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Prolactin and Calcium Balance In The American Eel, *Anguilla rostrata*

By

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UNIVERSITÉ D'OTTAWA
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ABSTRACT

The effects of prolactin on various aspects of calcium regulation were examined in the American eel, Anguilla rostrata.

It is known that all vertebrates strictly maintain extracellular plasma calcium concentrations through the regulation of calcium fluxes between various calcium pools. For terrestrial vertebrates the most important exchange occurs between blood and bone, whereas teleost fish continuously regulate Ca^{2+} through a direct exchange with the environment.

Intimately involved with this change in calcium pools are changes in the hormonal control of calcium homeostasis. But, the known hypercalcemic action of prolactin in various teleosts has yet to be confirmed in the American eel. Different approaches were employed in attempts to increase or decrease endogenous prolactin, and, under these opposing conditions, various physiological correlates were measured in order to quantify the calciotropic actions of prolactin.

Exogenous prolactin administration was associated with elevated plasma total calcium, increased whole body calcium accumulation, and, to a lesser extent, decreased whole body calcium efflux.

In addition, light and electron microscopic observations of eel prolactin cells suggested that increases or decreases in PRL cell activity, and, presumably circulating prolactin, were appropriately associated with increases or decreases in whole body calcium uptake and plasma total calcium levels.

Further, it was found that a single PRL injection in salt water

acclimated eels affected an increase in whole body calcium uptake within 24 hours, subsequently producing hypercalcemia at 48 hours post-injection.

Finally, evidence is provided that eel cellular bone may be one of the target organs for the calciotropic action of prolactin.

It is concluded that prolactin is involved in the regulation of plasma total calcium in the American eel, and that its role is chiefly hypercalcemic. The presumed action of this hormone at the gill to stimulate calcium uptake rates and decrease efflux rates resulted in a positive shift in plasma total calcium concentrations, and these effects took place in the course of one to two days.

RESUMÉ

Les effets de la prolactine sur une varieté des expositions de la réglementation de calcium été examiné chez l'anguille Amèricaine, Anguilla rostrata.

Il est connu que toutes les vertèbres maintenent la concentration de calcium extracellulaires du sang par la réglementation des fluxes de calcium entre quelques fonds communs. Pour les vertèbres terrestre le plus important échange se passe entre le sang et l'arête, puisque les téléostéens réglementent continument le calcium par une échange direct avec l'environment.

Les forces en jeu de cet changement en fonds communs de calcium sont des différences chez le contrôle hormonaux de la balance de calcium. Chez une varieté des téléostéens, il est vrais que la prolactine est hypercalcemique, mais ce fait n'etait pas ete invétéré chez l'anguille Américaine. Par conséquent, quelques approches été utilisé pour simuler des augmentations ou des diminutions des niveaus de la prolactine, et, sous ces deux situations opposé quelques aiguilles physiologiques été mesuré pour établir les actions calciotropiques de la prolactine.

L'administration de la prolactine par injection, infusion, ou par installation des lobes de la prolactine, été accompagné par une élévation de calcium de la plasma; et ce cas été supporté probablement par une augmentation de l'accumulation de la calcium dans le corps totale, et, moins d'une diminution de la perte de la calcium dans le corps totale.

En autre, les observations microscopique des cellules de la prolactine indiquent que les augmentations et diminutions en l'activité des cellules de la prolactine, et, probablement les niveaus de la prolactine dans la plasma, été proprement associé avec augmentations et diminutions dans

l'accumulation de la calcium dans le corps totale, et des niveaus de calcium totale de la plasma.

De plus, un seul injection de la prolactine chez les anguilles acclimaté dan l'eau de la mer a cause une augmentation dans l'accumulation de la calcium dans le corps totale avant 24 heures, et plus tard a causé une élévation de la calcium dans la plasma avant 48 heures après l'injection.

Finalement, quelque évidence été pourvu pour suggérer que l'arête cellulaires de l'anguille est peut-être parmi des organes objectif pour les action calciotropiques de la prolactine.

Pour conclure, la prolactine est engager dans la réglementation de la calcium totale du sang chez l'anguille Américaine, est que sa role est principalement hypercalcemique. L'action de la prolactine à la branchie pour augmenter l'accumulation de la calcium dans le corps totale, et pour diminuer la perte de la calcium a produit un changement positif dans la calcium de la plasma, et ces effets ont se passé avant un ou deux jours.

ABBREVIATIONS

ACTH	adremocorticotropic hormone
Ca^{2+}	plasma total calcium
$CaCl_2$	calcium chloride
CT	calcitonin
CO_2	carbon dioxide
dpm	disintegrations per minute
HC	hypocalcin
HCl	hydrochloric acid
HNO_3	nitric acid
Hypex	hypophysectomy
Jin	influx
Jout	efflux
LTH	luteotropic hormone (prolactin)
Mg^{2+}	plasma total magnesium
PRL	prolactin
PTH	parathyroid hormone (parathormone)
RBC	red blood cell
RPD	rostral pars distalis
Stx	Stanniectomy
SW	66% salt water
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone (thyrotropin)

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Introduction

The purpose of this study was to assess the putative hypercalcemic role of prolactin in the control of calcium homeostasis in the American eel, Anguilla rostrata. Various approaches including Stanniectomy, prolactin releasing factor treatments, prolactin "lobe" homografts, ovine prolactin injection and infusion, and SW to FW transfer were employed in order to measure the effects of prolactin by simulating or enhancing exogenous prolactin. Other treatments including hypophysectomy, treatment with prolactin release inhibiting factors, and adaptation to SW were used in attempts to measure the effect of simulated decreases in endogenous prolactin. Under these two opposing conditions accepted physiological correlates associated with the calciotropic actions of fish calcium regulating hormones (calcium influx rates, whole body efflux rates, plasma total calcium, plasma osmolarity, sodium, potassium and magnesium, prolactin cell cytology and morphometry and finally the monitoring of internal calcium pools), were measured in an attempt to identify and quantify the calciotropic actions of prolactin in the American eel.

All vertebrates, including teleosts, have the capacity to maintain extracellular calcium concentrations at levels distinct from that of the environment (Copp & Ma, 1978, Pang et al, 1980, Copp, 1982, Fenwick & Wendelaar Bonga, 1982, Flik et al, 1986). This is necessitated by the fact that the level of ionic calcium has a critical effect on such physiological processes as electrical signalling by nerve cells (Kuffler, Nichols & Martin, 1984), striated muscular contraction (Eckert & Randall, 1983), blood clotting (Schmidt-Nielson, 1983), the actions of hormones through "stimulus-secretion coupling" (Hadley, 1984 and Norris, 1985),

bone formation (Copp & Ma, 1978, Copp, 1982), and fish body permeability to water and ions (Copp, 1968).

Terrestrial vertebrates, despite intermittent feeding, maintain very stable plasma calcium concentrations (Carruthers et al, 1964) with the stability stemming from a balance of the rates at which calcium enters and leaves the plasma (Taylor, 1985). This balance involves the regulation of calcium fluxes between various calcium pools, namely, absorption from the gut, excretion at the kidney, and movement between the various internal pools with the most important exchange being that between blood and bone. There are several hormones that influence this balance. Parathyroid hormone (PTH) increases plasma calcium levels, primarily by increasing the mobilization of bone calcium but also by promoting calcium reabsorption at the kidney and by facilitating the formation of active vitamin D (Fischer, 1982). Vitamin D, in turn, stimulates intestinal calcium reabsorption and causes bone demineralization. Calcitonin acts to prevent post-prandial hypercalcemia and inhibits parathyroid stimulated bone demineralization (Bronner, 1982).

Whereas the rate of synthesis and secretion of PTH by the parathyroid cells is stimulated by a fall in plasma calcium concentration (Fischer, 1982, Taylor, 1985), calcitonin (CT) secretion from the ultimobranchial bodies is stimulated by an increase in plasma calcium (De Luise et al, 1972). The third major calciotropic hormone is $1,25(OH)D_3$, which is the best known metabolite of vitamin D_3 for which a clear physiological role has been confirmed (Fraser, 1980, Pang and Pang, 1986). PTH, the most important control of vitamin D_3 metabolism, stimulates the conversion of

$25(OH)D_3$ to the active 1,25 $(OH)D_3$ form, and calcium seems to inhibit this activation step (Kumar, 1984).

The major site of adaptation to changes in plasma calcium is the skeleton, and in all mammals examined bone resorption is more responsive than bone accretion (Bronner, 1982). PTH and 1,25 $(OH)_2D_3$ increase the net flux of calcium from bone to plasma through bone resorption (Vaughan, 1981). On the other hand, CT inhibits bone resorption and stimulates the osteocytic (bone cell) extrusion of a granular substance around which calcium phosphate precipitates (Baud & Boiuin, 1978). Gastrointestinal hormones released during feeding and digestion stimulate CT release, presumably serving to remove calcium from the plasma in preparation for influx from the gut (Taylor, 1985). In addition to the various effects on bone resorption both PTH (Parsons, 1976) and 1,25 $(OH)_2D_3$ (Massry, 1982) stimulate renal calcium reabsorption, whereas the effects of CT on the kidneys are less clear (Carney & Thompson, 1981). Further, transcellular absorption of calcium by the small intestine is stimulated by 1,25 $(OH)_2D_3$ (Pansu et al, 1981), however, at physiological concentrations neither CT nor PTH directly effects intestinal calcium transport (Taylor, 1985).

In mammals, calcium storage, excretion and absorption are all regulated, with the major site of regulation being the skeleton which contains over 98% of total body calcium (Bronner, 1982). The hormone primarily in control of this tissue is PTH, through its direct actions on bone and kidney, and through its effects on vitamin D_3 metabolism (Hermann-Erlee & Flik, 1989). In general, both PTH and CT are released at normal plasma calcium concentrations and therefore, increases and decreases

in plasma calcium will appropriately modify release of these two hormones (Taylor, 1985). Further, since the two hormones have antagonistic effects on bone, the difference between their concentrations determines their ultimate effects on plasma calcium.

In fish, however, the importance of bone as a major site for plasma calcium regulation is still uncertain (Taylor, 1985). Fish possess large gill surfaces that are continuously in contact with a surrounding medium that functions as a virtually limitless calcium reservoir, and this could greatly diminish the need for a major internal calcium store (Dacke, 1979). As a result, terrestrial vertebrates are thought to function as "closed" systems in terms of Ca^{2+} regulation, whereas most fish are considered "open" systems (Dacke, 1979). An open system in which the animal contains little or no internal Ca^{2+} reservoir, regulates Ca^{2+} through a direct exchange with the environment. Both cyclostomes and chondrichthyan fish do not possess calcified skeletons and are not considered to possess any major Ca^{2+} reserves (Urist, 1976). These fish exchange Ca^{2+} directly with the environment via the gut, skin and kidney (Urist, 1976). A closed system of calcium regulation involves the recycling of Ca^{2+} within the animal between the plasma and some internal Ca^{2+} reservoir (Dacke, 1979). Partially closed systems of regulation are found in those fish, which form bone, and of course terrestrial vertebrates. In practice teleosts probably utilize both systems to some degree, since clearly they are not mutually exclusive (Simkiss, 1974).

Osteichthyes, the bony fish, are the only fish to possess a large internal calcium store, the skeleton, and to possibly become independent of the environment in terms of calcium regulation. Osteichthyes and in particular the order Teleostei, can possess either cellular of acellular bone (Dacke, 1979). The acellular bone found in higher teleosts seems incapable of extensive remodelling (Moss, 1963), although Fleming (1967) considers that mineral can be withdrawn from acellular bone at a slow rate. It is probably only cellular bone and scales that can be responsive to the demands of plasma calcium homeostasis and indeed, cellularly boned fish repair bone fractures in all but the most hypocalcic of environments, indicating a role of this tissue as a Ca^{2+} reservoir (Moss, 1962). In fish, CT was shown to stimulate bone formation in Anguilla (Lopez et al, 1976) but the effect of CT on teleost blood calcium concentration is still not well understood (Barlet, 1982). Bone resorption is stimulated by $1,25(OH)_2D_3$ in eels (Lopez et al, 1977) and by pituitary extracts; the latter being attributed to the hypercalcemic action of prolactin (Pang et al, 1973) or possibly the PTH-like hypercalcin (Parsons et al, 1978). Despite the fact that teleost bone is responsive to calciotropic hormones, there is no convincing evidence that bone performs a major role in blood calcium homeostasis (Taylor, 1985). Indeed, even when waters are calcium deficient and their diet is calcium rich, teleosts preferentially obtain most of their calcium from the water (Taylor, 1985). Freshwater fish are known to drink little, and even in marine species, which do drink extensively, the gut is likely not a major site for calcium absorption (Simmons, 1971, Simkiss, 1974).

Gills probably represent the most important site of calcium regulation in many teleosts, apparently contributing over 80% of all whole body calcium accumulation in fish (Flik et al, 1985a). It is now known that calcium absorption takes place at the gill (Keys, 1931, Motais and Maetz, 1964, Berg, 1968, Simmons, 1971), and more specifically across the chloride cells of the gill lamellae (Girard & Payan 1980, Payan, Mayer-Gostan & Pang 1981). This absorption is active (Fleming, 1967, Mayer-Gostan et al, 1983, Flik, Van Rijs & Wendelaar Bonga 1985, Flik, Wendelaar Bonga & Fenwick 1985a), and involves a high affinity calcium-transporting enzyme (Flik, Wendelaar Bonga & Fenwick, 1983, 1984a,b). Further, Perry and Flik (1988) have suggested that calcium uptake via the gills is a two stage transcellular process; passive movement of calcium through apical cell membrane calcium channels, and then the calcium is actively pumped against an electrochemical gradient across the basolateral cell membrane from inside the chloride cell, into the blood, by a plasma membrane bound high affinity Ca^{2+}-ATP-ase (Perry & Flik, 1988). Presumably this enzyme is the biochemical correlate of the branchial Ca^{2+} pump (Flik Wendelaar Bonga & Fenwick, 1985a).

The transition from water to land has involved a switch from dependence on an open system of calcium regulation towards a closed system, and so to a dependence on an intermittent supply of dietary calcium. The effects of transition to land on hormonal control of calcium homeostasis are clear: whereas bone assumes the major role as target organ and buffer for terrestrial vertebrate calcium regulation, the gill serves as the target organ in the aquatic environment. Intimately involved with this change in target organs are the changes in hormonal control.

Whereas terrestrial vertebrate hypocalcemic control is generally achieved by CT, in fish, this activity is performed by the hormone of the corpuscles of Stannius (CS) variously referred to as hypocalcin (Pang, 1973, Pang, Pang and Sawyer, 1974), teleocalcin (Ma & Copp, 1978) or CS extract (Fontaine, 1964, Fenwick & Forster, 1972, Wendelaar Bonga and Pang, 1986). Purified CS extracts have consistently been shown to induce a hypocalcemic response in intact fish after infusion (Wendelaar Bonga and Pang, 1986, Lafeber & Perry, 1988b), and it is now accepted that the primary role of the CS is to produce a hypocalcemic factor (Fenwick, 1985) which acts to reduce branchial calcium uptake (Fenwick & So, 1974, So & Fenwick, 1979, Fenwick & Wendelaar Bonga, 1982, Lafeber & Perry, 1988b). Unfortunately, the mechanism involved in hypocalcin-mediated reduction of calcium uptake are as yet ill defined. Recent data from Perry & Flik, (1988), and Lafeber & Perry, (1988b) have excluded the possibilities that hypocalcin decreases apical chloride cell membrane permeability to calcium, or, that hypocalcin affects the kinetic properties of the basolateral membrane bound Ca^{2+}-ATPase (Flik *et al*, in press).

Calcitonin is also thought to be involved in hypocalcemic control, however, to date there is no clearly accepted role for this hormone in fish (Wendelaar Bonga, 1980, Feinblatt, 1982). Studies, involving CT administration in several species of fish have variously shown CT to have a hypocalcemic effect in eels (Chan *et al* 1968b), and both a hypercalcemic effect, or no effect at all in trout (Fouchereau-Peron *et al*, 1986, 1987).

As fish do not possess parathyroid glands, the source of the hypercalcemic hormone in terrestrial vertebrates, this control is achieved by the pituitary in fish (Pang et al, 1980). Fontaine (1956) was the first to show that hypophysectomy resulted in hypocalcemia suggesting that the pituitary contained a hypercalcemic factor. In fish, prolactin from the rostral pars distalis (RPD) of the pituitary has become recognized for its hypercalcemic actions (Pang et al, 1978, Wendelaar Bonga, & Flik, 1982). Indeed, when hypophysectomized killifish (Fundulus heteroclitis) are held in low calcium waters they become hypocalcemic and exhibit tetanic seizures, both of which are prevented by either the addition of calcium to the water (Pang et al, 1971), or by the administration of prolactin cell homogenates or ovine PRL (Pang et al, 1978, Pang, 1981a).

