increased markedly during the first 8 h. The increase became significant ($P < 0.05$) after 4 h and reached a maximum level (above 400 mOsm) after 8 h. Osmolality was restored to the initial level at 24 h and thereafter. On the other hand, plasma osmolality was reduced significantly ($P < 0.01$) 1 h after the first withdrawal in the fish in FW. A significant reduction ($P < 0.05$ or $P < 0.01$) in plasma osmolality was observed throughout the remainder of the experiment. No effect of blood withdrawal was seen on plasma osmolality in the fish in 30% SW.

(2) Plasma PRL levels (Fig. 2)

The initial plasma levels of PRL_{188} in the fish in FW (35 ng/ml) was significantly ($P < 0.05$) higher than in those in 30% SW (10 ng/ml) and in SW (5 ng/ml). It increased significantly ($P < 0.05$) after 1h, reached a peak level (more than 300 ng/ml) after 4 h, and declined thereafter. Significantly ($P < 0.05$ or $P < 0.01$) elevated PRL levels were maintained until the end of the experiment (after 120 h). In the fish acclimated to 30% SW, a significant ($P < 0.05$) increase in plasma PRL_{188} was observed after 120 h. However, no effect of blood withdrawal on PRL_{188} was seen in SW fish.

Plasma levels of PRL_{177} also increased in response to repeated blood withdrawal in the fish in FW, although the increases were less marked than in the case of PRL_{188}

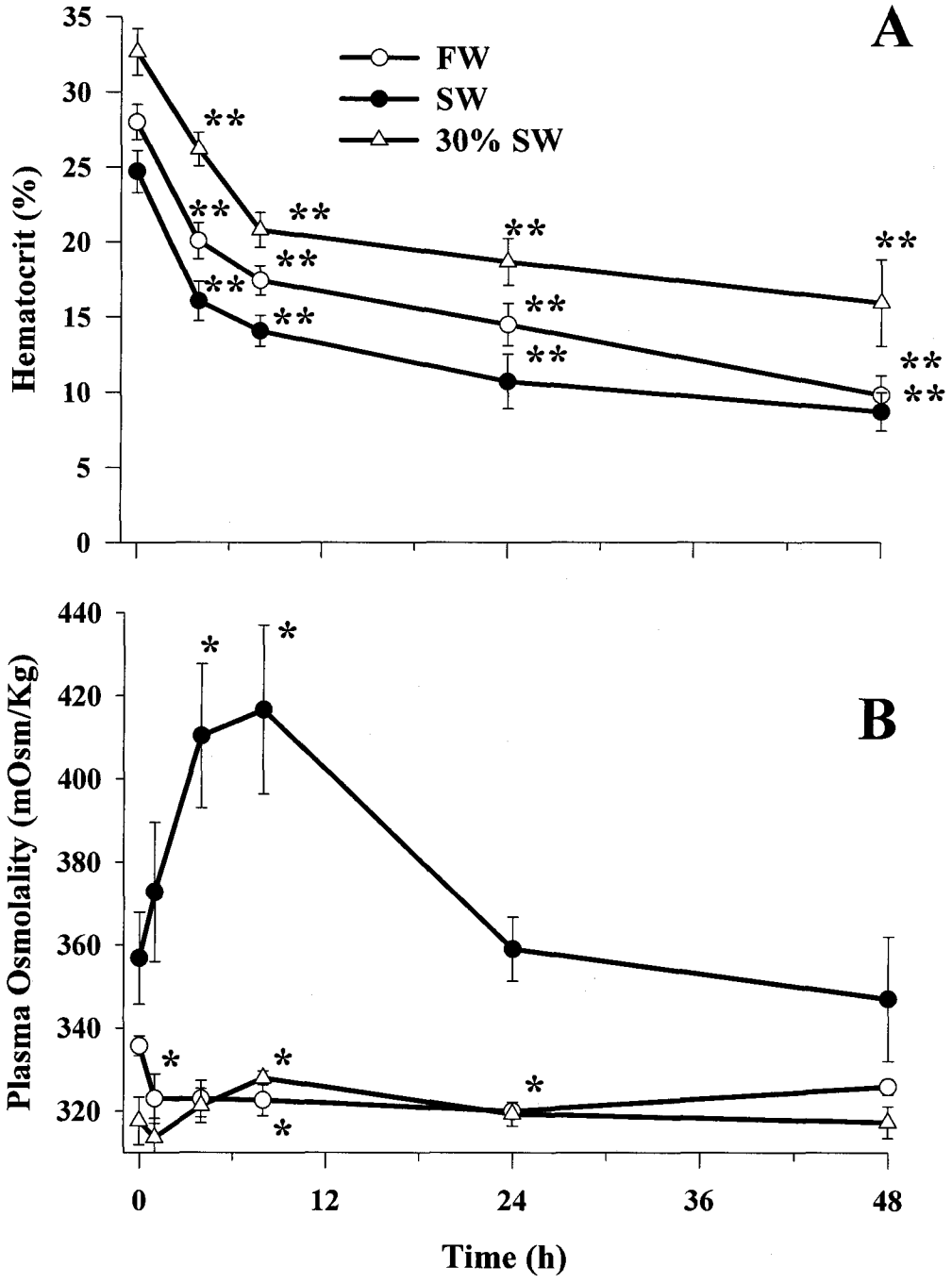

Figure 1. Effects of repeated blood withdrawal on hematocrit (A) and plasma osmolality (B) in tilapia. Blood was removed serially (ca. 5% of the estimated blood volume) from fish acclimated to fresh water, 30% seawater, and seawater. Samples were taken from 200-300 g tilapia at 0, 1, 4, 8, 24, 48, 96, and 120 h. Vertical bars indicate mean ± SEM (n = 8). *, **, Significantly different from the initial (time 0) at $P < 0.05$ and $P < 0.01$ respectively.

(Fig. 2B). Plasma PRL_{177} was increased significantly ($P < 0.05$) at 1 h after the first blood withdrawal, reached a peak (about 45 ng/ml) at 4 h, and was subsequently reduced at 24 h and thereafter to levels significantly ($P < 0.05$ or $P < 0.01$) lower than the initial level at time 0. In the fish in 30% SW, significant ($P < 0.05$) reductions in plasma levels of PRL_{177} were observed after 4 and 8 h, but the initial level was restored thereafter. Blood withdrawal had no effect on plasma PRL_{177} in SW-acclimated tilapia.

(3) Plasma GH and cortisol, and gill Na^+, K^+-ATPase activity (Figs. 3, 4)

In contrast with PRL_{188} and PRL_{177}, which increased markedly after blood withdrawal in FW, plasma GH levels increased only slightly with increases being observed at 8 h and thereafter ($P < 0.01$; Fig. 3A). In the fish in SW, on the other hand, plasma GH levels increased more markedly compared with the fish in FW. The response became significant ($P < 0.05$) after 4 h and thereafter ($P < 0.01$). Blood withdrawal had no effect on plasma GH in the fish acclimated to 30% SW.