In general, prolactin is considered the most versatile of the pituitary hormones in terms of its effects in vertebrates (Nicoll & Bern, 1972), with over 80 clearly established functions grouped into six categories including reproductive, osmoregulatory, growth and developmental, effects on ectodermal structures, metabolic effects, and synergism with steroids (Nicoll, 1980). Much of this versatility is derived from the effects prolactin has on osmoregulatory physiology. Significantly, the actions of PRL on ectodermal and integumentary structures are more prevalent in the aquatic vertebrates where these structures perform much more complex osmoregulatory functions than in terrestrial vertebrates (Bern, 1975).

The original finding by Pickford & Phillips (1959) that ovine PRL kept hypophysectomized Fundulus heteroclitis alive in fresh water led to the large volumes of literature on the effects of PRL in teleosts, and in turn, brought about the gradual recognition of the osmoregulatory roles of PRL in other vertebrate groups (Hirano, 1986). In fish, prolactin is primarily known for its effects on membrane permeability, specifically the regulation of water and electrolyte homeostasis (Nagahama et al, 1974, Wigham & Ball, 1977, Wendelaar Bonga & Greven, 1978); and, its effects on all osmoregulatory surfaces (gut, kidney, bladder and the gill) have been reported in fish (see reviews by Nicoll 1981, Bern 1983). Of significance to this study is the control prolactin (PRL) exerts on the teleost gill. In general, a reduction in gill osmotic permeability has been the best recognized role of PRL in fish; indeed, it was shown to prevent passive loss of Na^+ from the gills of hypophysectomized FW fish without altering active uptake (Bern 1983, Wendelaar Bonga et al, 1983, 1985). In addition, prolactin treatment was also reported to reduce water influx in the isolated gills of eels (Ogawa, 1974) and goldfish (Ogawa et al, 1973). More recently, it was shown that the isolated gills of hypophysectomized eels displayed high osmotic influx of water that was restored to normal by PRL injections or the addition of Ca^{2+} to the incubation medium (Ogaswara & Hirano, 1984).

The hypercalcemic potency of mammalian prolactins, usually ovine or bovine, has been confirmed for a number of teleosts, including SW adapted eels, Anguilla anguilla (Olivereau & Olivereau, 1978), SW tilapia

(Wendelaar Bonga & Flik, 1982), FW goldfish and tilapia (Wendelaar Bonga, Flik & Fenwick, 1984a, Flik et al, 1984, Flik et al 1986b), and in the American eel where both ovine and homologous eel prolactins have induced hypercalcemia (Flik et al, 1985a, 1986b). The physiological relevance of the hypercalcemic action of prolactin was demonstrated in tilapia Oreochromis mossambicus, and the stickleback Gasterostus aculeatus, where it was found that PRL cells were activated under low calcium (hypocalcic) conditions (Wendelaar Bonga et al, 1983, 1985). Together, these findings have led researchers to further investigate the calciotropic actions of prolactin; but even though the hypercalcemic nature of prolactin seems to be garnering acceptance, unfortunately the mechanisms of its action are not altogether understood (Flik et al, 1986a,b, Fenwick, in press, Flik, Fenwick & Wendelaar Bonga, in press).

Recently, for tilapia, Oreochromis mossambicus, it was demonstrated that prolactin-induced hypercalcemia was correlated with enhanced branchial Ca^{2+} uptake from the surrounding medium (Flik et al, 1986b). Previous studies by this group (Flik et al, 1984) provided evidence that during prolactin-induced hypercalcemia in the American eel, the activity of the gill plasma membrane bound high-affinity Ca^{2+} -ATPase or branchial Ca^{2+} pump, was stimulated. Moreover, it was shown in tilapia that prolactin receptors exist on the branchial epithelia (Edery et al, 1984). These observations together with recent evidence indicating that Ca^{2+} uptake th ugh the fish gill is a transcellular process (Perry & Flik, 1988), requiring an ATP energized Ca^{2+} stimulated ATPase enzyme (Flik et al, 1986b), indicate the gill is the primary target organ for PRL.

These reports suggest that fish may provide a model with which to study not only the calciotropic action of prolactin in fish, but which also may lead to understanding its role in calcium metabolism in higher vertebrates. For instance, in mammals, prolactin stimulated intestinal Ca^{2+} absorption in rats that were vitamin D deficient (Pahuja & DeLuca, 1981), and stimulated placental Ca^{2+} transport in pregnant ewes (Bartlet, 1985). If the mechanisms underlying the calciotropic actions of prolactin in higher vertebrates are similar to those in teleosts, then fish will provide a unique model that can be used to examine these possibilities in the absence of the major terrestrial vertebrate hypercalcemic hormone parathyroid hormone (Flik, Fenwick & Wendelaar Bonga, in press).

Although there may be other hypercalcemic hormones available to fish, as yet, none has demonstrated the important ability to stimulate branchial calcium uptake or Ca^{2+} ATPase activity. Estrogens (estradiol), present with the onset of sexual maturity in female teleosts (Fostier et al, 1978), have been known to induce hypercalcemia in the goby and tilapia (Nagahama, et al, 1975), and, in male killifish (Pang & Balbontin, 1978). The pars intermedia of the fish pituitary may also contain a hypercalcemic factor referred to as hypercalcin (Parsons et al, 1978, Pang, 1981a). However, it has been shown to have only acute effects on fish plasma calcium levels (Pang & Yee, 1980), and, as yet its identity has not been elucidated (Parsons et al, 1978, Ball, Batten & Pang, 1982a, Ball, Uchiyama &

Pang, 1982). As well, the fish liver contains hypercalcemic vitamin D metabolites in abundance (Fenwick and Wendelaar Bonga, 1982) and these have been reported to elevate plasma phosphate (MacIntyre et al, 1976), increase osteocytic resorption of bone (Chartier et al, 1977), and stimulate intestinal calcium absorption in eels (Lopez et al, 1980). But these findings are not well supported. In addition, no research has examined the effects of vitamin D on the branchial high affinity Ca^{2+} -ATPase, and, as yet its role in fish remains enigmatic (Taylor, 1985). Cortisol, produced in the interrenal tissue acts in synergistic fashion with prolactin to stimulate and maintain ion pumps at the gill (Hirano & Mayer- Gostan, 1978). Further, promising evidence has been provided implicating cortisol as a hypercalcemic factor by virtue of its ability to cause chloride cell proliferation (Foskett et al, 1983, Perry & Wood, 1985). Moreover, in trout, cortisol was shown to enhance branchial Ca^{2+} uptake presumably through an overall increase in the total amount of Ca^{2+}-ATPase and hence an increase in total activity of this enzyme (Flik & Perry, 1989).

PRL treatment has in fact been reported to elevate cortisol levels in various teleosts including tilapia (Flik et al 1986) and the American eel (Flik, Fenwick, & Wendelaar Bonga, in press). However in the eel, these elevated cortisol levels did not stimulate branchial Ca^{2+} -ATPase enzymic activity (Flik, Fenwick, & Wendelaar Bonga, in press).

The purpose of the present study was to assess the role of prolactin in calcium homeostasis in the American eel, emphasizing its effects on whole body calcium uptake. For this reason we employed the whole animal flux procedure introduced by Fleming, Brehe, and Hanson (1973). Flux experiments have been performed on perfused isolated gills in order to assess the effects on calcium uptake of various hormones such as hypocalcin in eels (Fenwick & So, 1974, So & Fenwick, 1977), the effects of estradiol in rainbow trout (Payan, Mayer-Gostan & Pang, 1981), characterization of the branchial Ca^{2+} pump in eels (Flik, Wendelaar Bonga & Fenwick, 1984 a,b, Flik, Van Rijs & Wendelaar Bonga, 1985a), and characterization of branchial transepitheleial calcium fluxes in rainbow trout (Perry & Flik, 1988).

More recently, whole animal fluxes have taken the place of the various isolated gill preparations in order to test the roles of hormones on whole body calcium uptake in several species including eels (Fenwick & Leung, 1981), Tilapia (Flik et al, 1985a, 1986) and rainbow trout (Wagner, Hampong & Copp, 1985, Perry & Wood, 1985, Flik & Perry, 1989). These studies provide fewer technical problems and, involve physiologically more relevant in vivo observations

Using this approach involved making a number of assumptions; (1) that in teleost fish the gill is the major route through which calcium is obtained (Berg, 1970, Flik et al, 1985a), (2) that whole body calcium influx rates reflect branchial flux rates (Flik et al, 1985a), and (3) that the primary target organ for prolactin and the other major calcium regulating hormones in fish is the gill (Flik et al, 1986b,

Fenwick, in press). Where possible, changes in calcium uptake or efflux rates, plasma total calcium, plasma sodium, potassium, magnesium and osmotic pressure were attributed to changes in the activity state shown either morphometrically or cytologically of the prolactin cells. The thesis is divided into three general sections: first, the effects on eel calcium metabolism of presumed increases in endogenous prolactin; secondly, the effects of simulated decreases in endogenous prolactin; and finally the interrelationship between the pituitary and the Stannius corpuscles. Within these sections various specific topics are covered.

One portion of this study attempted to take advantage of a possible relationship between the hypocalcemic factor, hypocalcin, (Wendelaar Bonga & Pang, 1986) and the major hypercalcemic factor, prolactin (Wendelaar Bonga & Greven, 1978, Flik et al, 1986). The effects of Stanniectomy, removal of the source of hypocalcin, or hypophysectomy, removal of the source of prolactin, or both, was examined in respect to their effects on the various physiological correlates associated with the calciotropic actions of hormones.

Another section of thesis involved the use of tropic hormones or drugs in order to stimulate or inhibit endogenous prolactin production from the in situ pituitary. Typically the pituitary gland in teleost fish differs from the terrestrial vertebrate gland in three ways; (1) the absence of a median eminence and portal system external to the pituitary (2) the pars

distalis is directly innervated by hypothalamic neurosecretory fibres, and (3) no fish possess a pars tuberalis (see review by Holmes & Ball, 1974). In addition, the eel pituitary is divided into the posterior pars intermedia and the anterior pars distalis, itself further divisable histologically into rostral and proximal regions (figure 1). The eel proximal pars distalis (PPD) is comprised of two cell types, STH cells (growth hormone cells) and gonadotropes (Peter & Fryer, 1983). The rostral pars distalis (RPD) contains three cell types, most numerous are the LTH or prolactin cells interspersed with TSH cells, and at the posterior border of the RPD with the neurohypophysis is a layer of ACTH cells (Holmes & Ball, 1974). Finally, the pars distalis (rostral & proximal) and the pars intermedia are directly innervated by hypothalamic aminergic neurosecretory fibres.

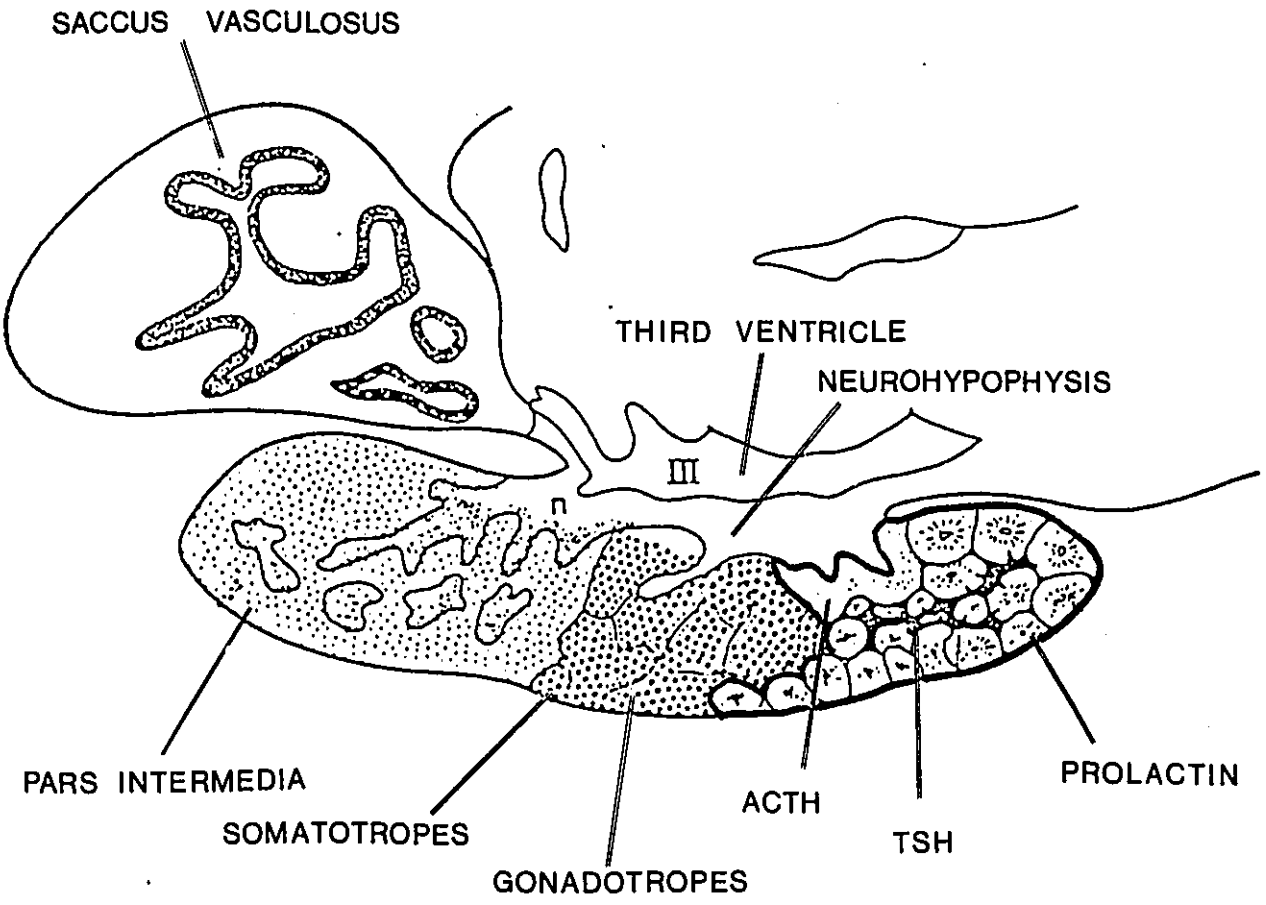
With this in mind, eels were treated with two known mammalian prolactin releasing factors (PRF's) haloperidol (Colles et al, 1987) and thyrotropin releasing hormone (TRH) (Haisenleder et al, 1986a,b); and the aminergic prolactin release inhibiting factors (PIF's), dopamine (Wigham & Ball, 1974), bromocryptine, ergocryptine (Olivereau, 1975, Peter & Fryer, 1983) and apomorphine (Caron et al, 1978), in an attempt to affect the endocrinological functioning of the rostral pars distalis.

In addition, the effects of eel PRL lobe implants were examined. As studies have shown that PRL secretion from the teleost pituitary is under tonic inhibitory control from the hypothalamus, and, as both TSH & ACTH cells degenerate (and only the prolactin cells become activated) when the fish pituitary is explanted (Ball, 1969) it would seem that the teleost

Figure 1

Anguilla anguilla. Diagram of a midsagittal section through the eel pituitary, anterior to the right. The follicles of lactotropes (PRL) mixed with TSH cells and posteriorly bordered by ACTH cells form the rostral pars distalis (RPD). The posterior pars distalis (PPD) is comprised of cells of the neurohypophysis, but is mainly composed of somatotropes and gonadotropes. Posteriorly the pars intermedia is deeply invaded by the neurohypophyseal processes and contains PAS (periodic acid-Schiff) positive cells which the source of so-called hypercalcin and PBH (lead hemotoxylin) positive cells.

Diagram after Olivereau (1969)


SACCUS VASCULOSUS
THIRD VENTRICLE
NEUROHYPOPHYSIS
III
PARS INTERMEDIA
SOMATOTROPES
GONADOTROPES
ACTH
TSH
PROLACTIN

provides a unique model with which to study the effects of homologous prolactin treatments using implantation surgery. In addition to this, the eel in particular provides an excellent means of obtaining grafts consisting largely of prolactin cells. Figure 1 illustrates that the eel rostral pars distalis is comprised primarily of prolactin cells, and our experience and that of others (Flik, Fenwick & Wendelaar Bonga, in press) has shown that this white "prolactin lobe" is easily separable both visually and surgically from the grey posterior pars distalis (PPD) and pars intermedia. Intact, hypophysectomized, and hypophysectomized plus stanniectomized eels were treated in this manner in various experiments exemplifying either exposure to sources of excess prolactin (PRL lobes) or residual pituitary grafts (PPD/pars intermedia).