Fig. 3B illustrates the changes in plasma cortisol in response to repeated blood withdrawal. The initial plasma cortisol levels were correlated with acclimation salinity, the highest levels being observed in the fish in SW (291 ng/ml) and the lowest in those in FW (126 ng/ml). In the fish acclimated to FW, no marked change was seen except for slight but significant decreases after 72 h ($P < 0.05$) and 120 h ($P < 0.01$). Plasma cortisol was reduced in response to blood withdrawal with the exception of an ephemeral increase in the fish in 30% SW at the 48-hour time point. In the SW fish, plasma cortisol fluctuated considerably during the first 24 h, returned to the initial level after 48 h, and

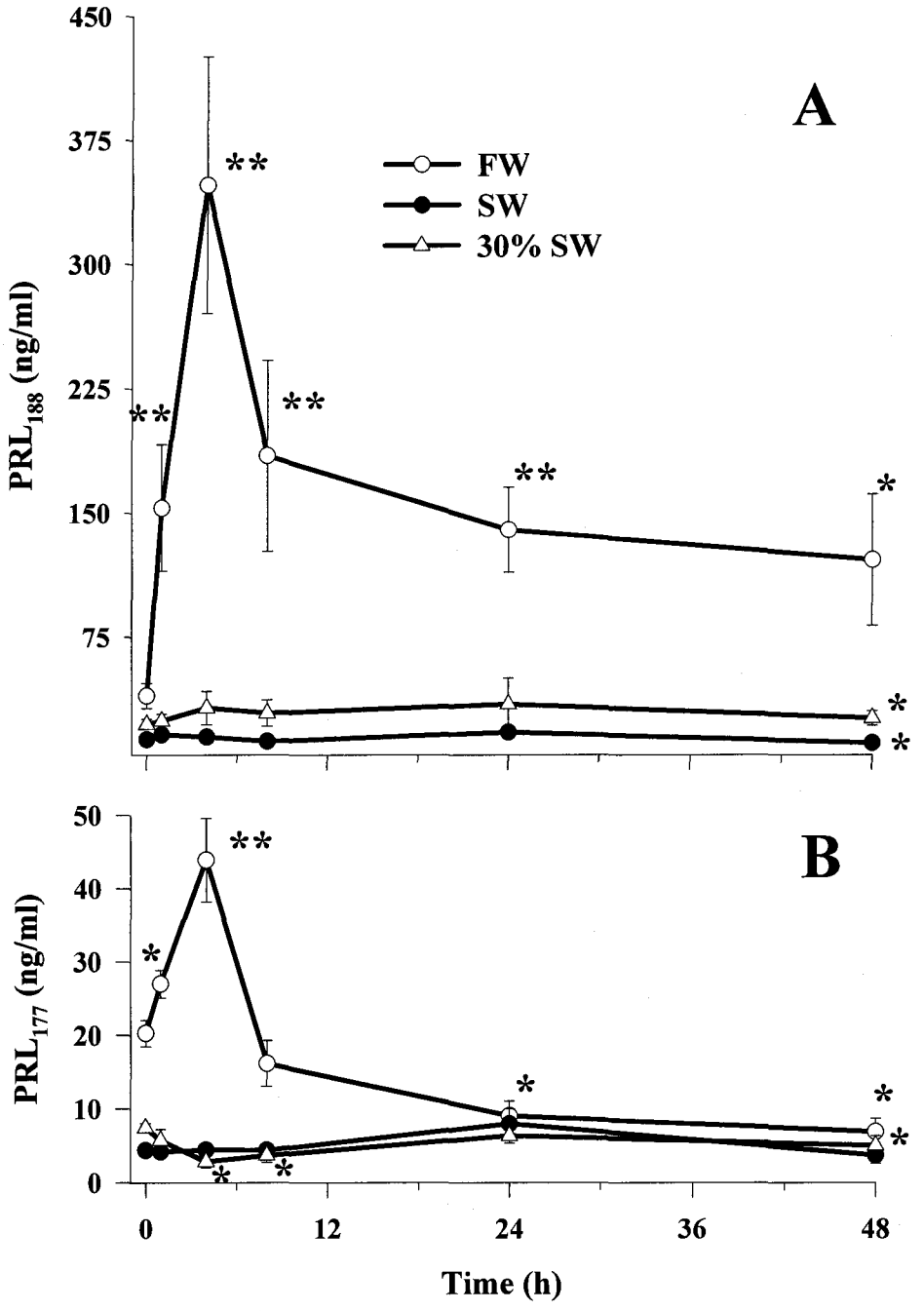

Figure 2. Effects of repeated blood withdrawal on plasma levels of PRL177 (A) and PRL188 (B) in tilapia. Blood was removed serially (ca. 5% of the estimated blood volume) from fish acclimated to fresh water, 30% seawater, and seawater. Samples were taken from 200-300 g tilapia at 0, 1, 4, 8, 24, 48, 96, and 120 h. Vertical bars indicate mean ± SEM (n = 8). *, **, Significantly different from the initial (time 0) at $P < 0.05$ and $P < 0.01$ respectively.