Further, ovine prolactin treatments were administered to FW and SW adapted eels either by injection or continuous infusion. Most studies, even on fish, employ ovine PRL because of its availability in pure form. Research has shown that fish homogenates also have activities similar to ovine PRL (Clarke & Bern, 1980), although recently, Hasegawara et al (1986) reported that chum salmon PRL was up to 100 times more potent than ovine PRL in the hypophysectomized Fundulus sodium retention bioassay (see Grau et al, 1984). This may be the reason behind the typically high doses of ovine PRL used in various studies (see Pang et al, 1978, Flik et al, 1986 a,b), and which one could argue evoke pharmacological rather than physiological responses. Nevertheless, these doses (0.15-0.30 IU/g fish/day) seem necessary. Further, prolactin characteristically requires 3-5 days for its effects to become noticeable (Bern et al, 1981b, Flik et al, 1986 a,b, Flik, Fenwick,

al, 1986 a,b, Flik, Fenwick, Wendelaar Bonga, in press).

Until recently, studies on regimen or schedule dependence have not been a part of assessing drug or hormone action, however, interest has grown on the impact of drug administration methods, as well as, on the expression of hormone and drug studies. Smaller animals, including fish, generally metabolize and/or excrete drugs much more rapidly than do humans (Nau et al, 1981). As a result, most hormone half lives are a fraction of what they are in man. A daily injection regimen then, produces high peak blood concentrations followed by lower often undetectable levels (Urquhart, 1982). In order to avoid fluctuations in blood prolactin levels, the use of mini osmotic pumps was employed. Two other studies have used this method; in tilapia (Wendelaar Bonga & Flik, 1982) and more recently in the eel (Flik et al, 1986b, Flik, Fenwick & Wendelaar Bonga, 1986, in press) to demonstrate the calciotropic actions of prolactin in fish.

Another section of this study represents an attempt to identify the time frame within which both in situ circulating prolactin and ovine (exogenous) PRL may work to stimulate calcium uptake, and, to elevate plasma calcium levels. To test the effects of ovine PRL, SW adapted eels were used so that endogenous PRL levels would presumably be depressed (Wendelaar Bonga & Greven, 1978, Hirano, 1986) without the use of pharmacological or surgical intervention. As a result, changes in calcium uptake and plasma calcium would be due only to exogenously administered PRL and the time it takes to elicit these changes would be evident.

In conjunction with this, a second set of experiments was performed in which, SW acclimated eels were used to test the time frame required for *in situ* or endogenous prolactin to affect these changes. This test attempted to mimic the catadromous migrations (SW to FW) displayed by the American eel (Vladkykov 1964). Apparently, immature eels leave their spawning areas south of the Bermuda Island in Sargasso Sea and make their way north travelling east of the Bermuda Island, to the freshwaters of eastern Canada and the northeastern U.S.A. The Gulf Stream, Florida and North Atlantic Streams play important roles in the distribution of the eels on their way to the coast and during autumn return to the Sargasso Sea (Tesch, 1977). Clearly, the eels are faced with large salinity changes during this migration which must be compensated for by the osmoregulatory mechanisms available to it, and, ultimately the endocrine factors responsible for maintaining these mechanisms. During the migration process calcium homeostasis is strictly maintained (Bjornsson & Nilsson, 1986) largely due to the actions of the gills (Maetz *et al*, 1967a,b, Shuttleworth and Freeman, 1973, a,b,c,). It was decided this same process be imitated in SW adapted fish in order to possibly identify the time required for stimulation of PRL cells. An attempt was made to link these two factors with the stimulation of calcium uptake and elevation of plasma calcium. The aim of the test was to stimulate PRL cell activity using conditions similar to what the eels would face during a catadromous migration.

The ability of prolactin to prevent calcium ion loss or calcium efflux (Hirano et al, 1976, Flik et al, 1986b) was also assessed. Efflux rates were determined in stanniectomized eels, hypophysectomized eels, and in intact eels treated with PRL lobes or ovine PRL.

The final section of the thesis is a record of calcium influx rates, plasma total calcium, plasma sodium, potassium and total magnesium in intact fish throughout the length of study. Care was taken to identify possible fluctuations or cycles in these parameters (Fleming, Brehe & Hanson, 1973, Wagner Hampong & Copp, 1985, Flik et al, 1985a).

MATERIALS AND METHODS

1. Experimental Animals

Immature Americans eels, Anguilla rostrata Le Sueur (75-200 grams) were obtained during the summers of 1986 and 1987 from an eel ladder located at the W.H. Saunders hydroelectric dam, Cornwall, Ontario. In the laboratory eels were held in fibreglass tanks ($4m^3$) supplied with well aerated, running, dechlorinated City of Ottawa tap water (Ca^{2+}=0.4mM). The fish were not fed and were kept under 12 hours of light alternating with 12 hours of darkness at a temperature which fluctuated during the year (4-13° C).

Prior to any experiment fish were transferred to white 200 litre plastic tanks supplied with the same water as described above and were held for at least a week. For salt water (SW) studies fish were acclimated to 66% SW in glass 45 litre aquaria and were kept under constant light conditions for at least three weeks, and at a temperature of 5°C. Saltwater in the aquaria was aerated (Hagen-Elite 800, Montreal, Quebec), filtered continuously (Aqua Clear Jr.), and replaced on a regular basis. Constant light was used to keep eels in the tanks as they become active in the dark (Tesch, 1977).

2. Surgical Techniques

(a) Anaesthetization

All surgical procedures were performed on anaesthetized fish. For recovery operations, eels were anaesthetized in a solution of two grams/litre MS222 (Sigma) buffered (1:1) with Trizma base (Sigma) to give a pH of 7.8. For non-recovery or terminal procedures the anaesthetic used

consisted of an unbuffered solution of (DH 4.5) 4-5 grams/ litre MS222. For all anaesthetizations eels were placed in a 10 litre tub for 5-7 minutes in a total of 5 litres of the appropriate anaesthetic solution.

(b) Fish Marking For Identification

For experiments involving two experimental groups sham operated fish (or appropriate controls) were distinguished from experimental eels by installing a wound clip (Clay Adams, division of Becton Dickinson, Parsippany, N.J.) on the dorsal fin. For three group experiments, shams were clipped, experimentals were unmarked, and intact eels received one stitch with 00 silk (Ethicon Inc., Somerville, N.J.) on the dorsal fin.

(c) Stanniectomy (Surgical Removal of the corpuscles of Stannius).

A 1.5 cm longtitudinal incision was made approximately 1 cm lateral to the vent. The corpuscles were removed from beneath the ventro-lateral surface of the kidney capsule using fine forceps (Fenwick & Forster, 1972). Sham operations were performed in the same manner excepting corpuscle removal in order to simulate the wounding caused by exposing the corpuscles.

All suturing was done using surgical silk (Ethicon) and using the surgeon's knot. Muscle tissue was stitched with 000 silk, skin was held together with 00 silk.

(d) Hypophysectomy (Surgical Removal of the Pituitary)

The rostral pars distalis (RPD) of the eel anterior pituitary contains

a large mass of prolactin (PRL) secreting cells and its removal is facilitated by the fact that it can easily be seen through the cartilage on the roof of the mouth (see also figure 1 - introduction). To remove the pituitary an operating tray was modified such that two holes about the width of the fish's head were placed at one end, and two retort stands were placed at the opposite end. The eel was held ventral side up, and the upper jaw fastened to the tray with a length of surgical silk run through the two holes placed in the tray. The lower jaw was held open with another piece of silk fastened to both retort stands as far caudal as possible allowing access to the roof of the mouth. The cartilage on the roof of the mouth was carefully removed with a drill (Dremel moto-tool model 395) save for a thin layer directly over the white RPD. A pair of curved fine forceps was used to make a small perforation in this final cartilage layer and the pituitary was removed by applying low suction with the use of a suction apparatus consisting of a glass pasteur pipette bent 90° at the tip. A hole placed on the pipette shaft was used to adjust the suction strength by either covering or uncovering the hole with a finger. The pipette was connected by polyethylene tubing (Tygon) to a vacuum pump (Vactorr Direct Drive 100, GCA Precision Scientific, Chicago, 511). The success of the procedure was determined by observing the pituitary in the glass pipette. A small piece of fibrin impregnated gel foam (Upjohn Inc., Toronto) was then inserted into the cavity left after the removal of the pituitary. Sham operated fish were treated in the same way except that the pituitary was not removed. As an additional verification of successfull hypophysectomy plasma cortisol assays were run on several occasions. Low

plasma cortisol was taken as indicative of successful hypophysectomy (see section 8-C for assay and section 9 for individual experimental protocols).

(E) Hypophysectomy Plus Stanniectomy

Fish upon which both these operations were performed underwent the identical procedures as previously described for hypophysectomy (d) and Stanniectomy (c). Hypophysectomy was always performed first. The time elapsed for both operations was 20-25 minutes. Control fish were sham operated for both procedures and were prepared as above.

(F) Removal and Isolation of Prolactin Lobes and Residual Pituitary for Implantation Experiments.

The process of pituitary removal from "donor" fish for use as implants differed from removal by hypophysectomy. Donor eels of about the same weight as recipient fish were anaesthetized for about 5-7 minutes in terminal anaesthetia. The eel was then held firmly with one hand around the opercular region just posterior to the skull. Starting at the caudal end of the skull (epiotic bone) a firm stroke was made anteriorly completely sheering off the top of the skull. The medulla oblongata was lifted dorsally and rostrally with fine forceps to reveal the pituitary which was removed with another pair of fine forceps. The pituitary was placed in a watch glass in physiological saline and, under a microscope the RPD containing the prolactin cells was separated from the rest of the pituitary ("residual" portion). These prolactin lobe were implanted within 15 minutes of removal from donor fish.

(G) Implantation of Prolactin Lobes and Residual Pituitary

Prospective recipients of prolactin lobe or residual pituitary homografts were weighed to ensure that they were about the same weight as donor fish and were then anaesthetized in recovery anaesthetic for 5-7 minutes. A longitudinal incision through skin and muscle was made lateral to the vent to expose the renal capsule. The peritonium covering the mesonephric kidney was cut and the homografts from donor eels were inserted with fine forceps. The hole in the peritoneum was then plugged with a small fragment of hemostatic gelfoam and a single stitch of 000 surgical silk was installed. The surrounding muscle tissue was sewn with 000 silk and the integument closed with 00 silk. Eels that were sham operated for implants underwent the identical procedure except that fragments of dorsal musculature from donor fish were used as homografts.

(H) Criteria for Successful Prolactin Lobe or Residual Pituitary Homografts

The success of homografts were tested by performing autopsies at the end of the experiments. Vascularization of the lobe or residual pituitary as seen under a dissecting microscope was taken as the criteria for a successful graft (Nagahama, Nishioka & Bern, 1974). Preliminary observation showed that vascularization occurred within 4 or 5 days.

(I) Implantation of Mini-Osmotic Pumps

Intraperitoneal implantation of 200 ul Alzet mini-osmotic pumps (model 200, Alza Corporation, Palo Alto, California) was accomplished by making a 1 cm incision running anterior/posterior just lateral to the

ventral midline and 3 cm anterior to the vent. Pumps were filled with the appropriate solution and inserted delivery portal facing anteriorly. The muscle was sutured with 00 silk and skin with 000 surgical silk.

(J) Validation of Perfusion

At the end of experiments the infusion rates of the pumps were checked by measuring the pump contents and comparing this to the calculated infusion rates for the pumps at the ambient temperature. In all cases the difference between actual and predicted infusion rates was less than 5%.

(K) Blood Sampling

Eels were anaesthetized in terminal anaesthetic (see section 2-A) for about five minutes after which a ventral 2 cm incision was made longitudinally just anterior to the heart. Skin and muscle were spread with tissue spreaders and the ventral aorta was exposed. A hemostat was used to hold the vessel taut so that a needle could puncture the aorta. The hemostat was then clamped around both needle and aorta and from 0.5 to 1.0 ml of blood was withdrawn. Blood was placed in an eppendorf centrifuge cup and centrifuged for two minutes at 5000 x g. The plasma was transferred to a clean centrifuge cup (containing 10 ul of ammonium heparin solution) using a pasteur pipette and the plasma was stored at -20°C. The needle used to take blood was a 1 inch 23 guage needle fixed to 1 ml tuberulin syringe (Becton Dickinson). Previously the needle and syringe were soaked in ammonium heparin solution 1000 units/ml and then air dried.

3. Administration of Hormones and Drugs

(A) Bolus Injections

All fish were injected intraperitoneally (i.p.) 1.5-2.5 cm anterior to the vent just lateral to the ventral midline. All drugs used were prepared no more than one hour prior to the experiment and injections were performed with a one inch 23 guage needle (Becton Dickinson & Comp.) fixed to a 1cc tuberculin syringe. Various experiments employed either single or multiple injections of different hormones or drugs and these injection schedules and hormone/drug preparations are outlined in Section 8 (Experimental Protocols).

(B) Implanted Osmotic Pumps

Experiments utilizing infusion as a means of hormone or drug delivery involved surgical implantation of osmotic pumps as previously described in section 2-I. Infusion schedules and hormone/drug preparation for specific experiments are outlined in Section 8 (Experimental Protocols)

4. Calcium Flux Studies

(A) Measurement of Calcium Influx

(i) Influx Protocol

Eels were placed individually into 1.5 litre plastic flux boxes the day before the experiment. Each box was supplied with running dechlorinated water but was not aerated. Just prior to the experiment the water to the boxes was disconnected and the volume was adjusted to 1 litre. At this point a 5 ml water sample was taken to measure background radioactivity. Enough aqueous solution of ^{45}Calcium (as $CaCl_2$; specific activity 2.1 mCi/ml, Amersham, Intl.) was added to each box to give an

external count of 35,000 dpm/ml in FW and 400,000 dpm/ml in 66% SW. The eels were fluxed for four hours. Water samples were taken hourly during the flux period to ensure that the ambient specific activity remained stable throughout during the course of the experiment.

At the end of the flux period each eel was rinsed in tapwater and then placed in terminal anaesthetic containing 10 mM $CaCl_2$ to facilitate the removal of surface bound tracer. Each fish was weighed, a terminal blood sample was taken, and the exact time recorded.

Plasma samples were prepared from the blood samples and stored frozen at -20°C in labelled centrifuge cups for later ion analysis and ^{45}Ca determination. Likewise, fish carcasses were placed in plastic bags, labelled and stored at -20°C for subsequent whole body ^{45} calcium determinations. It has been suggested that fish mucus plays an important role in osmoregulation (Johnson, 1973) and, since the mucous cell matrix is known to concentrate cations from the environment (Kirschner, 1977) it was felt that fish should be rinsed in anaesthetic containing $CaCl_2$ in order to remove surface bound isotope. Data collected from mucus scrapings (see results Table 14) indicate that after going through the typical flux procedure the mucus of eels contains somewhat less than 2% of the total radiocalcium of the fish

(ii) Determination of Whole Body ^{45}Calcium and Influx Rates

Frozen eels were placed in a 1 litre pyrex beaker and microwaved (Sharp carousel microwave oven) at medium high setting for four minutes. Each whole eel was then homogenized for three minutes in 200 ml

of dH_2O using a Waring blender. Duplicate samples, about 10 g each, were taken from each homogenate with the blender running to facilitate the aquisition of homogeneous samples and were placed in preweighed 25 ml high form ceramic crucibles. The crucibles were then weighed to obtain the exact weight of the homogenate samples.

Homogenate samples were dried for 12 hours at 70°C in a Fisher Isotemp oven, ashed at 550°C for 4-6 hours in a Fisher Isotemp muffle furnace model 184A, and the ash dissolved in 5 ml of 1N HCL for 24 hours. This acid extract was neutralized with 833 ul of 6N NaOH. Three 500 ul aliquots of each dissolved sample were taken (total of 6 per fish), placed in separate plastic scintilation vials, and 4.5 ml of dH_2O and 10 ml of scintillant (ACSII, Amersham) were added to each. This mixture was counted for ^{45}Calcium activity by liquid scintillation counting using an LKB Rackbeta LSA with a built in DPM program. From this, whole body ^{45}Ca content was calculated.

Calcium influx rates (Jin) were calculated from whole body counts (WBC) and ambient specific activity (SA) as follows:

$$Jin = \frac{WBC \times 1000 \text{ grams}}{SA \times \text{fish wt.} \times T} = \text{umol } kg^{-1}hr^{-1}$$

Where WBC = whole body ^{45}Ca counts; SA = specific activity of the ambient medium (DPM/ml H20) - umole ^{40}calcium/ml H20); fish weights are in grams; T = duration of the flux period for that particular fish in hours; and 1000 is the conversion factor to kilograms.

B) Measurement of Whole Body Calcium Efflux

(i) Efflux Protocol

For efflux studies fish were injected intraperitoneally (i.p.) with 25 uCi of ^{45}Ca in 0.5 ml of 0.9% NaCl per fish 72 hours before the start of the four hour efflux period. This ensured stable plasma tracer levels during the efflux period between 72 and 76 hours post injection (see Table 1). This enables one to use the blood specific activity at the end of the four hour efflux as representative of the specific activity of the plasma throughout the four hours.

TABLE 1 ^{45}Calcium Levels In Eel Plasma Following Different Intervals

	Time After Tracer Loading	Mean + SEM, n=20 ^{45}Ca Levels (DPM/ml)	% Decrease in Amount of Tracer
Flux Period	12	94.92 ± 6.69	0
	24	84.64 ± 5.43	10.87
	36	74.10 ± 5.49	12.45
	48	70.80 ± 6.96	4.45
	72	65.46 ± 6.33	7.54
	76	64.83 ± 5.51	0.96

The day before the efflux experiment eels were placed individually into 1.5 litre plastic flux boxes. Just prior to the experiment the water to the boxes was disconnected and the volume adjusted to 1 litre. At this point the first 5 ml water sample was taken and this represented time zero of the efflux. Water samples were then taken hourly for four hours so that the appearance of tracer could be followed. The relative quantity of isotope leaving the fish was monitored to ensure that efflux rates were even and could be attributed to branchial and not urinary routes of efflux.

At the end of the flux period (exact time recorded) eels were placed in terminal anaesthetic. Each fish was then weighed and a terminal blood sample was collected. Plasma samples were prepared from the blood samples and stored frozen at -20°C in labelled centrifuge cups for later ion analysis and ^{45}Ca determination.

There was only one exception to this protocol, that being the efflux experiment performed June 8, 1987. Here eels were administered ovine PRL by continuous infusion via osmotic pumps. Simultaneous with this the fish were continuously infused with ^{45}calcium (instead of the usual bolus injection 72 hours prior to the experiment). This experiment then, saw fish exposed to both the isotope and PRL solutions for ten days. Plasma ^{45}calcium levels were assumed to remain stable under these conditions since pump infusion rates were constant (see section 2-J).

(ii) Determination of Whole Body ^{45}Ca Efflux Rates

The measurement of whole body ^{45}calcium efflux rates (Jout) was based on the rate of appearance of the tracer in the ambient medium

over the four hour period and was calculated as.

$$Jout = \frac{DPM_f - DPMmi^{-1} \times 1{,}000}{SA\ blood \times fish\ wt \times T} = umol\ kg^{-1}\ hr^{-1}$$

Where DPM_f = final DPM/ml of ambient medium; DPM;= initial DPM/ml of ambient medium; SA blood = DPM(^{45}Ca)/ml blood .(umol (^{40}Ca)/ml blood)$^{-1}$; fish weights are in grams; T = time of flux for that fish in hours; and 1000 is the conversion factor to kilograms.

5. Histological and Morphometric Studies of Eel Prolactin Cells

(A) Location and Removal of the Pituitary: In Situ and Final Fixation

Pituitaries were exposed as for pituitary removal (refer to earlier section 2-F) and then flooded in situ with fixative (see section 5E) for 60 seconds. The fixed pituitaries were removed with fine forceps and were placed in separate 1 dram vials containing fresh fixative (see part E below). This fixative was replaced after 1 to 2 hours and the pituitaries were left in fixative at 4°C over night. The fixative was then replaced with cacodylate buffer (see part E) containing 7% dextrose. Tissues could be stored indefinitely at 4°C after this point.

B) Final Fixation, Dehydration, Infiltration, Embedding

Previously fixed tissues were post fixed for two hours in a 1% osmium tetroxide solution and then rinsed in dH2O for 10 minutes.

Tissues were then dehydrated in the usual way.

- 1 rinse with 50% ethanol - (10 minutes)
- 1 rinse with 75% ethanol - (10 minutes)
- 1 rinse with 95% ethanol - (10 minutes)
- 3 rinses with absolute ethanol (10 minutes each)
- 2 rinses with propylene oxide (15 minutes each)

Epoxides are alkylating agents causing cancer by disrupting DNA, also are effective dehydrating agents reacting with H2O to form alcohols.

Following this tissues were infiltrated overnight in a 1:1 resin/propylene oxide solution. After 12 hours the resin/propylene oxide solution was replaced with resin mixture only, and the tissues were allowed to sit in open vials for 4 hours.

For embedding, fresh resin was centrifuged (Sorvall super speed RC2-B Auto Refrigerated centrifuge) for 10 minutes at 2000 x g and placed in a flat embedding mold (J.B. EM). The tissues were placed in the mold and properly oriented under a dissecting microscope and labelled. Molds were then placed in a drying oven at 60°C (Thelco Precision Scientific Co,) for 36 hours to allow the resin to polymerize.

C. Sectioning and Staining For Light and Electron Microscopy

Blocks were trimmed using the trimming station on an LKB ultratome III microtome or using a dissecting microscope (Wild Hferbrugg). Thick sections (1 um) were cut on the LKB ultratome III with glass knives (Fisher) made using an LKB knife maker, type 7801 B. Thick sections were placed on pre-cleaned specimen slides, heat dried using a Corning PC-35 hot plate, then heat stained with an Azure II/Methylene blue stain. Thin sections (60-80nm) were cut on the same microtome, and placed on 200 mesh copper EM grids (J.B. EM) using copper pick-up grids (J.B. EM). Grids were stained for 30 minutes with uranyl acetate (light sensitive) and 10 minutes with lead citrate (with NaOH pellets added to absorb CO_2), on petri dishes lined with dental wax.

Light micrographs of thick sections were obtained on a Zeiss light microscope using a planapo 63x/1.4 oil immersion lens, with a red filter on the condenser. Electron micrographs were obtained on a Phillips 300 electron microscope.

D. Morphometric Analysis of Prolactin Cells

To obtain an objective measure of cellular activity nuclear measurements of prolactin cells were made. Prospective cells were observed under the light microscope (Zeiss) using a planapo 63 x 1.4 oil immersion lens with a red filter on the condenser. The eye pieces of the scope were equipped with scalar measurements through which both the "length" and "width" of any given ovoid nucleus were measured.

The lengths and widths for each nucleus were averaged to obtain a radius, and the nuclear volume was calculated using the formula $V=4/3\ r^3$. Within any given treatment group of fish, four fish were selected and 20 cells from each fish (total of 80 cells) were measured.

E. Make up of Histological Solutions

1) Cacodylate Buffer (0.1 M containing 0.05% CaCL2, pH 7.4).

To 200 ml of distilled water were added 4.28 g sodium cacodylate (BDH chemicals) and 0.13 g calcium chloride dihydrate (BDH). The pH was adjusted to 7.4 with several drops of 5N HCL and stored at 4°C.

2) Fixative

To 100 ml of cacodylate buffer was added 3 g of paraformaldehyde. This was heated to 60°C with a constant stirring then cooled to room temperature in a water bath and stored at 4°C. To 70 ml of this solution was added a 2 ml ampule of glutaraldehyde (also stored at 4°C) immediately before use of the fixative. The fixative was kept covered and on ice throughout the duration of any experiment.

3) Storage Buffer

Fixative was discarded and replaced with cacodylate buffer containing 7% dextrose. Tissues were stored at indefinitely 4°C.

4) Azure II/Methylene Blue Stain (Light Microscopy)

To 100 ml of dH2O were added 1 g each of methylene blue (Sigma) and sodium borate (BDH). To another 100 ml of dH2O was added 1 g of Azure II (sigma). These were stored refrigerated and mixed 1:1 in the appropriate amounts when needed.

5) a) Uranyl Acetate

Uranyl Acetate (J.B. Em) was made up to a final conc of 3.5% in 50% ethanol.

b) Lead Citrate

To 30 ml of dH2O were added 1.33 g lead nitrate ($Pb(NO3_2$) - BDH, and 1.76 g sodium citrate ($NO_3C_6H_5O_7$ - $2H_2O$-BDH). This was shaken for 1-2 minutes (cloudy). 8 ml of 1N NaOH was then added (solution cleans) and was set to stand for 30 minutes with occasional shaking. The solution was then made up to 50 ml with dH2O (pH=12.5).

6) Osmium Tetroxide Fixative

The final solution was a 1% osmium tetroxide solution (J.B. EM) in 0.1M cacodylate buffer (without CaCl2) with 7% dextrose. Toward this end, a 2% OsO_4 solution (volume varied depending on number of samples) was mixed 1:1 with a 0.2M cacodylate buffer (without $CaCl_2$) with 15% dextrose (again volume varied).

6. Tissue Sampling and Preparation

A) Blood Sampling - see 2-k

B) Pituitary Removal - see 2-F

C) Soft Tissue (Muscle, Skin, Organs) and Bone Separation and Sampling

Two mucous samples were obtained immediately after a terminal blood sample was taken from each anaesthetized fish that had been fluxed . Two four square inch areas per fish were scraped with a scalpel, and the mucous weighed and measured for ^{45}calcium content (see section 7-B). Frozen eels were placed in a 1 litre pyrex beaker with 200 ml dH20 and microwaved (Sharp Carousel microwave oven) at medium high setting for six minutes. Microwaving facilitated the separation of skin and muscle from the skeleton. Towards this end a dorsal slit lateral and parallel to the backbone was made the length of the eel. The soft tissue was carefully removed by hand from the backbone and cranial skeleton. The cartilage and bone of the cranium as well as the branchial basket were included in the bone samples. Bone and soft tissue were weighed separately for each fish. Bone samples (typical wt. 5-7g per fish) were digested in 25 ml of 15N HNO_3 and soft tissue (typical wt.-75g per fish) in 200 ml of 15 N HNO_3. Both were placed covered in a Fisher Isotemp drying oven overnight at 50°C to liquify. The next day samples were neutralized with equal amounts of 15N NaOH. Three to four samples (1 ml each) were counted for ^{45}calcium content (see section 8-B).

7. Analytical Techniques

A) Plasma Parameters (Total Calcium, Sodium, Potassium, Magnesium & Osmolarity).

Plasma samples (50 ul) were diluted 200 x in 10 ml of dH_2O and agitated in a vortex. Levels of total calcium, sodium and potassium were determined by emission spectrophotometry and total magnesium was measured using atomic absorption spectrophotometry (Varian Spectra AA-10). Plasma osmolarity was determined using a Wescor, Inc. 5100 C Vapor Pressure Osmometer.

B) ^{45} Calcium Measurement

(i) General

The flux of calcium was followed using the radiotracer ^{45}calcium (Amersham) as $CaCl_2$ in aqueous solution with a specific activity of 2.1 mCi/ml. The activity of ^{45} calcium was measured by liquid scintillation counting using an LKB Rackbeta LSA with a DPM program.

(ii) Whole Body ^{45}Ca Activity

Samples of tissue homogenate were weighed, dried, ashed, and dissolved in acid and neutralized (see section 4A). Following this, three 500 ul aliquotes of each dissolved sample were taken (total of 6 per fish) placed in separate plastic scintillation vials, and 4.5 ml of dH2O and 10 ml flour (ACS II, AMersham) were added to each. This mixture was counted for ^{45}calcium activity. From these, whole body ^{45}calcium content was calculated.

(iii) Plasma and Water

Plasma samples, 50 ul, were mixed with 5 ml of distilled water and 10 ml ACS II for counting. Water samples, 5 ml were counted by adding 10 ml of ACS II to each sample.

(iv) Soft Tissue, Bone, Mucous

To 1 ml samples of digested soft tissue or bone, were added 4 ml dH2O and 10 ml of ACS II scintillation flour. Mucous samples of a known weight were treated in the same manner and counted for ^{45}calcium.

(C) Plasma Cortisol Determination

Plasma samples, 20 ul, were used in determination of the plasma cortisol levels of fish from various experiments. A commercial ^{125} iodine based cortisol assay was used (Radioimmunoassay Incorporated, Scarborough, Ontario, Canada).

9) Experimental Protocols

(A) Effect of Stanniectomy (5 day - Sept. 20 &,23, 1986; 10 day Sept. 30 & Oct.2, 1986; 14 day Oct. 6 & 8, 1986; 21 day - Oct. 16 & 29, 1986; 56 day - Dec. 4, 1986)

The effect of Stanniectomy over various periods of time (5,10,14,21 & 56 days) on calcium influx rates, whole body calcium efflux rates, plasma total calcium, plasma sodium, potassium and total magnesium levels and prolactin cell morphometry was examined.

Sham Stanniectomy had no significant effect on any parameters measured relative to unoperated control fish (see results). As a result, the effect of Stanniectomy was tested only against a sham operated group. There were then, two groups of eels per experiment, sham stanniectomized and stanniectomized groups.

Surgery was performed under recovery anaesthetic producing N=6 fish for each of the two groups listed and for all periods of time except for the 56 day experiment where N=7 for both groups.

Following surgery, eels were transferred to 200L recovery tanks and held for the desired period. The survival rate was extremely high with but one mortality occurring in the sham group of the 5 day influx experiment and one in the stanniectomized group from the 10 day efflux experiment. On the day of any experiment the influx or efflux regimen for FW was followed and thereafter plasma thereafter analyses and cell morphometry were performed.

(B) Effect of Hypophysectomy (5 day - Jan. 15 and 29, 1987; 10 day - Oct. 4, 1987)

The effect of hypophysectomy for five and 10 day intervals on unidirectional calcium influx rates, whole body calcium efflux rates, plasma total calcium, plasma sodium, potassium, total magnesium and osmolarity, and plasma cortisol levels was examined.

Sham hypophysectomy had no effect on any parameter measured (see Table 2) and therefore only two groups of eels were tested for the five day experiments, sham hypophysectomized and hypophysectomized groups; however a third unoperated group was added for the ten day influx experiment.

Five or ten days prior to the chosen experiment dates eels were either sham hypophysectomized or hypophysectomized. Surgery was performed under recovery anaesthetic such that N=6 eels for all groups in all experiments.

After surgery the fish were placed in recovery tanks for the appropriate length of time. Two fatalities resulted, one each in the sham and hypophysectomized groups from the Jan. 29, 1988, five day efflux experiment. On the days of the experiments the typical influx or efflux procedure was followed and later plasma analyses performed.

A number of problems are inextricably linked with studies of teleost calcium metabolism and have been reviewed previously (Pang, 1973, Fleming, Brehe & Hanson, 1973). However, several authors report significant plasma cortisol levels in Fundulus grandis (Srivastava & Meier, 1972), and F. Kansae (Fleming, Brehe & Hansen, 1973) even after hypophysectomy. As such, studies examining calcium homeostatic alterations in the absence of

prolactin could possibly be suspect. Also as previously mentioned cortisol may play a role in branchial calcium uptake in Salmo gairdneri (Perry & Wood, 1985, Flik and Perry, 1989), therefore, testing for cortisol was performed (see section 8-C).

(C) Effect of Both Hypophysectomy and Stanniectomy (5 day - March 7, 1987)

The effect of both hypophysectomy and Stanniectomy for five days on unidirectional calcium influx rates, plasma total calcium, plasma sodium, potassium, total magnesium and osmolarity was examined.

There were two experimental groups in the experiment, one group both sham hypophysectomized and sham stanniectomized and a second group consisting of eels both hypophysectomized and stanniectomized. Fish subjected to both hypophysectomy plus Stanniectomy underwent these procedures in the manner described previously in sections 2-C and 2-D. Hypophysectomy was always performed first, the time elapsed for both operations was 25 minutes. Prior to surgery eels were placed in recovery anaesthetic and N=6 fish were operated on for both groups of fish. After the surgery, fish were transferred to the recovery tanks where they remained for the five days, no mortalities resulted. Typical influx procedures were followed the day of the experiment and various plasma analyses performed thereafter.

(D) Treatment with Prolactin Releasing Factors (TRH - July 22, 1987; Haloperidol - August 12, 1987).

The effects of putative prolactin releasing factors (PRF) on unidirectional calcium influx rates, plasma total calcium, plasma sodium, potassium and total magnesium were tested.

Each experiment involved an independent solvent injected group. TRH was injected as a single bolus and the flux was performed the same day. Haloperidol was injected daily for five days and the flux rates measured on the fifth day. Fish were not anaesthetized for the injections but were held in a net, and injections were performed in the usual manner (see section 3-A). N=7 eels for both treatment groups from each experiment.

Following the injection of TRH, fish were placed directly into the flux boxes as the flux was performed the same day. However, since haloperidol treated fish were injected daily for five days, they were held in the recovery tanks each day after the injections. On the day of the experiment the fluxes were performed in the usual manner and thereafter typical plasma analyses carried out.

Stock TRH (L-Pyroglutary 1-L-Histidyl-L-Prolinamide, 250 mg, Lot #125F-5920,m.w. 362.4, SIGMA) solution consisted of 10 mg of TRH dissolved in 1 ml of 0.1 N/HCL (after Haisenleder et al, 1986a). For experimentation 10 ul of stock TRH (containing 100 ug of TRH) was diluted and added to 490 ul of physiological saline. Of this 50 ul maximally was injected (containing 10 ug TRH) per fish per day. Stock TRH solution was lyophilized in a Labanco Freeze Dryer - 3 and stored at -40°C.