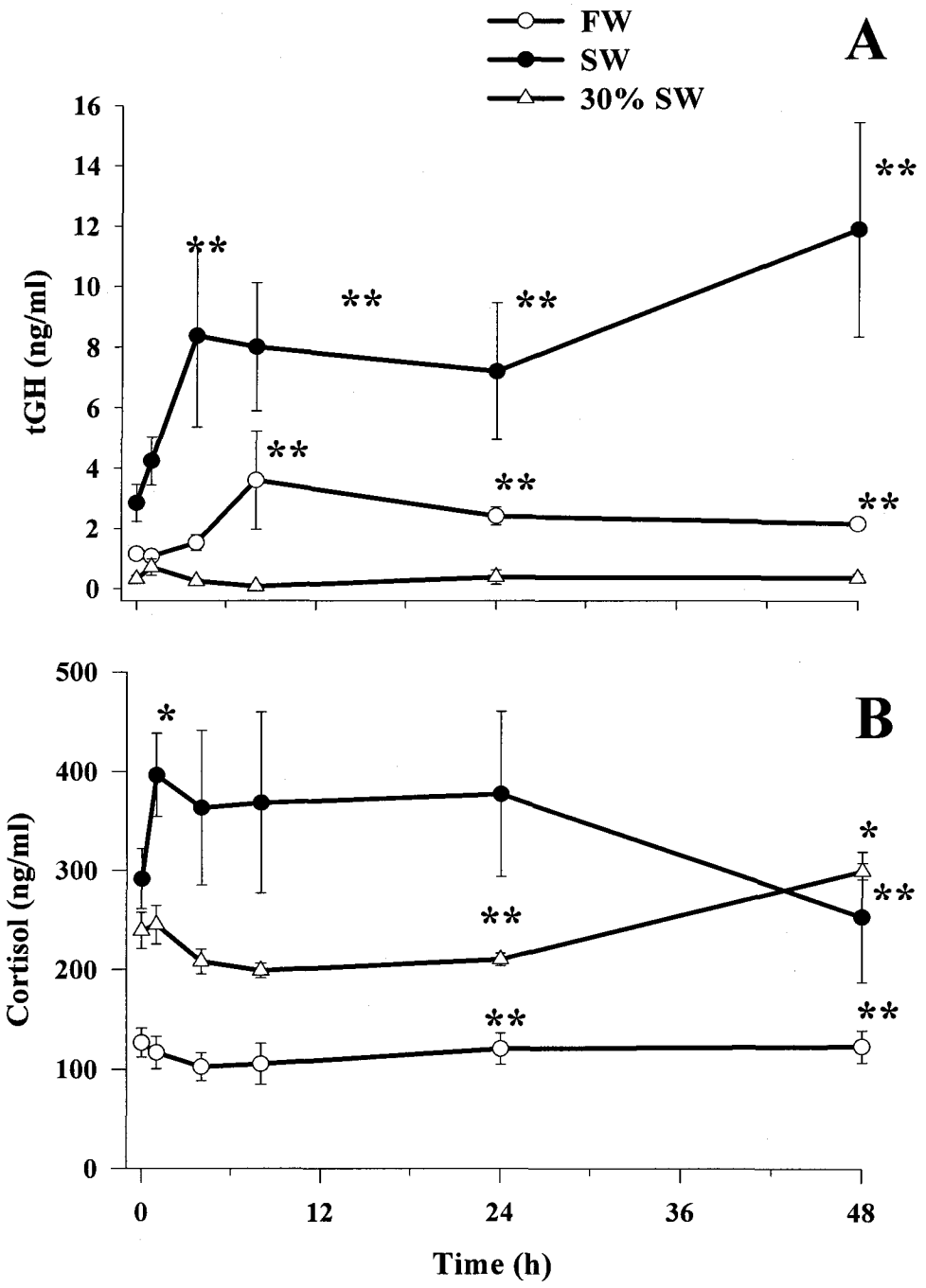

Figure 3. Effects of repeated blood withdrawal on plasma levels of GH (A) and cortisol (B) in tilapia. Blood was removed serially (ca. 5% of the estimated blood volume) from fish acclimated to fresh water, 30% seawater, and seawater. Samples were taken from 200-300 g tilapia at 0, 1, 4, 8, 24, 48, 96, and 120 h. Vertical bars indicate mean ± SEM (n = 8). * Significantly different from the initial (time 0) at $P < 0.05$. decreased significantly ($P < 0.01$) after 72 and 120 h. A significant ($P < 0.05$ or $P < 0.01$) reduction was also observed in the fish in 30% SW after 8, 24, 72 and 120 h.

gill Na^+, K^+-ATPase increased gradually after blood withdrawal in FW-acclimated fish, becoming significant ($P < 0.05$) after 120 h (Fig. 4). Blood withdrawal in SW fish had no effect on the enzyme activity, which was maintained at significantly ($P < 0.05$) higher levels than in the fish in FW throughout the experiment with the exception after 120 h.

Effect of a single blood withdrawal (Table 1)

In the next experiment, the effect of a single blood withdrawal of 20% of the estimated blood volume was examined with special reference to its effect on drinking rate. As shown in Table 1, 5 h after the blood withdrawal significant increases were observed in plasma PRL_{188} ($P < 0.01$) and PRL_{177} ($P < 0.05$) in the fish acclimated to FW. There was no change in plasma PRL levels in the fish in 30% SW or in those in SW, except for a significant ($P < 0.05$) increase in PRL_{177} level in the fish in SW. Osmolality of the fish in SW (341 mOsm) was significantly ($P < 0.05$) higher than that in the fish in FW (315 mOsm) or in 30% SW (313 mOsm). A single blood withdrawal had no effect on plasma osmolality in any of the fish, either in FW, 30% SW or SW.

On the other hand, drinking rates in the control groups were correlated to environmental salinity, highest in SW fish (0.35 ml/100 g/h) and lowest in FW fish (0.06 ml/100 g/h). Blood withdrawal produced significant ($P < 0.05$ or $P < 0.01$) increases over the control levels in all the fish treated with ANG II regardless of environmental salinity.

Table 1

Effects of blood withdrawal of 20% of total blood volume on plasma PRLs, GH, and drinking rate in tilapia acclimated to fresh water, 30% seawater, and seawater.[a]

	Envirnomental Salinity	PRL_{188} (ng/ml^{-1})	PRL_{177} (ng/ml^{-1})	Plasma Osmolality (mOsm)	Drinking Rate (ml 100 g^{-1} h^{-1})	GH (ng/ml^{-1})
Control	FW	6.0 ± 0.4	49.7 ± 12.3	314 ± 4.2	0.06 ± 0.01	0.69 ± 0.116
Blood Withdrawal	FW	9.1 ± 0.7**	96.9 ± 28.6*	319 ± 4.1	0.23 ± 0.012**	0.687 ± 0.121
Control	30% SW	4.5 ± 0.4	8.2 ± 1.1	313 ± 0.3	0.26 ± 0.058	0.56 ± 0.095
Blood Withdrawal	30% SW	4.3 ± 0.6	9.6 ± 2.1	315 ± 1.2	0.45 ± 0.061*	0.48 ± 0.122
Control	SW	1.8 ± 0.2	13.6 ± 3.4	341 ±3.5	0.35 ± 0.042	0.589 ± 0.109
Blood Withdrawal	SW	2.1 ± 0.2	18.3 ± 1.9*	339 ± 2.3	0.49 ± 0.032*	0.463 ± 0.062

[a] Data are presented as mean ± SEM (n = 5 for all treatments)

*, ** Significantly different from the corresponding control level at 5, and 1%, respectively.