Haloperidol (Halo-teratogen, 5 g, Lot #85F-0008, m.w. 375.9, SIGMA) solutions were made up fresh on the day of the experiment. Toward this end the daily dose was 2.5 mg per fish per day dissolved in a solution 95% of which was physiological saline, and 5% of which was acetic acid. Na^+ in the saline is required for activation of this drug. The injected volume was 100 ul maximally. Controls received an equal volume of the carrier solution.

(E) Treatment With Prolactin Release Inhibiting Factors

DA-(intact Fish) - July 14, 1987; DA(Stx) - July 20;

Bromo-I-Aug. 7 ; Apo-I-Aug. 14; Ergo - I-Aug, 18;

Bromo-II-Nov. 6; Apo-II-Nov. 27, Ergo-II-Dec. 5, 1987).

The effect of putative prolactin release inhibiting factors (PIF) on unidirectional calcium influx rates, plasma total calcium, plasma sodium, potassium and total magnesium and in some cases plasma osmolarity and prolactin cell cytology and morphometry was examined.

There were two treatment groups per experiment, a carrier injected infused control group, and a PIF treated group. Dopamine (DA) was administered by continuous infusion for five days in intact or stanniectomized eels. Bromocryptine, apomorphine and ergocryptine were all administered by daily injection for five consecutive days. In all cases influx rates were measured on the fifth day of treatment followed immediately by plasma analyses and cell morphometry.

Dopamine infusion was used in two experiment, one on intact eels and one on stanniectomized eels; and experiments involving PIF injections (bromocryptine, apomorphine, ergocryptine) were performed in duplicate.

Fish were not anaesthetized for injections but were held with a net during injection; osmotic pumps for DA infusion were implanted in the aforementioned fashion (see section 2-I). N=7 in most cases except: BROMO-I-Aug. 7, where N=6 for BROMO treated fish and N=5 for shams; BROMO-II-Nov, 6, where N=8 for both groups and these data were pooled with those of Aug. 7 to create N=13 for shams and N=14 from BROMO treated fish; Apo-II-Nov. 27 where N=6 for APO treated fish; and finally ERGO-II-Dec. 5 where N=6 for both groups. Fish treated with DA were held in the white recovery tanks throughout the infusion period of five days. Similarly, bromocryptine, apomorphine and ergocryptine treated fish were held in these same tanks each day after daily injections.

Dopamine (3,4 dihydroxyphenylalanine Hydrochloride - m.w. 189.6-Sigma) infusion lasted five days. Pumps were implanted in the usual manner, all experiments were carried out as 12°C using the same pump type and implanting them in the same manner. DA dosage was as follows: a stock solution of DA was made up of 2.5 g DA made up to 1 litre in 0.07% acetic acid (after Haisenleder et al, 1986a). This was further diluted to give a DA infusate dosage of 15 mg/kg/day (after Olivereau, 1975). Seven experimental fish were tested and seven controls. Control fish received 0.1 mM acetic acid.

For the first experiment (August 7, 1987) bromocryptine (2-Bromo-2-ergocryptine Mesylate 100 mg, lot #44F-0297, m.w. 750.7, SIGMA) was dissolved in 50% ethanol and injected i.- p. at a dose of 2 mg/fish/day (Haisenleder et al, 1986a). The total injected volume per fish was 200 ul,

control fish received an equal volume of carrier. For the second experiment (Nov. 2-6, 1987) Bromocryptine was dissolved in 0.3% tartaric acid containing 30% ethanol (after Arita and Kimura, 1986) at the same dose and injected volume.

Apomorphine (Apomorphine Hydrochloride 1g, Lot #55F-0080 m.w. 303.8-SIGMA) was dissolved in a solution consisting of 30% of absolute ethanol and 70% physiological saline, and injected i.- p. at a dose of 3.5 mg/fish/day. The higher dose as compared to other drugs was necessary because of the relatively lower potency of apomorphine (Caron et al, 1978). This same regime was followed for the second experiment as well. Total injected volume was 150 ul.

Ergocryptine (Ergocryptine, 100 mg. Lot #108C-0264 m.w. 575.7, SIGMA) was dissolved in a 1:1 solution of 95% Ethanol and 0.9% NaCl containing 5 mg/kg fish weight tartaric acid [(D-) Tartaric Acid, 5 g, Lot #75F-3484, SIGMA]. Daily dose was 2.8 mg/fish/day and the total injected volume was 150 ul. (after Brewer & McKeown, 1978).

(F) Effect of Prolactin Lobe And Residual Homografts

(PRL lobes-intact fish-Feb. 2 and 12, and March 9 and Oct. 9, 1987; hypophysectomized fish - March 28; hypophysectomized and stanniectomized - April 4; and residual grafts - intact - Nov. 20, 1987).

The effect of prolactin lobe grafts in intact hypophysectomized, and both hypophysectomized and stanniectomized eels; and the effect of residual

pituitary grafts in intact eels, on unidirectional calcium influx, plasma total calcium, plasma sodium, potassium and total magnesium and in some cases plasma osmolarity, and prolactin cell morphometry, and whole body calcium efflux were examined.

There were two groups of fish per experiment, eels sham operated for implants and those receiving PRL lobe or residual pituitary implants, except for the lobe implant experiment on intact fish October 9 where a third unoperated group was included. All surgery was performed under recovery anaesthetic, so that N=6 for the sham and PRL lobe implant groups of February 2nd and March 9th and the sham and residual implant groups of November 20, however N=7 for those groups on February 12th, and N=7 for both shams and implants and N=4 for the intact group on October 9th. Data from Feb. 2 and 12 were pooled so that N=12 per group (see Table 9).

PRL lobes used for implantation were recovered from donor fish as described in section 2-F. The influx experiments on Feb. 2 and March 9 and the efflux experiment Feb. 12 involved the installation of 2 PRL lobes per fish, whereas for the influx on Oct. 9 four lobes per fish were used. Further, 2 residual pituitary fragments were implanted at any one time.

For experiments where hypophysectomized eels received implants, PRL lobes were initially implanted into intact fish. Eels were not hypophysectomized until five days later so that the grafts could vascularize before the <u>in situ</u> pituitary was removed. Similarly, eels were not hypophysectomized plus stanniectomized until five days after they too had received implants. For all implant experiments control fish were

sham operated for lobe implantation only. Shams received dorsal musculature fragments (from a common donor fish) as implants.

All fish were held in recovery tanks for the desired time post-surgery. Survival rates were high with only 1 mortality occurring in each group. On the day of the experiment typical influx or efflux procedures were followed, plasma analyses were done later and, where applicable, PRL cell morphometry performed.

G) Effect of Ovine Prolactin

Most studies on fish employ ovine PRL. Tilapia PRL has however been used and such studies showed that fish pituitary homogenates etc. have similar activities as ovine PRL (Clarke & Bern, 1980). But recently Hasegawara et al, 1986 reported that chum salmon PRL is up to 100 times more potent than ovine PRL in the hypophysectomized Fundulus sodium retention bioassay (see Grau et al, 1984). This may be the reason behind the typically high doses of ovine PRL used in various studies (see Pang et al, 1978, Flik et al, 1986, a,b) which one could argue evoke pharmacological rather than physiological responses. Nevertheless these doses (in the ug/g range) seem necessary, and, it takes 3-5 days for the effects of PRL to be noticeable (Bern et al, 1981, Flik et al, 1986 a,b Flik, Fenwick, Wendelaar Bonga - in press). The effect of ovine PRL administration by single injection in SW acclimated eels, by multiple injection in both FW and SW acclimated fish, and by continuous infusion via implantable osmotic pumps in FW fish on unidirectional calcium influx rates, plasma total calcium, plasma sodium, potassium and total magnesium, and occasionally whole body calcium efflux rates and prolactin cell morphometry were examined. (pump implants (FW fish)- June 8, July 17,27, 1987; multiple injections (FW fish) - Oct. 24, 31, 1987; multiple

injections (SW fish) - Nov. 16, 1987 & Jan. 9, 1988; single injections (SW fish) Dec. 30, 31, 1987, Jan. 6, 17, 18, 1988)

Every experiment included both carrier injected or infused and ovine PRL injected or infused groups. Injections were performed without anaesthetic as described (see section 3-A) and pump implantation under recovery anesthetic as outlined in sections 2-I and 3-B. For influx experiments fish received only 1 pump implant, however for the efflux experiment (June 8, 1987) 2 pumps were needed as both ^{45}calcium and ovine PRL were simultaneously infused throughout the ten days. N=6 for both experimental groups in most experiments except that N=14 for shams and 13 for PRL groups for continuous infusion experiments where influx rates were measured (pooled data from July 17 and 27) N=12 for both groups for PRL injections in FW fish (pooled data from Oct. 24 and 31), and N=5 for both groups of the continuous infusion experiment where efflux was measured on June 8, 1987.

After surgery or injection eels were held in the usual recovery tanks. Mortality was very low with only one death occurring in the sham groups of July 17, Dec. 30, 1987 and Jan. 18, 1988 and 1 each in the PRL treated groups of Jan. 9 and 18, 1988.

The usual influx procedures for FW or SW were used to determine flux rates and the appropriate plasma analyses and PRL cell morphometry performed. However, the typical efflux protocol was modified for the infusion experiment June 8, 1987. Instead of injecting fish i.p. with ^{45}calcium 72 hours prior to the flux period as per usual, these fish

were simultaneously infused with ^{45} calcium and ovine prolactin for 10 days. Additionally, although N=5 for both carrier and PRL infused groups in terms of the numbers of fish used to calculate efflux rates, originally the n value was 10 for both groups. Five fish from each group were dissected to determine the amounts of ^{45} calcium deposited into several calcium pools (Table 14) and the other five eels from each group were treated in the usual manner to calculate efflux rates (Table 12).

As mentioned the influx experiments using implantable pumps employed a single pump per fish. The multiple injection experiments, whether performed using FW or SW acclimated eels, consisted of injecting fish every other day (Monday, Wednesday, Friday) and measuring flux rates on Saturday, the sixth day (after Flik et al, 1986b). Only influx experiments were done. The single injection experiments performed used SW acclimated eels. On the day of injection five groups of 12 fish (within which each group consisted of six shams and 6 PRL treated fish) were injected. Thereafter at 0,9,24,48 and 72 hours post-injection, influx rates were measured. The prolactin used for the influx experiments in which fish were infused, was purchased from the Sigma Chemical Company, St. Louis Mo. (31 IU/mg total of 36 mg or 1000 IU, lot #26F-0076). A total of 101 mg of PRL was dissolved in 50 mM HCl (Flik et al, 1986) to a total volume of 1389ul, filling seven pumps each with a reservoir of 213 ul. At 12°C and a delivery rate of 0.298 ul/hr this provided a daily dosage of 1.5 IU of prolactin per fish per day; leaving 141.5 ul of excess PRL solution left in the pump. Controls were infused with 50 mM HCl (after Flik et al, 1986).

For the efflux experiments using PRL infusion the dose was the same, however, the protocol was changed slightly by spiking the hormone solution (infusate) with ^{45}calcium so that fish were infused with both PRL and tracer for 10 days. 10 fish were infused with PRL plus ^{45}calcium and 10 with 50mM HCl plus ^{45}calcium. Each "experimental" pump contained 7.21 mg of PRL, 59 ul of stock ^{45}calcium (Amersham, aqueous solution as $CaCl_2$, specific activity 2.1 mCi/ml) and 147ul of 74mM HCl (to give a final acid concentration fo 50mM. Two pumps were implanted per experimental fish. Control fish received only 1 pump each, containing 118 ul of stock ^{45}calcium and 95 ul of 111mM HCl (to give a final acid concentration of 50mM HCl). A variation on this technique has shown PRL to be stable under these conditions (Flik et al, 1986). Of the original 10 fish in the two experimental groups five controls and 5 PRL treated fish were dissected into wet tissue, mucous, and bone samples as described in section 6-C. The amounts of radiocalcium deposited into each "pool" were determined as described in section 7-B (see results - Table 14).

Prolactin used for all the multiple or single injection experiments was identical to that used for infusion.

Both SW and FW acclimated groups were held in tanks under constant light conditions (see section 1). SW groups being held in filtered sea water. On the day of transfer all fish were transferred to the recovery tanks supplied with running FW. Thereafter flux experiments were performed daily throughout the week.

(H) The Effect of SW - FW Transfer (transfer flux experiments performed daily)

Dec. 7 through Dec. 13, 1987)

There were always three groups of fish per experiment, the two transfer groups, SW acclimated and FW acclimated controls held under constant light and a third group from the main laboratory stock held under natural photoperiod, in the event that photoperiod affected the results.

Generally 6 SW to FW transfer fish, 6 FW to FW controls and 4 or 5 of the eels held under natural photoperiod were used each day. On the day of the experiment typical influx protocol for FW was used.

(I) Temporal Variations In Unidirectional Influx Rates and Some Plasma Parameters (September 1986 through December 1987).

There is some evidence of seasonal variations in whole body calcium influx. Fleming, Brehe & Hanson, (1973) reported several variations in calcium uptake rates of Fundulus Kansae (Wagner, Hampong & Copp, 1985) reported an 11 day cycle for Ca^{2+} uptake in rainbow trout while Flik et al (1985) report no seasonal variation in uptake rates of Tilapia. Nevertheless this factor must be accounted for in experimental designs.

That the parameters measured in this study could undergo temporal variations was investigated. Either valid control data from previous experiments was used, or fish were put through the flux procedures periodically whenever the time between flux experiments was lengthy.

These data represent only 1 group and represent either sham groups (where it was shown shamming had no effect) or carrier injected controls from the various injection experiments (when the solvent was composed mainly of physiological saline).

STATISTICAL ANALYSIS

All data were analyzed using either Student's t test or One or Two Way Analyses of Variance (ANOVA). $P \leq 0.05$ was taken as the accepted limit of significance.

RESULTS

Effect of sham-Stanniectomy and Stanniectomy (Stx) on uni-directional calcium influx, prolactin cell nuclear volume, plasma osmolarity and plasma total, Na^+, K^+, Mg^{2+} and Ca^{2+} in FW acclimated eels.

Sham Stanniectomy had no significant effect on any of the parameters measured relative to unoperated controls (data not shown). Consequently, the effect of Stanniectomy was tested only against a sham operated group. Figure 2 shows that stanniectomized eels exhibited significantly higher plasma total calcium 5 days after Stanniectomy. The hypercalcemia persisted throughout all tested intervals post-Stanniectomy (Figure 2B) and at 5, 10, 21, and 56 days following Stanniectomy calcium influx rates were significantly higher in stanniectomized eels. But the influx rates at the 14 day interval were not significantly different (Figure 2-A). Prolactin cell nuclear volumes were lower in stanniectomized eels after four of the five intervals but only two of these differences were significant. This was taken to indicate that, in general, prolactin cell activity declined following Stanniectomy. Plasma osmolarity and Mg^{2+} were not consistently affected by Stanniectomy but a significant hyponatremia was noted after 14, 21 and 56 days together with significant hyperkalemia at the 14 and 21 day test periods (Figure 3).

Figure 2

Effect of sham-Stanniectomy and Stanniectomy (STX) on unidirectional calcium influx (A), plasma total calcium (B) and prolactin cell nuclear volumes (C) in FW acclimated American eels, Anguilla rostrata, at 5, 10, 14, 21 and 56 days post-Stanniectomy. N=6 fish in all cases except for nuclear volume measurements where each point represents a mean of 80 cells from 4 fish in all cases. * $P \leq 0.05$ relative to control groups. Control groups (sham STX) are open bars and circles, stanniectomized groups, solids bars and circles. Values are means (bars or circles) $\pm$ S.E.M.

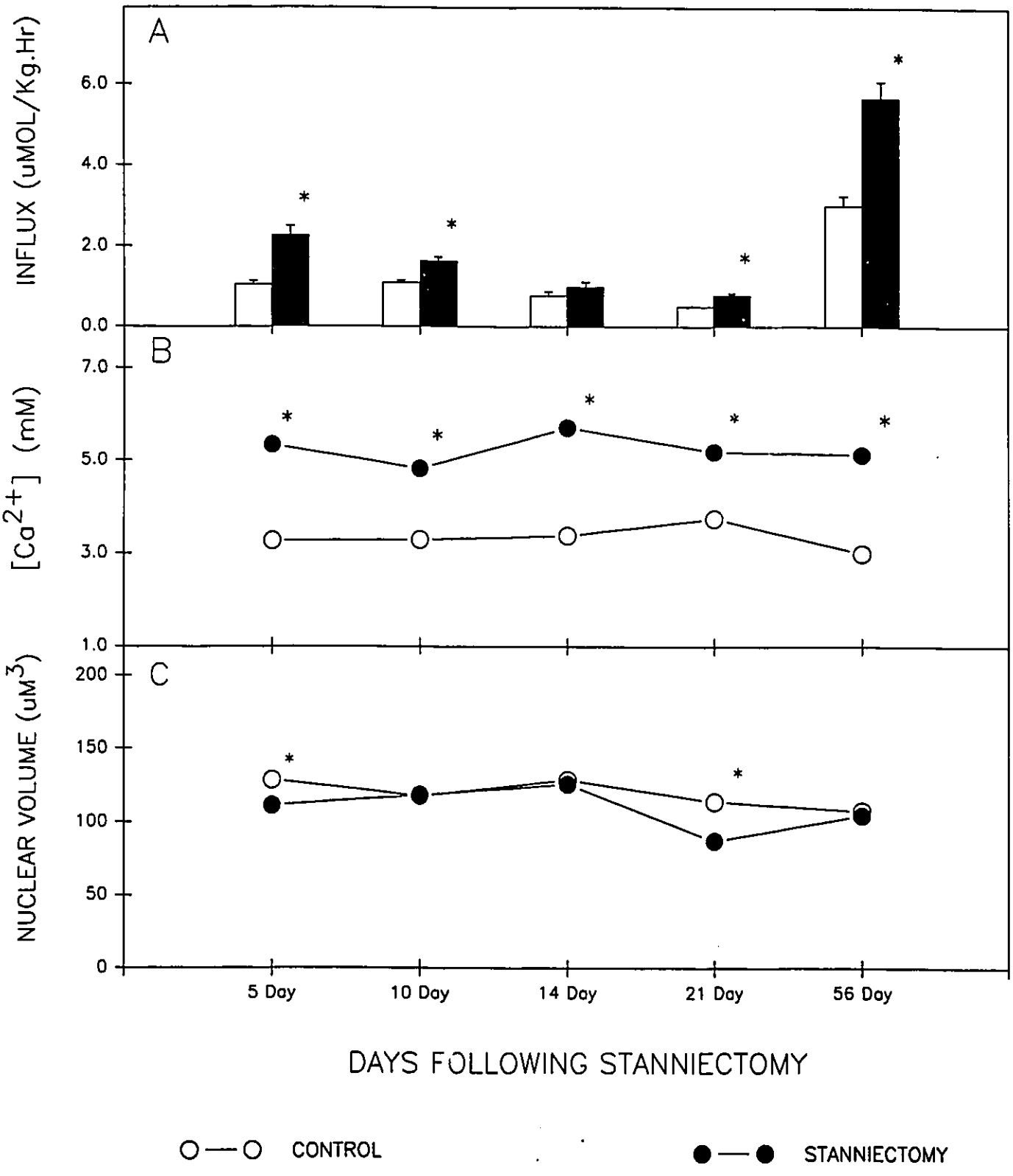
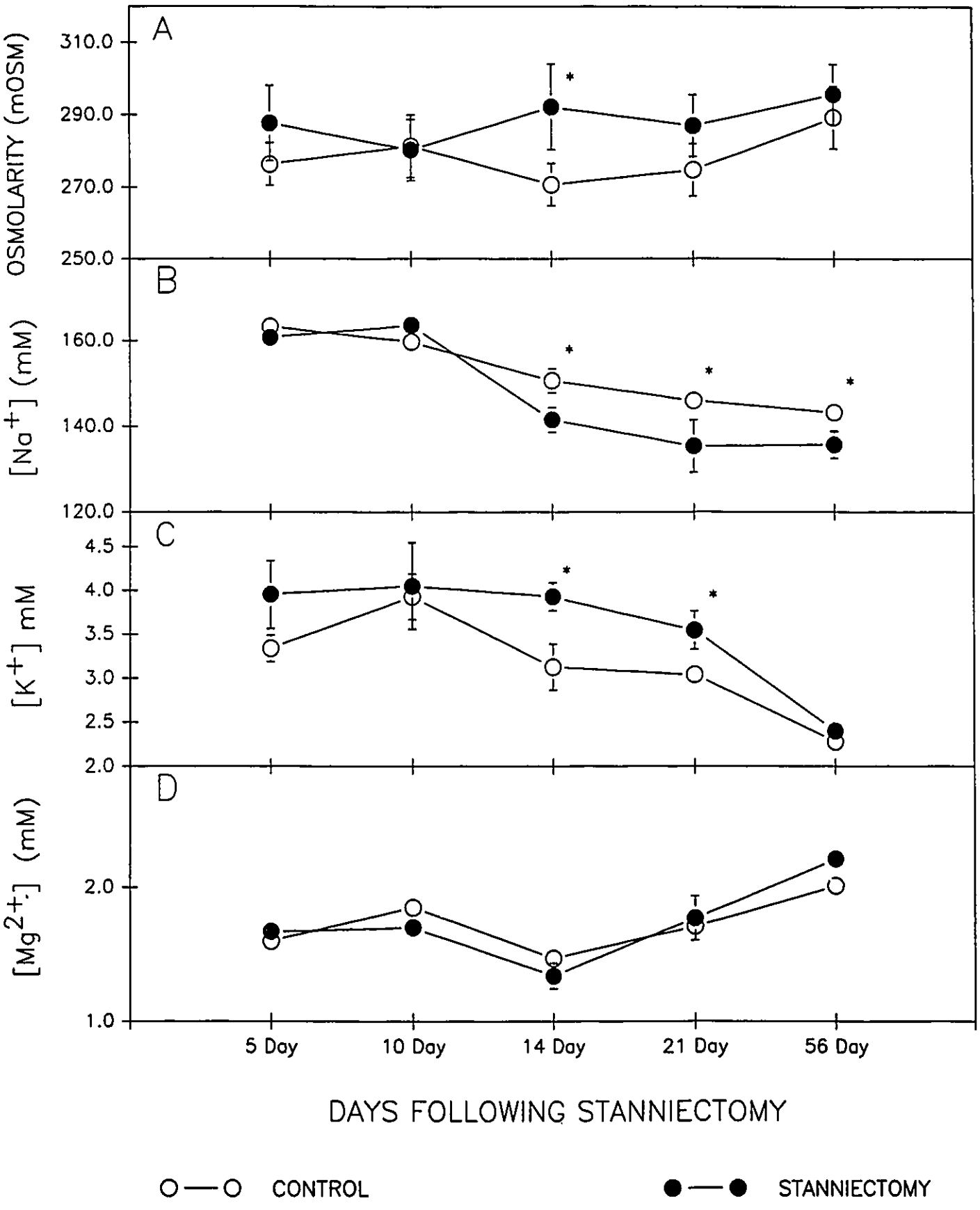

A
INFLUX (uMOL/Kg.Hr)
6.0
4.0
2.0
0.0
B
[Ca2+] (mM)
7.0
5.0
3.0
1.0
C
NUCLEAR VOLUME (uM3)
200
150
100
50
0
5 Day
10 Day
14 Day
21 Day
56 Day
DAYS FOLLOWING STANNIECTOMY
CONTROL
STANNIECTOMY

Figure 3

Effect of sham-Stanniectomy and Stanniectomy on various plasma parameters. Blood osmolarity (A), plasma sodium (B) plasma potassium (C) and plasma total magnesium levels (D) in FW acclimated American eels, Anguilla rostrata, at 5, 10, 14, 21 and 56 days post Stanniectomy. Means (circles) and S.E.M. (lines). N=6 in all cases. * $P \leq 0.05$ relative to control groups. Control groups open circles, stanniectomized fish solid circles.


A
B
C
D
OSMOLARITY (mOSM)
310.0
290.0
270.0
250.0
[Na+] (mM)
160.0
140.0
120.0
[K+] mM
4.5
4.0
3.5
3.0
2.5
2.0
[Mg2+] (mM)
2.0
1.0
5 Day
10 Day
14 Day
21 Day
56 Day
DAYS FOLLOWING STANNIECTOMY
CONTROL
STANNIECTOMY

Effect of sham hypophysectomy, hypophysectomy and both hypophysectomy and Stanniectomy on uni-directional calcium influx, plasma total calcium and various plasma parameters in FW acclimated eels.

Neither sham hypophysectomy nor hypophysectomy had any significant effect on any parameter measured after 5 days (Table 2). But 10 days after hypophysectomy whole body calcium uptake was significantly lower. Blood potassium and osmolarity were also lower compared to either the sham hypophysectomized or intact groups after 10 days hypophysectomy (Table 3). No differences in plasma calcium, sodium, or magnesium were noted (Tables 2 and 3). Uni-directional calcium influx rates were unaltered five days after eels were both hypophysectomized and stanniectomized (Table 2). Similarly, no changes were observed in the various other parameters measured after this treatment (Tables 2 and 3). Plasma cortisol levels 10 days after hypophysectomy were extremely low (Table 2).

Table 2 Effect of Hypophysectomy (Hypex) and both Hypophysectomy and Stanniectomy (Stx) on whole body calcium uptake rates and plasma $[Ca^{2+}]$[a].

Treatment	Group	N	Date	Cortisol[b]	Ca^{2+} Uptake Rates[c]	Plasma $[Ca^{2+}]$[d]
Hypex (5 days)	intact	6	Jan. 8/87	N.M.	2.001 ± .387	2.467 ± .269
	sham	6	Jan. 8/87	N.M.	2.299 ± .222	2.513 ± .200
	sham	6	Jan. 15/87	N.M.	2.212 ± .441	2.216 ± .241
	hypex	6	Jan. 15/87	N.M.	1.535 ± .105	2.703 ± .267
Hypex (10 days)	intact	6	Oct. 4/87	302.0	5.554 ± .635	2.496 ± .097
	sham	6	Oct. 4/87	495.7	6.668 ± .492	2.688 ± .081
	hypex	6	Oct. 4/87	5.3	4.699 ± .672[e]	2.872 ± .197
Hypex & Stx (5 days)	sham	6	Mar. 17/87	N.M.	0.327 ± .059	1.798 ± .077
	hypex & stx.	6	Mar. 17/87	N.M.	0.386 ± .048	1.900 ± .079

N.M. = not measured

[a] Values as $\bar{X}$ ± S.E.M.; N=6

[b] Cortisol levels in nmol/litre

[c] Uptake rates in umol-kg^{-1}-hr^{-1}

[d] $[Ca^{2+}]$ in mM/litre

[e] Significantly different than shams (p .05)

Table 3 Effect of Hypophysectomy (Hypex) and both Hypophysectomy and Stanniectomy (Stx) on various plasma parameters[a].

Treatment	Group	N	Date	Osmolarity [b]	Na^{+c}	K^{+c}	Mg^{2+c}
Hypex (5 days)	Intact	6	Jan. 8/87	275.69 ± 5.26	151.87 ± 2.12	2.752 ±1.639	2.424 ± .101
	Sham	6	Jan. 8/87	269.99 ± 4.68	151.23 ± 1.22	2.669 ±2.129	2.500 ± .099
Hypex (5 days)	Sham	6	Jan.15/87	268.67 ± 4.89	150.80 ± 3.53	2.620 ± .474	2.342 ± .672
	Hypex	6	Jan.15/87	269.00 ±14.59	143.10 ± 1.95	2.560 ± .210	2.560 ± .112
Hypex (10 days)	Intact	6	Oct. 4/87	281.50 ± 6.35	147.42 ± 1.79	2.848 ± .422	1.230 ± .052
	Sham	6	Oct. 4/87	283.80 ± 6.57	150.06 ± 1.08	3.102 ± .506	1.322 ± .018
	Hypex	6	Oct. 4/87	273.00 ± 6.75[d,e]	148.28 ± 1.87	1.969 ± .105[d,e]	1.293 ± .083
Hypex & Stx (5 days)	Sham	6	Mar.17/87	275.34 ± 6.96	147.23 ± 2.75	2.655 ± .079	2.157 ± .082
	H/S	6	Mar.17/87	281.69 ± 7.13	146.34 ± 2.06	2.826 ± .176	2.41 ± .067

[a] Values as $\bar{X}$ ± S.E.M.; N=6

[b] Osmolarity in milliosmoles

[c] ion concentrations in mM/litre

[d] Significantly lower (p .05) relative to shams

[e] Significantly lower (p .05) relative to intact

Effects of putative prolactin releasing factors thyrotropin releasing hormone (TRH) and haloperidol on uni-directional calcium influx, plasma total calcium, plasma Na^+, K^+ and total Mg^{2+} and PRL cell morphometry, in FW acclimated eels.

Neither injection of TRH or haloperidol, had any significant effect on uni-directional calcium influx, plasma total calcium or plasma sodium or potassium concentrations (Table 4 and 5). Table 5 also shows that TRH but not haloperidol treatment was associated with an increase in plasma total Mg^{2+} relative to solvent injected controls.

Table 4 Effect of PRL Releasing Factor Injections on whole Body Ca^{2+} uptake and plasma $[Ca^{2+}]$ in intact FW eels[a].

Treatment	Group	N	Dates	Ca^{2+} Uptake Rates [d]	Plasma $[Ca^{2+}]$ [e]
TRH[b]	Sham	7	July 22/87	10.689 ± 2.076	3.099 ± .077
	TRH	7	July 22/87	9.150 ± 1.029	3.149 ± .099
Haloperidol[c]	Sham	7	Aug. 12/87	7.387 ± .828	2.247 ± .078
	Halo	7	Aug. 12/87	7.346 ± 1.694	2.321 ± .060

[a] Values are $\bar{X}$ ± S.E.M. N=7 in all cases

[b] L- Pyroglutaryl, -L-Histidyl, -L-Prolinamide (Thyrotropin releasing hormone or TRH); dose 10 ug/fish, single injection

[c] Dose 2.5 mg/fish/day for five days

[d] umole $kg^{-1}hr^{-1}$

[e] mM/litre

Table 5 Effect of PRL Releasing Factor Injections on various Plasma Parameters In Intact Fish"[a].

Treatment	Group	Plasma $[Na^+]$[b]	$[K^+]$[b]	$[Mg\ 2^+]$[b]	Nuclear Volumes[c]
TRH	shams	138.67 ± 2.30	3.555 ± .181	1.434 ± .023	75.86 ± 2.38
	TRH	143.01 ± 1.90	3.323 ± .168	1.515 ± .037[d]	77.10 ± 1.97
Haloperidol	shams	146.80 ± 2.74	2.534 ± .070	1.491 ± .047	81.99 ± 1.99
	HALO	145.19 ± 1.94	2.874 ± .256	1.410 ± .027	83.23 ± 2.02

[a] Values are $\bar{X}$ ± SEM; N=7 in all cases

[b] Ion concentrations in mM/litre

[c] Nuclear volumes in um^3

[d] Significantly higher (p .05)

Effects of the putative prolactin release inhibiting factors dopamine, apomorphine, ergocryptine and bromocryptine on uni-directional calcium influx, plasma total calcium, various plasma parameters and prolactin cell cytology and morphometry.

Dopamine administration using implanted mini-osmotic pumps in intact fish had no effect on calcium influx, plasma total calcium or any other parameter measured (Tables 6 & 7). Interestingly, the same treatment in stanniectomized fish significantly increased unidirectional calcium influx, plasma total calcium, plasma K^+ and Mg^{2+}. Because of the lack of effect in intact fish and the unusual effects in stanniectomized fish further studies with dopamine were not pursued.

Apomorphine or ergocryptine administered as a series of five daily bolus injections significantly reduced calcium influx relative to solvent injected groups in two separate experiments (Tables 6 & 7). This effect was coupled with a clear degranulation of the prolactin cells which also showed reduced cell and nuclear volumes (Figures 4, 5 and Table 7). Prolactin cells of the ergocryptine treated eels exhibited a greater degree of degranulation and vacuolation relative to the cells of solvent injected fish (Figure 5) than did the cells of the apomorphine treated fish (Figure 4). The ergocryptine treated fish also displayed pycnotic nuclei (Figure 4). Finally, the prolactin cells of apomorphine and ergocryptine treated fish exhibited qualitatively less prominent nucleoli, and diminutive, less basophilic nuclei than control fish. Neither apomorphine nor ergocryptine affected plasma total calcium or any other parameter measured. Bromocryptine had no effect except for a significant increase in plasma total Mg^{2+} concentration (Tables 6 and 7).

Table 6 Effect of PIF (PRL release inhibiting factor) administration via injection or osmotic pump on whole - Body Ca^{2+} uptake rates and Plasma total $[Ca^{2+}]$ in intact or Stanniectomized Eels[a].