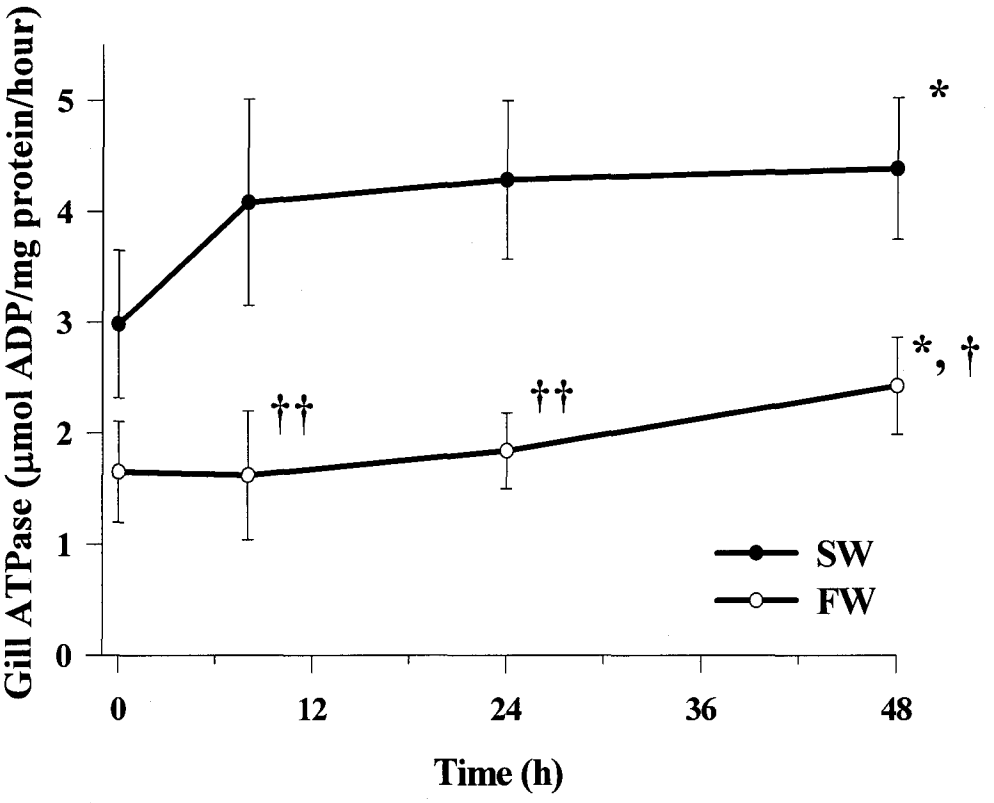

Figure 4. Effects of repeated blood withdrawal on gill Na+, K+-ATPase activity. Blood was removed serially (ca. 5% of the estimated blood volume) from fish acclimated to fresh water and seawater. Samples were taken from 200-300 g tilapia at 0, 8, 24, 48, 96, and 120 h. Vertical bars indicate mean ± SEM (n = 8). * Significantly different from the initial (time 0) at $P < 0.05$. †, ††, significantly different from the SW treatment at $P < 0.05$ and $P < 0.01$ respectively.

In vivo effects of ANG II (Table 2)

Effects of intraperitoneal injection of ANG II on plasma PRLs, GH, osmolality and drinking rate were examined in FW-and SW-acclimated tilapia. Blood samples were taken at 1 h after the injection. In the fish in FW, a significant ($P < 0.05$) increase in PRL_{177} was observed after injection of the low dose (0.1 μg/g) of ANG II. Plasma levels of both PRL_{188} and PRL_{177} were increased significantly ($P < 0.01$) after injection of the high dose (1.0 μg/g) in the fish in both FW and SW. The high dose of ANG II significantly reduced ($P < 0.01$) plasma GH in the fish in SW. No significant change was seen in plasma osmolality or in drinking rate (Table 2).

On the other hand, plasma levels of both PRLs were significantly ($P < 0.05$) higher in the fish in FW compared with those in SW, and the high dose of ANG II caused a significant ($P < 0.05$) increase in PRL_{188} level. There was no effect of ANG II on PRL_{177}. Drinking was stimulated significantly ($P < 0.05$) after injection of the high dose of ANG II. Significant ($P < 0.01$) increases in plasma osmolality were seen after low and high doses of ANG II.

In vitro *effects of ANG II (Figs. 5, 6)*

In vitro effect of ANG II on PRL secretion was examined using pituitaries isolated from the tilapia in FW. Pituitaries were incubated for 8 h with 0, 10, 100, and 1000 nM ANG II (^{1}Asp, ^{5}Val - angiotensin II) in isosmotic (330 mOsm) or hyperosmotic (355 mOsm) medium. The medium was replaced at 1, 2, and 4 h. Under isosmotic conditions, ANG II stimulated the release of both PRLs over time in a dose-related manner ($P < 0.05$; Fig. 5). The effect was already significant ($P < 0.01$) after 1 h at a

Table 2

Effects of ANG II on plasma PRLs, osmolality and drinking rate of tilapia acclimated to fresh water (FW) and seawater (SW).[a]

	Environmental Salinity	PRL_{188} (ng/ml^{-1})	PRL_{177} (ng/ml^{-1})	Plasma Osmolality (mOsm)	Drinking Rate (ml $100\ g^{-1}\ h^{-1}$)	GH (ng/ml^{-1})
Control	FW	15.6 ± 5.6	22.8 ± 3.1	320.0 ± 8.6	0.20 ± 0.06	2.9 ± 0.72
ANG II (0.1 mg/g)	FW	20.0 ± 6.6	33.6 ± 2.2*	334.8 ± 11.2	0.16 ± 0.04	1.2 ± 0.22
ANG II (1.0 mg/g)	FW	34.6 ± 7.3**	81.5 ± 14.6**	315.8 ± 4.8	0.21 ± 0.08	0.9 ± 0.10**
Control	SW	1.7 ± 0.2	0.61 ± 0.1	353.8 ± 9.5	0.21 ± 0.02	1.2 ± 0.13
ANG II (0.1 mg/g)	SW	2.0 ± 0.2	0.48 ± 0.1	386.3 ± 3.4**	0.29 ± 0.07	1.0 ± 0.14
ANG II (1.0 mg/g)	SW	2.6 ± 0.1**	0.87 ± 0.2	405.5 ± 7.5**	0.35 ± 0.04*	0.9 ± 0.12*