Treatment	Group	N	Dates	Weights [h]	Ca^{2+} Uptake Rates[i]	Plasma (Ca^{2+})[j]
Apomorphine [b] - I	Solvent	7	Aug. 14/87	103.14 ± 7.45	15.997 ± 1.503	2.785 ± .112
(intact fish)	APO	7		97.71 ± 3.44	9.443 ± 1.775[k]	2.866 ± .060
Apomorphine - II	Solvent	6	Nov. 27/87	105.43 ± 7.02	2.682 ± .270	4.074 ± .078
(intact)	APO	6		111.83 ±13.41	1.749 ± .344[k]	4.156 ± .118
Ergocryptine - I [c]	Solvent	7	Aug. 18/87	101.86 ±20.44	16.791 ± 1.760	3.205 ± .201
(intact)	ERGO	7		93.57 ±10.29	8.209 ± 1.473[k]	3.112 ± .097
Ergocryptine - II	Solvent	6	Dec. 5/87	123.83 ±13.58	27.036 ± 2.635	3.159 ± .215
(intact)	BROMO	6	Nov. 6/87	140.00 ±13.53	18.204 ± 2.408[k]	3.440 ± .244
Bromocryptine [d,g]	Solvent	13	Aug. 7/87	103.62 ± 6.27	3.577 ± .390	2.697 ± .183
(intact)	BROMO	14	Nov. 6/87	102.86 ± 7.42	2.987 ± .306	2.311 ± .134
Dopamine [e] (pumps)	Solvent	7	July 14/87	69.57 ± 6.51	9.448 ± 2.109	2.887 ± .066
(intact)	DA	7		77.71 ± 8.61	7.440 ± 1.069	3.006 ± .092
DA (pumps) [f]	Solvent	6	July 20/87	70.50 ± 5.89	51.842 ± 4.937	6.795 ± .267
(STX fish)	DA	6		77.40 ± 6.99	82.180 ±15.855[k]	9.175 ± .395[k]

[a] Values are $\bar{X} \pm$ S.E.M.
[b] Apo dose - 3.0 mg/fish/day for 5 days intact fish (injected)
[c] Ergo dose - 2.8 mg/fish/day for 5 days intact fish (injected)
[d] Bromo dose - 2.0 mg/fish/day for 5 days intact fish
[e] DA dose - 3.2 mg/fish/day for 5 days intact fish (via pumps)
[f] DA dose - 3.2 mg/fish/day for 5 days (via pumps)
[g] Data pooled from two experiments (no sig. diff. between controls)
[h] Mean weight in grams
[i] Mean fluxes (umol. $kg^{-1}hr^{-1}$)
[j] Mean $[Ca^{2+}]$ (unmole/litre)
[k] Significantly diff. than shams (p .05)

Table 7 Effect of PIF (PRL release inhibiting factor) administration via injection of osmotic pump on PRL cell morphometry and various plasma parameters [a]

Treatment	Group	Nuclear Volume[b]	Osmolarity[c]	Plasma $[Na^+]^d$	$[k^+]^d$	$[Mg^{2+}]^d$
APO - I	Solvent	99.99 ± 1.28	301.57 ± 16.94	135.10 ± 2.09	2.706 ± .091	1.177 ± .063
	APO	93.53 ± 1.32[e]	291.00 ± 23.52	135.26 ± 3.92	2.609 ± .135	1.235 ± .046
APO - II	Solvent	101.35 ± 2.67	312.63 ± 8.24	141.63 ± 2.57	1.967 ± .080	1.953 ± .027
	APO	95.01 ± 1.99[e]	294.54 ± 9.69[e]	138.17 ± 3.45	2.001 ± .085	1.740 ± .076[e]
ERGO - I	Solvent	83.32 ± 1.52	301.86 ± 7.76	135.76 ± 4.42	2.232 ± .097	1.201 ± .063
	ERGO	72.48 ± 2.16[e]	277.43 ± 24.97	139.01 ± 4.46	2.757 ± .368	1.559 ± .089[e]
ERGO - II	Solvent	89.69 ± 1.23	299.59 ± 6.66	134.65 ± 2.74	1.857 ± .086	1.735 ± .065
	ERGO	74.68 ± 2.02[e]	275.21 ± 14.60[e]	133.68 ± 1.76	1.798 ± .205	1.783 ± .139
BROMO	Solvent	102.67 ± 2.68		159.31 ± 7.88	3.071 ± .147	1.317 ± .010
	BROMO	99.97 ± 1.78	n.m.	152.59 ± 2.24	3.219 ± .052	1.483 ± .055[e]
DA - intact (pumps)	Solvent	n.m.	n.m.	135.24 ± 2.33	3.694 ± .156	1.320 ± .053
	DA			134.26 ± 3.53	3.886 ± .109	1.342 ± .049
DA - Stx	Solvent	n.m.	n.m.	122.22 ± 2.06	4.768 ± .110	1.353 ± .054
	DA			120.94 ± 9.37	6.598 ± .318	1.568 ± .031[e]

[a] Values are $\bar{X}$ ± S.E.M.

[b] Nuclear volume ($.um^3$)

[c] $mOsm.L^{-1}$

[d] $mM.L^{-1}$

[e] Sig. diff. from shams (p .05)

n.m., not measured

Figure 4

Light micrograph of prolactin cells of FW adapted A rostrata after apomorphine (APO) injection (A) and solvent injection (B). APO induces degranulation (fewer cytoplasmic granules - arrows) and a reduction in overall cell and nuclear (N) volumes (see also Table 7).

Control cells (B) show more prominent nuclei with nucleolus (nu) and extensive cytoplasmic granulation (arrows).

Typically prolactin cells of the eel RPD are arranged in spherical or ovoid follicles surrounding a central oval or elongated follicular lumen (FL) and always contiguous with basally located blood vessels (V) for hormone delivery. At the light microscopic level one observes that the cells are typically elongated with basally located nuclei (x 3000).

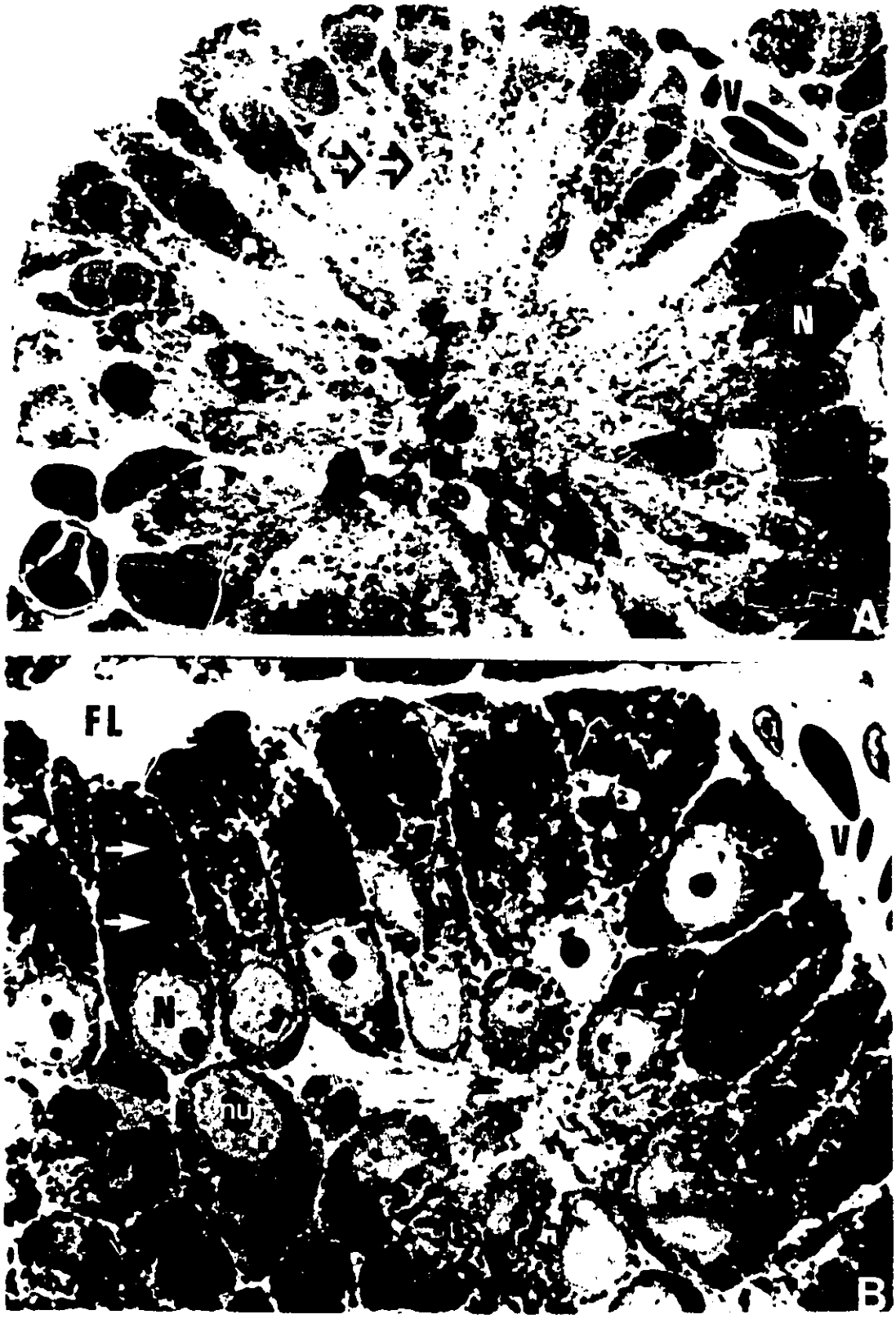
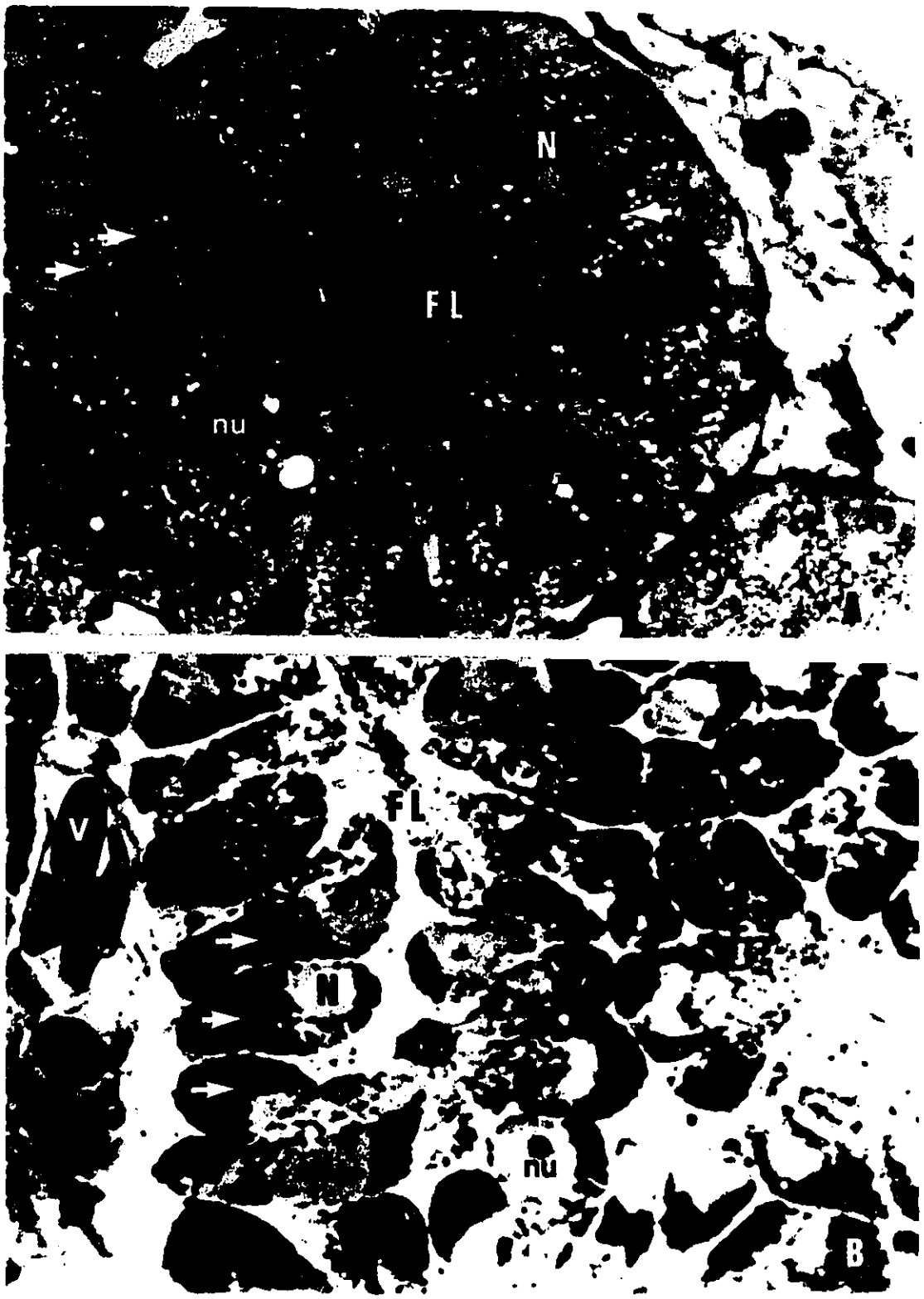

V
N
A
FL
V
N
nu
B

Figure 5

Light micrograph showing effects of ergocryptine (A) and solvent injections (B) on the prolactin cell cytology of A. rostrata. Ergocryptine exerts an inhibitory effect on cellular activity typically resulting pycnotic nuclei (N) containing barely visible nucleoli, and marked degranulation of the cytoplasm (arrows). Solvent injections (B) show typically healthy cells exhibiting prominent nuclei, nucleoli, and granulation (x 3000).


N
FL
nu
FL
V
N
nu
B

Effect of prolactin lobe grafts in intact, hypophysectomized and both hypophysectomized and stanniectomized eels; and the effect of residual pituitary grafts in intact eels, on uni-directional calcium influx, plasma total calcium and various other plasma parameters.

Calcium uptake rates and plasma total calcium were significantly higher in fish 10 days after they had received two extra PRL lobes (Table 8). Plasma sodium, potassium and magnesium showed no changes. Prolactin cell nuclear volumes were unaltered (Table 9). Fish which received four extra lobes showed no change in calcium influx rates although plasma total calcium rose significantly, and this was coupled with reduced PRL cell nuclear volumes (Tables 8 and 9).

Neither branchial calcium uptake rates nor plasma total calcium levels were altered in fish in which 2 residual pituitary grafts (devoid of PRL cells) were implanted (Table 8). Likewise, this treatment did not affect any other parameter measured (Table 9).

Table 8 Effect of PRL lobe grafts in intact, hypophysectomized, and both Hypex'ed + Stx'ed eels; and effect of Pars intermedia grafts in intact eels on calcium uptake rates and plasma $[Ca^{2+}]$[a]

Treatment	Group	Date	N	Plasma[c] Cortisol	Whole body[d] Ca^{2+} Uptake	Plasma $[Ca^{2+}]$[e]
PRL lobes (2 lobes)[b] (10 days)	Shams	Feb. 2/87 & March 9/87	12	335	1.265 ± .178[f]	2.275 ± .182[f]
	Implants		12	247	2.028 ± .536[f]	3.403 ± .162[f]
PRL lobes (4 lobes) (14 days)	Intact	Oct. 9/87	4	264	7.676 ± 1.061	3.266 ± .134
	Shams		7	235	7.141 ± 0.866	2.974 ± .099[f]
	Implants		7	139	6.288 ± 0.629	3.255 ± .078[f]
Hypex (5 days) PRL Lobes (2 lobes - 10 days)	Shams	Mar. 28/87	6	n.m.	0.414 ± 0.068	1.852 ± .049[f]
	Implants		6		0.358 ± 0.070	2.088 ± .068[f]
Hypex & Stx (5 - days) & Lobes (2 lobes-10 days)	Shams	April 4/87	6	n.m.	0.379 ± 0.069	2.304 ± .168
	Implants		6		0.441 ± 0.072	2.321 ± .152
Pars intermedia grafts (2 lobes - 10 days)	Shams	Nov. 20/87	7		1.796 ± 0.499	2.312 ± .157
	Implants		7	n.m.	1.633 ± 0.241	2.627 ± .090

[a] Values are $\bar{X} \pm$ S.E.M.