[a] Data are presented as mean ± SEM (n = 8 for FW and SW

*, ** Significantly different from the corresponding control level at 5,

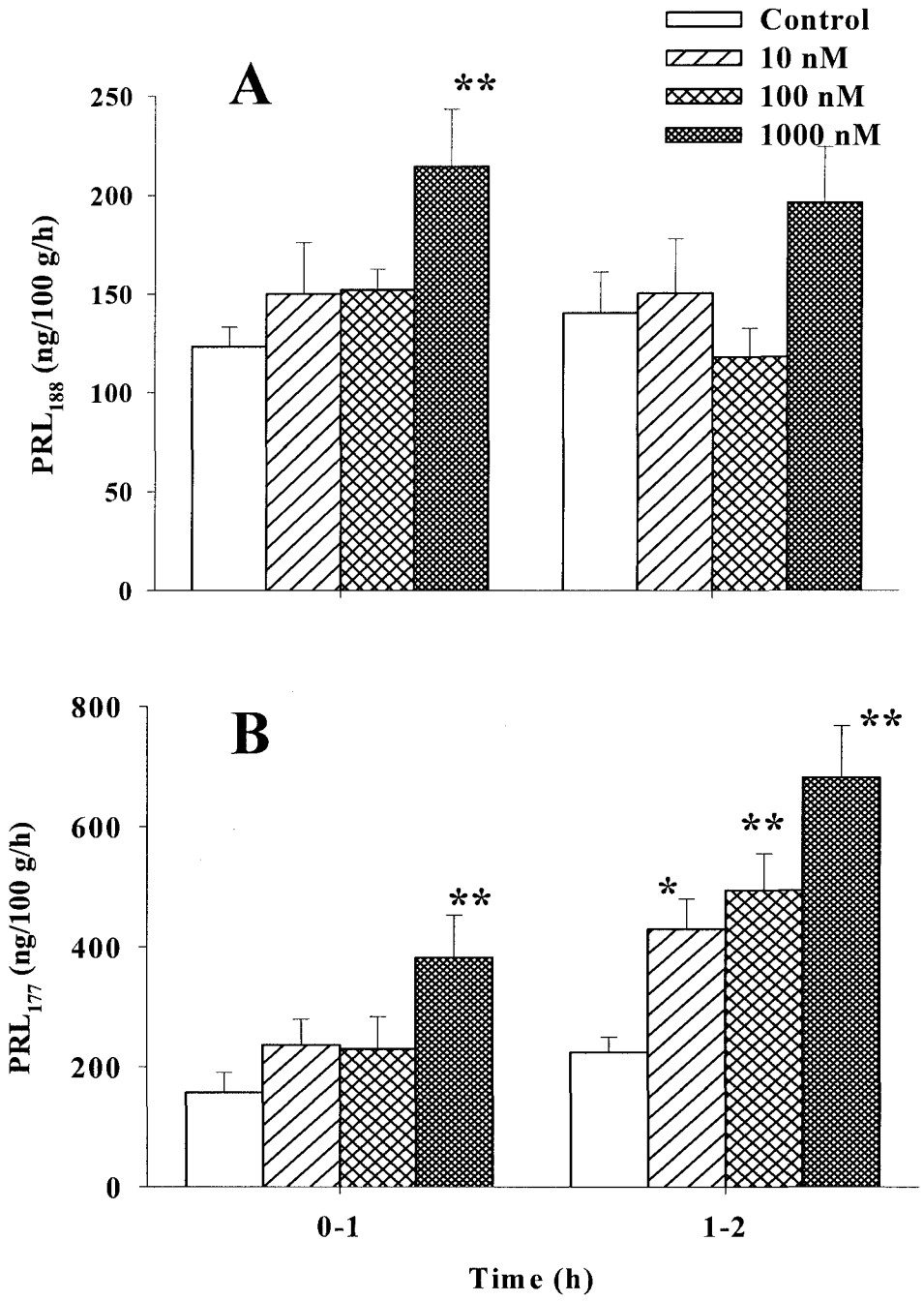

Figure 5. Effects of ANG II on release of PRL188 (A) and PRL177 (B) from the tilapia pituitary *in vitro*. Pituitaries were pre-incubated for 18 h, and then exposed to 10 -1000 nM ANG II (1Asp, 5Val – angiotensin II). Incubation medium (330 mOsm) was changed at 1, 2, 4, and 8 h. PRL release is normalized to body weight and expressed as a rate during each time interval. Vertical bars indicate mean ± SEM (n = 8). *, ** Significantly different from the control (0 nM) at each time point at $P < 0.05$ and $P < 0.01$ respectively.

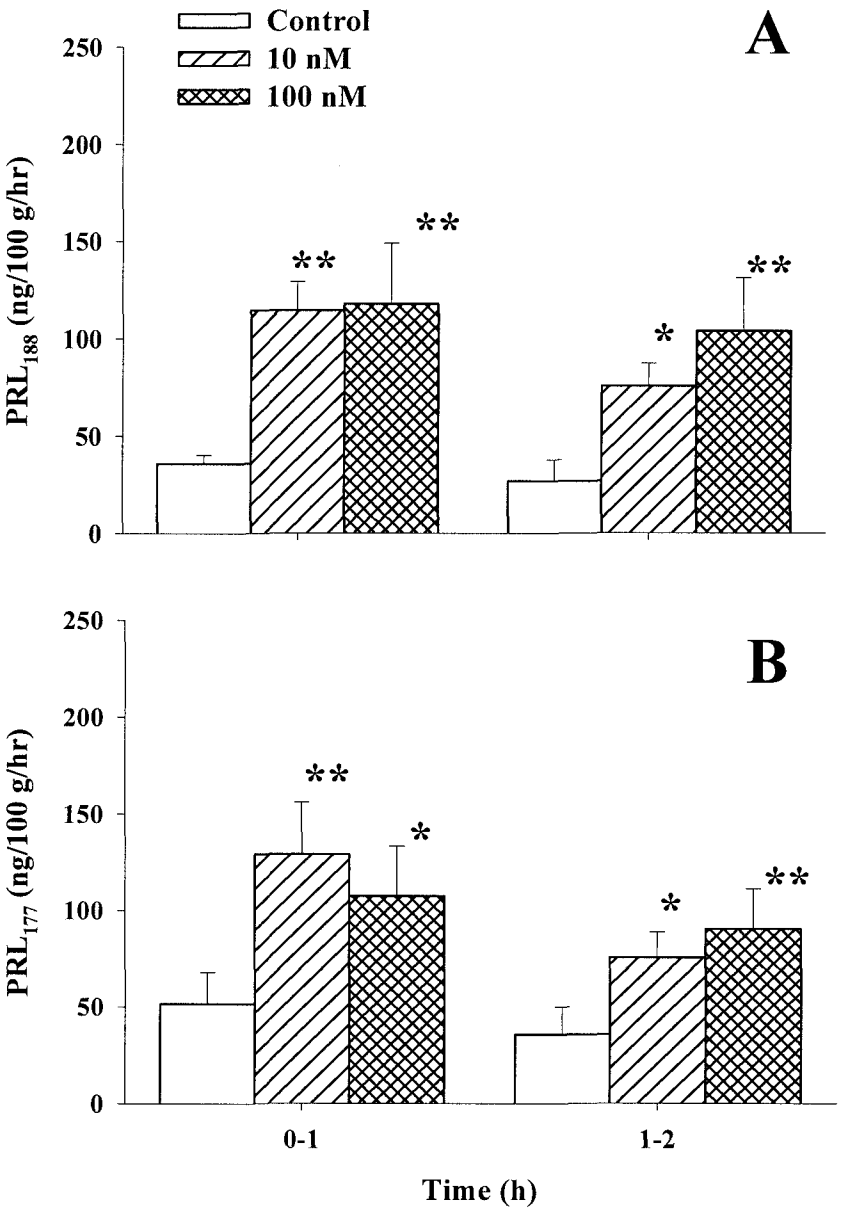

Figure 6. Effects of ANG II on release of PRL188 (A) and PRL177 (B) from the tilapia pituitary *in vitro*. Pituitaries were pre-incubated for 18 h, and then exposed to 10 - 100 nM ANG II (1Asp, 5Val – angiotensin II). Incubation medium (355 mOsm) was changed at 1, 2, 4, and 8 h. PRL release is normalized to body weight and expressed as a rate during each time interval. Vertical bars indicate mean ± SEM (n = 8). *, ** Significantly different from the control (0 nM) at each time point at $P < 0.05$ and $P < 0.01$ respectively.

dose of 1000 nM, and a significant effect ($P < 0.05$ or $P < 0.01$) was observed after 2 and 4 h. However, no stimulation was seen during the 4-8 h incubation. A significant ($P < 0.05$) dose-related response to ANG II was observed during the 0-1 and 2-4 h incubation intervals for PRL_{188} and for PRL_{177}.

Angiotensin II was also effective under hyperosmotic conditions (Fig. 6). Compared with the release of PRL_{188} and PRL_{177} in the isosmotic medium, the release rates were significantly less ($P < 0.01$) in hyperosmotic medium. ANG II at a concentration of 10 and 100 nM produced significant ($P < 0.01$ or $P < 0.05$) increases in PRL_{188} and PRL_{177} release after 1 h. Significant effects ($P < 0.05$ or $P < 0.01$) were also observed after 2 and 4 h. No stimulation was seen on PRL_{177} during the 1-2 and 4-8 h incubation intervals. A significant ($P < 0.05$) dose-response effect of ANG II was observed during the 1-2 and 4-8 h incubation for PRL_{188} and during the 1-2, 2-4, and 4-8 h incubation for PRL_{177}.

DISCUSSION

It is well established that PRL is essential in many euryhaline teleosts, including the tilapia, *O. mossambicus*, to the maintenance of hydromineral balance in FW (Hirano, 1986; Brown and Brown, 1987; Grau *et al.*, 1994). Consistent with earlier observations (Ayson *et al.*, 1993; Yada *et al.*, 1994; Shepherd *et al.*, 1997a), plasma levels of PRLs in the present study were always higher in the tilapia acclimated to FW than in those in 30% SW or SW. Repeated blood withdrawal (5% of the estimated blood volume for 5 times in 24 h) from the tilapia in FW resulted in a marked increase in plasma levels of PRLs. Plasma PRL_{188} was increased to more than 300 ng/ml after 4 h, or 2 h after the second

withdrawal. Our previous studies (Shepherd *et al.*, 1999) suggest that a reduction in plasma osmolality *in vivo* exerts a direct regulatory action on PRL release and gene expression in the tilapia pituitary. Although the increase in plasma PRLs seemed to be correlated with a decrease in plasma osmolality, the increases in PRL levels after blood withdrawal were far greater than those expected from the reduction in plasma osmolality (from 335 mOsm to 320 mOsm during the first 24 h). For example, when tilapia were transferred from SW to FW, the maximum levels of PRL_{188} observed were 25-35 ng/ml, while plasma osmolality decreased from 330 mOsm to 300 mOsm (Yada *et al.*, 1994). Therefore, factors other than plasma osmolality would appear to be involved in the marked increase in plasma PRLs after repeated blood withdrawal in FW. Repeated blood withdrawal from the fish in 30% SW or SW, on the other hand, did not cause any change in plasma PRL levels. This is consistent with the Na-retaining effect of PRL which is counter to SW adaptation (Hasegawa *et al.*, 1986; Hirano, 1986), and also with the fact that PRL release from the tilapia pituitary is suppressed by increased extracellular osmolality both *in vivo* and *in vitro* (Ayson *et al.*, 1993; Auperin *et al.*, 1994; Yada *et al.*, 1994; Shepherd *et al.*, 1997a).

Blood withdrawal resulted in a significant reduction in hematocrit values regardless of environmental osmolality. A reduction in hematocrit indicates a decrease in the number of blood cells in the blood or with hemodilution associated with a restoration in plasma volume. Although the blood cells were not reintroduced in the present study, it is unlikely that the observed change in hematocrit is due to a decrease in the blood cell number alone. Furthermore, since plasma osmolality was increased in the fish in SW and decreased in those in FW, it is also unlikely that the observed change in hematocrit can

be attributed to shrinking or swelling of the cells in response to changes in osmotic pressure. Hemodilution following hemorrhage, accompanied by a reduction in plasma osmolality in the case of fish in FW, has been observed in several teleost species (Nishimura *et al.*, 1979; Carroll *et al.*, 1984; Ogilvy *et al.*, 1988; Takei, 1988). Therefore, dilution with extravascular fluid is likely the cause for the reduced hematocrit observed in the present study.

The essential role that drinking plays during SW adaptation has been well characterized in fish (Hirano, 1974; Fuentes and Eddy, 1996; Takei *et al.*, 1998). Fish in SW are faced with constant dehydration from water lost osmotically across the body surfaces (Evans, 1993; Takei, 2000). In the present study, drinking rate increased in proportion to the environmental salinity. Drinking rates were increased further 5 h after a single blood withdrawal of 20% of the estimated blood volume, regardless of plasma or environmental osmolality, clearly indicating hemorrhage induced drinking in the tilapia. Furthermore, the decrease in plasma osmolality after blood withdrawal in FW tilapia and the increased osmolality in the fish in SW suggest that blood volume is restored at least in part through drinking water from the environment. Thus, tilapia seem to restore blood volume by drinking environmental water at the expense of regulating plasma osmolality.

Activation of the renin-angiotensin system after blood withdrawal and the dipsogenic action of ANG II is well established in fish (Kobayashi and Takei, 1996; Takei, 2000). In the present study, fish ANG II (^{1}Asp, ^{5}Val-ANG II) stimulated drinking and PRL release both *in vivo* and *in vitro*. As in mammals, drinking in fish seems to be stimulated following activation of the renin-angiotensin system by hypotension or an increase in plasma osmolality (Hirano, 1974; Bath and Eddy, 1979; Evans, 1979; Takei *et*

al., 1979; Hirano and Hasegawa, 1984; Balment and Carrick, 1985). Therefore, the increased drinking observed after blood withdrawal in the tilapia in SW is likely to be the result of activation of the renin-angiotensin system. In the present study, however, drinking was apparently not stimulated by ANG II in FW-adapted tilapia. While Kobayashi *et al.* (1983) also observed that ANG II had no dipsogenic effect at doses of up to 1 μg/g in this species, the dipsogenic actions of ANG II have been observed in FW-adapted salmon and rainbow trout using more sensitive methods to estimate drinking (Fuentes and Eddy, 1996; Fuentes and Eddy, 1997). It is likely that the phenol red method used in this study and by Kobayashi *et al.* (1983) may not be sensitive enough to detect the small change in the drinking rate after ANG II injection into FW fish. Further study is needed to determine the effectiveness of ANG II in stimulating drinking in the FW-acclimated tilapia.

The PRL cells of tilapia are osmosensitive. In fact, PRL release is stimulated by less that a 5% reduction in extracellular osmolality both *in vivo* and *in vitro* through the passive influx of water into the PRL cell (Grau *et al.,* 1988; Shepherd, *et al.,* 1999). The subsequent increase in cell volume likely initiates an increase in extracellular Ca^{2+}, possibly through stretch gated channels, and a consequent rise in intracellular Ca^{2+}, thus mediating PRL release. Similarly, ANG II stimulates PRL release through a calcium-dependant process in mammals by interaction with the AT1 receptor (Malarkey *et al.,* 1987; Diaz-Torga *et al.,* 1998; Iglesias *et al.,* 2000). Pituitary stimulation of the AT1 receptor is mediated through phospholipase-C hydrolysis of membrane phosphoinositides, leading to the formation of diaglycerol and inositol phosphates and subsequently to the activation of protein kinase C and to an increase in intracellular free

[Ca^{2+}] (Malarkey *et al.,* 1987; Canonico and MacLeod, 1986). Thus, PRL release appears to be mediated through the same calcium-dependant pathway by ANG II and osmolality.

We have reported earlier that ANG II stimulates PRL release from the tilapia pituitary *in vitro* (Grau *et al.*, 1984). In mammals, stimulation of PRL secretion by ANG II has been well established (Diaz-Torga *et al.*, 1998; Iglesias *et al.*, 2001). Results of the present study clearly indicate that ANG II is a potent stimulator of PRL secretion from the tilapia pituitary *in vitro*. Similarly, intraperitoneal injection of ANG II stimulated PRL secretion in the tilapia in FW and SW. However, repeated blood withdrawal from the fish in SW did not affect the PRL levels, which were maintained at lower levels than FW fish, suggesting that increased plasma osmolality after blood removal in SW fish inhibits PRL secretion and overrides the stimulating effect of Ang II *in vivo*. It is well established that increases in extracellular osmolality attenuate PRL secretion from the tilapia pituitary both *in vivo* and *in vitro* (Grau and Helms, 1990; Grau *et al.*, 1994). However, ANG II was still effective in stimulating PRL secretion *in vitro* from the tilapia pituitary incubated not only in isosmotic medium (330 mOsm) but also in hypertonic medium (360 mOsm). Therefore, inhibitory factors such as somatostatin may be operating to inhibit PRL secretion in the tilapia in SW (Grau *et al.*, 1982; Grau *et al.*, 1985; Helms *et al.*, 1991). This is conceivable since the sodium-retaining action of PRL in teleosts is antagonistic to SW adaptation (Hasegawa *et al.*, 1986; Hirano, 1986).

In the tilapia, plasma concentration, pituitary content and mRNA levels of PRL_{188} and PRL_{177} are differentially regulated by changes in plasma osmolality. The underlying mechanisms for this differential regulation are unknown (Borski *et al.*, 1992; Ayson *et*

al., 1993; Yada *et al.*, 1994). The present study clearly indicates a differential processing between PRL_{188} and PRL_{177} in response to repeated blood withdrawal and subsequent changes in plasma osmolality (Fig. 2). While pituitary PRL_{188} increased nearly ten fold after 8 h, PRL_{177} peaked only at twice that of the initial level. In addition, while PRL_{188} remained significantly elevated, PRL_{177} declined to below initial concentrations over the remainder of the experiment. Plasma levels of both PRLs, on the other hand, were unaffected with the exception of an ephemeral elevation of PRL_{177} after 24 h. Intraperitoneal injection of ANG II at a dose of 1.0 μg/g increased plasma levels of both PRLs by nearly three fold in the fish in FW. In the fish in SW, however, ANG II was only effective in increasing PRL_{188}. According to Nishioka *et al.* (1993), both PRL_{188} and PRL_{177} appear to be produced within the same cells of the tilapia pituitary. If PRL secretion by ANG II in the tilapia is mediated through a Ca^{2+}-dependent process by interacting with the AT1 receptor as in mammals (Malarkey *et al.*, 1987; Diaz-Torga *et al.*, 1998; Iglesias *et al.*, 2001), both PRLs would be similarly released. Thus, the differential secretion of the two PRLs observed in this study may be due to differences in cell populations containing relatively more PRL_{188} than PRL_{177} within the pituitary. The cause of such uneven distribution of the two PRLs among different cells, if it exists, is unknown.

In this study, withdrawal of 5% of the estimated blood volume from FW tilapia resulted in a significant reduction in plasma osmolality from 335 mOsm to 320 mOsm after 1 h. The low osmolality was maintained despite 7 additional withdrawals over the next 120 h. This indicates that mechanisms are operating to restore plasma osmolality, in spite of the effects of continued ingesting of environmental water on osmolality

reduction. There is overwhelming experimental support for the role of PRL in promoting ion uptake not only in euryhaline fish in FW but also in stenohaline FW fish (Hirano, 1986; Brown and Brown, 1987). On the other hand, ANG II has been known to modulate Na^+, K^+-ATPase activity in various tissues in mammals (Therien and Bolstein, 2000). In the eel, ANG II has been suggested to play a role in osmoregulation by regulating Na^+, K^+-ATPase activity in the gills, kidney and intestine (Marsigliante *et al.*, 1997, 2000, 2001). According to Tsuchida and Takei (1999), however, infusion of physiological doses of ANG II into the eel in FW did not affect plasma Na^+ concentration or osmolality, although it induced drinking. By contrast, plasma Na^+ concentration was decreased by high doses of ANG II. Therefore, a direct effect of ANG II in retaining Na^+ in the blood-depleted tilapia may be unlikely.

Angiotensin II is a major regulator for aldosterone synthesis and release in all tetrapod species examined to date (Kobayashi and Takei, 1996). ANG II is also a potent secretagogue for cortisol in trout (Arnold-Reed and Balment, 1994). Although cortisol has been identified largely as a SW-adapting hormone acting primarily through stimulation of ion excretion by the gills, an increasing body of evidence indicates that cortisol is also involved in ion uptake in FW (McCormick, 2001). Recently, we have observed a synergistic action of PRL and cortisol in restoring the decreased plasma osmolality in the hypophysectomized catfish in FW (Eckert *et al.*, 2001). Although no significant change in plasma cortisol was observed after repeated blood withdrawal in the FW-acclimated tilapia, Na^+, K^+-ATPase activity in the gills increased gradually during the course of the experiment. The effect became significant ($P < 0.05$) over the initial level at 120 h (Figs. 3 and 4). Since no particular attention was paid to avoid handling

stress during the blood withdrawal, the observed cortisol levels could be an over-estimation of the physiological levels (Wendelaar Bonga, 1997). Stimulation of gill Na^+, K^+-ATPase activity by cortisol has been reported repeatedly in several teleost species including tilapia (McCormick, 1995; Wendelaar Bonga, 1997; Marsigliante *et al.*, 2001). Thus, the reduced plasma osmolality after blood withdrawal in FW tilapia would be restored by the ion-retaining action of PRL, cortisol and several other hormones involved in fish hydromineral balance such as neurohypophysial hormones (Acher, 1996) and natriuretic peptides (Takei, 1999).

Recently, GH and its mediator, insulin-like growth factor I (IGF-I), have been implicated in the control of SW adaptation in several euryhaline teleosts such as salmonids, tilapia and killifish (Sakamoto *et al.*, 1993; McCormick, 1996; Sakamoto *et al.*, 1997). Injection of GH increased hyposmoregulatory ability not only of euryhaline tilapia, *O. mossambicus* (Sakamoto *et al.*, 1997; Shepherd *et al.*, 1997b) but also of less euryhaline Nile tilapia, *O. niloticus* (Xu *et al.*, 1997). In *O. mossambicus*, plasma GH increases in correlation with an elevation of plasma osmolality following acclimation to SW (Ayson *et al.*, 1993; Yada *et al.*, 1994). Reflecting its role in SW-adaptation, plasma GH increased significantly after repeated blood withdrawal in the tilapia in SW and to a lesser extent, in FW fish (Fig. 3). However, the response in SW was more rapid and marked than in the fish in FW. As described above, several lines of evidence indicate a role for cortisol in SW acclimation of euryhaline teleosts, including increased circulating levels and metabolic clearance after exposure to SW (Bern and Madsen, 1992; McCormick, 1995; 2001). Although the plasma levels of cortisol fluctuated greatly after blood withdrawal, plasma levels were highest in the fish in SW, followed by the fish in

30% SW, and lowest in FW (Fig. 3). In salmonids, GH acts in synergy with cortisol to increase SW tolerance (McCormick, 1996). Therefore, it seems possible that the increased plasma osmolality after blood withdrawal in the tilapia in SW and 30% SW is restored, at least in part, by the synergistic actions of GH and cortisol. On the other hand, the inhibitory effects of somatostatin and high extracellular osmolality on PRL secretion are well established in tilapia (Grau *et al.*, 1982; Grau *et al.*, 1985; Helms *et al.*, 1991). The inhibitory effects of somatostatin and increased plasma osmolality on PRL secretion may act to override the stimulatory effect of ANG II on PRL secretion in the tilapia in SW.

In conclusion, repeated blood withdrawal from the tilapia acclimated to FW resulted in a marked increase in plasma levels of PRLs. The increased PRL levels were more pronounced than levels expected from the decrease in plasma osmolality alone. Blood withdrawal resulted in a significant reduction in hematocrit values regardless of the environmental osmolality suggesting hemodilution. Tilapia seem to restore blood volume by drinking environmental water at the expense of regulating plasma osmolality. Activation of the renin-angiotensin system after blood withdrawal and dipsogenic action of ANG II is well established in fish. ANG II was effective in stimulating PRL secretion both *in vivo* and *in vitro*. There was no effect of ANG II on GH release. Thus, the marked increase in PRL concentration after blood withdrawal from the fish in FW may be due to a facilitative effect between ANG II and a reduced plasma osmolality. The reduced plasma osmolality after blood withdrawal in FW tilapia would be restored in due course by the ion-retaining action of PRL, cortisol and other hormones such as neurohypophysial hormones and natriuretic peptides.

On the other hand, repeated blood withdrawal from the fish in SW did not cause any change in plasma PRL levels, although ANG II stimulated PRL secretion *in vitro* from the pituitary incubated in hyperosmotic medium. Blood withdrawal and ANG II injection stimulated drinking in SW tilapia. Significant increases in plasma GH and cortisol were observed after blood withdrawal accompanied with an increase in plasma osmolality. The increased plasma osmolality after blood, withdrawal in SW would be restored, at least in part, by synergistic actions of GH and cortisol. Secretion of PRL, the Na^+-retaining action of which is inhibitory for osmoregulation in SW, may be suppressed possibly by the synergistic action of increased plasma osmolality and somatostatin.

Chapter III

General Conclusions

In the present study I have revealed a facilitative role for ANG II and osmotic pressure in the release of PRL in tilapia faced with hyposmotic stress. The dramatic increase in plasma PRL associated with blood withdrawal and reduction in plasma osmolality implicates a mechanism in addition to osmotic pressure responsible for the observed PRL release. Moreover, the lack of effect of blood withdrawal on PRL in SW-adapted tilapia indicates a role unique to hyposmotic conditions, consistent with the critical role of PRL in sodium conservation. The apparent hemodilution observed in parallel with blood withdrawal in both FW and SW demonstrates active blood volume regulation of mechanisms to restore blood volume, namely the renin angiotensin system. In further studies, I confirmed a role for the RAS in PRL release as ANG II the increased PRL release *in vi*vo and *in vitro*.

Tilapia have recently enjoyed remarkable worldwide distribution due largely to their unique ability to adapt to unpredictable changes in environmental salinity. Therefore, it is essential that tilapia, when faced with such challenges, be capable of rapidly manipulating the regulation of salt and water balance in order to maintain appropriate blood volume and osmolality. The substantial hemodilution observed following blood withdrawal clearly reveals the replacement of blood volume by of non-RBC containing fluids. Furthermore, the observed changes in plasma osmolality which occurred in parallel to blood withdrawal suggest such fluid is strongly influenced by the environment. Indeed, my studies demonstrated drinking immediately following a single blood withdrawal that revealed a route for fluid to be sequestered for blood volume

maintenance even at the expense of plasma osmolality. Thus, blood volume seems to be regulated more strictly than osmolality.

To conclude, when faced with a reduction in blood volume, tilapia restore blood volume at the temporary expense of blood osmolality through drinking water from the environment. Such relationships represent an adaptive mechanism allowing for the simultaneous regulation of both body fluid osmolality and volume during osmotic stressors such as FW transfer. In the present series of experiments, I have demonstrated that following a reduction in blood volume, hemodilution occurs as well as a transient reduction in blood osmolality in tilapia adapted to FW. Accordingly, plasma PRL markedly increases to levels that cannot be explained by the observed reduction in osmotic pressure alone. Furthermore, I have demonstrated PRL release by ANG II *in vivo* for the first time in fish and described a common molecular pathway for PRL release by ANG II and osmotic pressure. It is therefore likely that the marked increase in plasma PRL demonstrated in these experiments is due to the synergistic effects of ANG II and osmotic signaling on PRL release.

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