[b] Pooled data from 2 experiments

[c] Cortisol levels (nmol/l)

[d] Mean fluxes in umol $kg^{-1}hr^{-1}$

[e] Calcium levels (mM)

[f] Significantly higher (p .05) than shams

n.m., not measured

Table 9 Effect of PRL lobe grafts in intact, hypophysectomized, and both Hypex'ed and Stx'ed eels; and effect of Pars intermedia grafts in intact eels on PRL cell morphometry and various plasma parameters[a]

Treatment	Group	Nuclear Volumes [b]	Osmolarity [c]	Plasma $[Na^+]$ [d]	$[K^+]$ [d]	$[Mg^{2+}]$ [d]
2 PRL lobes (10 days)	SH	96.89 ± 2.42	n.m.	146.892 ± 2.495	2.389 ± .134	2.138 ± .126
	Implants	98.67 ± 2.90		149.158 ± 2.259	2.259 ± .171	2.343 ± .138
4 PRL lobes (14 days)	Intact	107.93 ± 2.69	n.m.	161.225 ± .960	2.030 ± .158	1.448 ± .051
	SH	112.65 ± 2.98[e,f]		154.343 ± .956[f]	2.375 ± .175	1.381 ± .039[f]
	Implants	89.96 ± 2.93[e,f]		156.114 ± 1.789[f]	2.163 ± .136	1.284 ± .040[f]
5 day Hypex 10 day 2 PRL lobes	SH	n.m.	222.00 ± 13.16	142.530 ± 2.746	1.843 ± .095	1.232 ± .075
	Implants		240.60 ± 3.91	150.431 ± 4.278	2.224 ± .209	1.308 ± .060
5 day Hypex/Stx 10 day 2 PRL lobes	SH	n.m.	238.00 ± 8.53	147.100 ± 3.368	3.817 ±1.562	1.475 ± .106
	Implants		231.67 ± 5.79	151.960 ± 1.573	1.663 ± .119	1.498 ± .130
2 Pars intermedia "lobes" - 10 days	SH	n.m.	n.m.	152.501 ± .929	1.835 ± .059	1.300 ± .042
	PI-implants			149.661 ± .901	2.103 ± .117	1.216 ± .028

[a] Values are $\bar{X} \pm$ S.E.M.

[b] Nuclear volumes in um^3

[c] Osmolarity in mosm

[d] ion conentrations (mM)

[e] Significantly diff. from intact group (p .05)

[f] Signficantly diff. from "shams" (p .05)

n.m., not measured

Effect of continuous infusion or injections of exogenous ovine prolactin on uni-directional calcium influx and various plasma parameters, in FW and SW adapted eels, and PRL cell morphometry in SW adapted eels.

The continuous infusion of ovine prolactin using implanted mini-osmotic pumps increased whole body calcium uptake rates and plasma total calcium in FW fish (table 10). In addition, blood sodium and magnesium were lowered significantly while potassium levels rose (table 11). Similarly, daily injections of ovine PRL over a 1 week period caused calcium influx rates to rise significantly, however, no alterations in blood total calcium, potassium or magnesium were noted. Plasma sodium levels were significantly higher in PRL injected animals (table 11).

In two experiments, daily ovine PRL injections over a 1 week period decreased whole body calcium influx rates but increased plasma calcium levels in SW acclimated eels (table 10). In both of these experiments, PRL treatment caused blood sodium to rise significantly (table 11). Morphometric analyses of the PRL cells showed that in both these experiments nuclear volumes were significantly smaller in the PRL treated fish.

In SW adapted eels, a single PRL injection induced a significant elevation in uni-directional calcium influx rates 24 hours post injection (Figure 6). This same figure shows that the increase in uptake rates at 24 hours (Figure 6A) is followed by a significant increase in plasma total calcium at the 48 hours interval post injection (Figure 6B). Finally, Figure 7 shows that a single PRL injection in SW fish increased both plasma osmolarity and potassium at 24 hours, and augmented plasma sodium at 72 hours following injection.

Table 10 Effect of Ovine PRL administration via mini-osmotic pumps in FW adapted eels and i-p injections in FW and SW adapted eels on Ca^{2+} uptake, Plasma Ca^{2+} and PRL cell morphometry[a]

Treatment	Group	N	Dates	Whole Body Ca^{2+} Uptake[e]	Plasma $[Ca^{2+}]$[f]	Nuclear Volumes
PRL infusion [b,c] (pumps - 10 days)	Sham	14	July 17/87 and 27/87	3.744 ± .355[h]	2.630 ± .055[h]	n.m.
	PRL	13		6.439 ± .693[h]	2.951 ± .047[h]	
PRL injections (FW-fish) [b,c]	Sham	12	Oct. 24&31 1987	1.912 ± .458[h]	2.601 ± .123	n.m.
	PRL	12		5.886 ±1.224[h]	2.736 ± .051	
PRL injections [d] SW fish - I	Sham	6	Nov. 16/87	19.872 ±4.461[h]	2.423 ± .101[h]	91.58 ± 1.07[h]
	PRL	6		6.497 ±1.418[h]	2.854 ± .099[h]	82.27 ± 1.94[h]
PRL injections [d] SW fish - II	Sham	6	Jan. 9/88	4.003 ±1.056[h]	2.567 ± .110[h]	100.79 ± 1.02[h]
	PRL	6		1.388 ±0.099[h]	2.928 ± .087[h]	91.22 ± 1.86[h]

[a] Values are $\bar{X}$ ± S.E.M.

[b] Pooled data from 2 expts.

[c] FW adapted eels $[Ca^{2+}]$ = 0.4mM

[d] SW adapted eels $[Ca^{2+}]$ = 7.0mM

[e] Mean fluxes (umol . $kg.^{-1}hr.^{-1}$)

[f] ion concentrations in mM/litre

[g] Nuclear volumes in um^3

[h] Significantly diff. (p .05)

Table 11 Effect of Ovine PRL administration via mini-osmotic pumps in FW adapted eels and i-p injections in FW and SW adapted eels on plasma various parameters[a].

Treatment	Group	$[Na^+]$ [b]	$[K+]$[b]	$[Mg^{2+}]$[b]
PRL Infusion (FW)	SH	127.450 ± .510	2.913 ± .026	1.542 ± .029
	PRL	140.417 ± .485[c]	3.206 ± .089[c]	1.413 ± .030[c]
PRL injection (FW)	SH	149.267 ± 1.265	2.560 ± .121	1.261 ± .070
	PRL	153.816 ± .952[c]	2.511 ± .109	1.258 ± .031
PRL injections SW - I	SH	154.210 ± 3.832	1.899 ± .212	1.813 ± .175
	PRL	166.610 ± 2.538[c]	1.921 ± .107	1.767 ± .160
PRL injections SW - II	SH	170.001 ± 4.189	2.538 ± .073	1.777 ± .169
	PRL	193.671 ± 3.751[c]	2.587 ± .249	2.053 ± .176

[a] Value as $\bar{X} \pm$ S.E.M.

[b] Ion concentrations in mM

[c] Significantly diff. (p 0.5)

Figure 6

Effect of a single injection of ovine PRL on calcium influx rates (A) and plasma total calcium in SW acclimated American eels, Anguilla rostrata at time zero and 9, 24, 48 and 72 hours post injection. Means (bars, circles) and S.E.M. (lines) are shown. N=7 in all cases. * indicates a significant difference ($P \leq 0.05$) relative to the control groups. Control groups (solvent injected) open bars and circles, prolactin injected groups solid bars and circles. Ovine prolactin dose 0.15 IU g^{-1} fish day^{-1}.

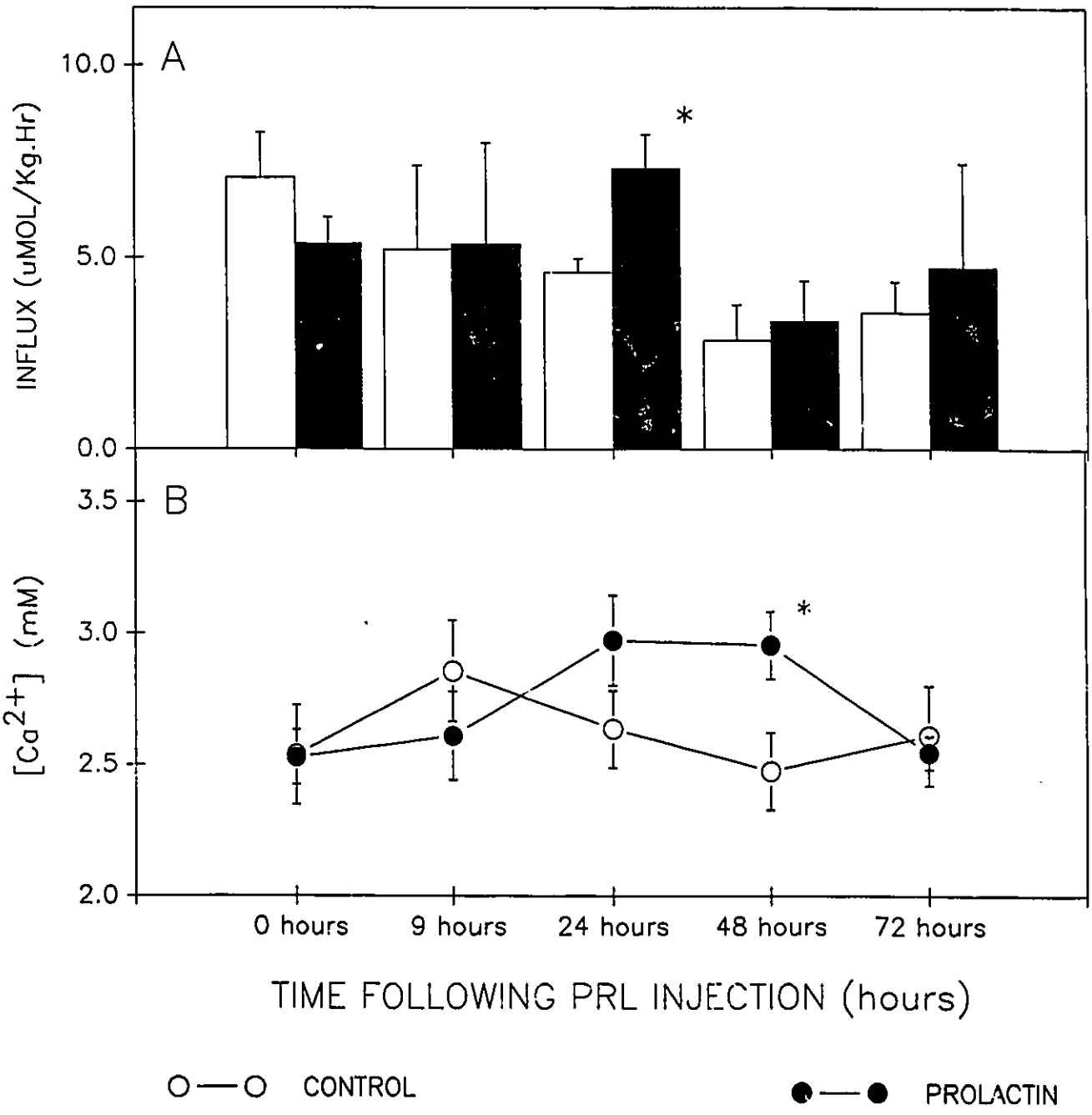
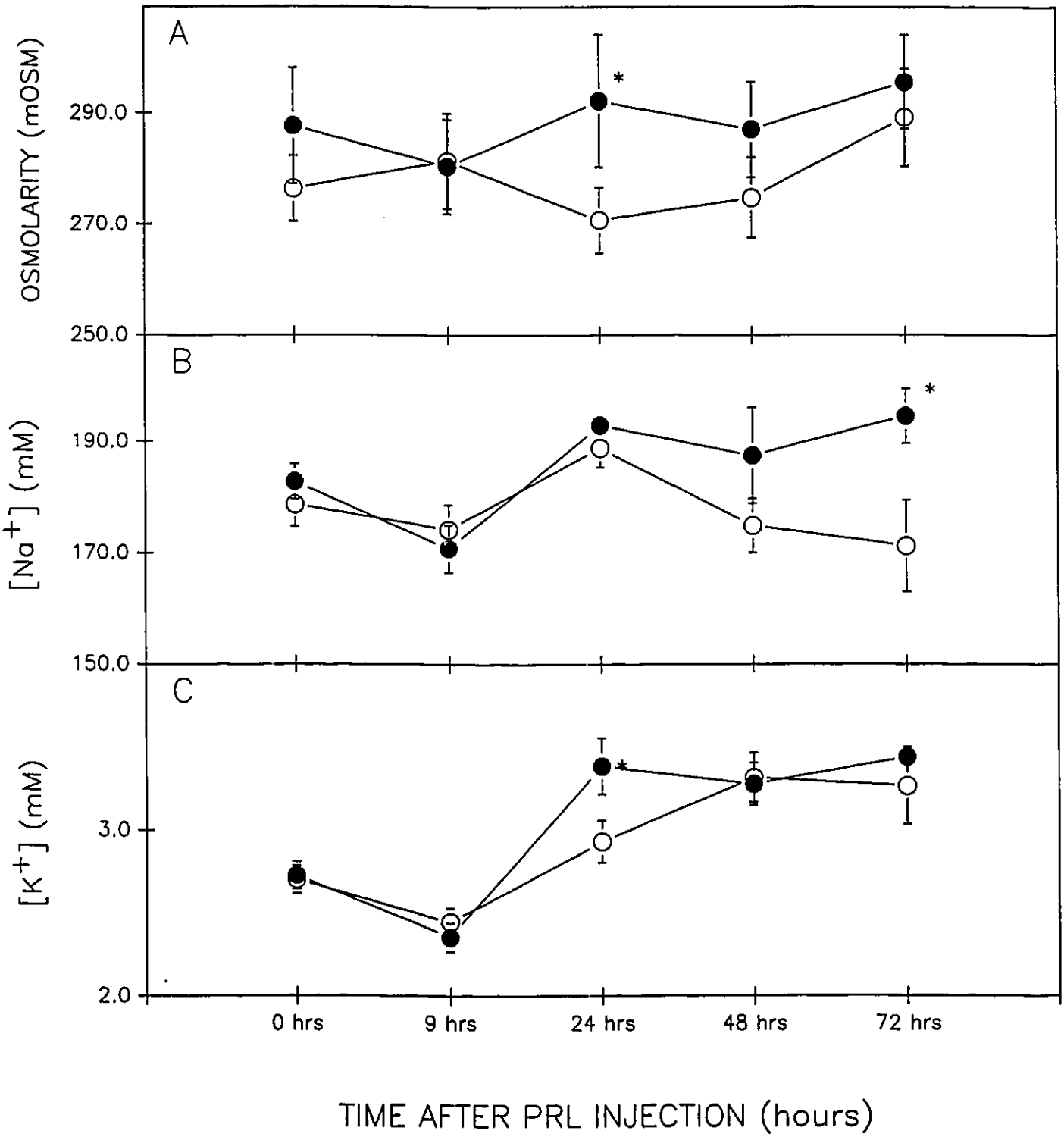

A
10.0
5.0
0.0
INFLUX (uMOL/Kg.Hr)
*
B
3.5
3.0
2.5
2.0
[Ca2+] (mM)
*
0 hours
9 hours
24 hours
48 hours
72 hours
TIME FOLLOWING PRL INJECTION (hours)
CONTROL
PROLACTIN

Figure 7

Effect of a single injection of ovine PRL on various plasma parameters, including blood osmolarity (A), plasma sodium (B) plasma potassium (C), in SW adapted American eels, Anguilla rostrata, at time zero and 9,24,48 and 72 hour post injection. Means (bars, circles) and S.E.M. (lines) are shown. N=7 in all cases. * indicates a significant difference ($p \leq 0.05$) relative to control groups, open circles, prolactin injected groups solid circles. Prolactin dose 0.15 I.U. g^{-1}fish day^{-1}.


A
B
C
OSMOLARITY (mOSM)
290.0
270.0
250.0
[Na+] (mM)
190.0
170.0
150.0
[K+] (mM)
3.0
2.0
*
0 hrs
9 hrs
24 hrs
48 hrs
72 hrs
TIME AFTER PRL INJECTION (hours)
CONTROL
PROLACTIN

Effect of SW to FW transfer on uni-directional calcium influx rates, plasma total calcium, various plasma parameters and PRL cell cytology and morphometry in both SW and FW acclimated eels.

Figures 8 and 9 show the process that takes place when a SW acclimated eel is placed in FW. Initially, calcium influx rates were similar to those of FW controls but were increased significantly after 3 and 5 days (Figure 8A). By days 6 and 7 the calcium influx rates in the transfer fish had dropped back to the levels of FW controls (Figure 8A). Concomitantly, plasma total calcium levels in transfer fish were consistently lower than controls until days 6 and 7 when they appeared to increase. In general, the blood osmotic pressure of transfer fish was higher than control fish until day 7 (Figure 9A). It too increased while total plasma magnesium remained low in transfer fish (Figure 9D). Plasma sodium and potassium showed no changes.

PRL cell and nuclear volumes of tender fish tended to be higher for the first five days but this difference disappeared at the same time as did the differences in calcium influx and plasma calcium. In addition, the PRL cells of transfer fish became quantitatively more granulated as time progressed and as the animals presumably acclimated to the FW environment (Figure 10). Specifically, at the light microscopic level the first day after transfer from SW to FW transfer fish displayed surprisingly large prolactin cell volumes with significantly larger nuclear volumes in spite of the fact that they exhibited less cytoplasmic granulation than FW controls (Figure 10 - Day 1). Two days after transfer the cells and nuclei of transfer fish were larger than those of controls, and, patches of cytoplasmic hormone granules were evident (Figure 10 - Days 2,3). On the

fourth day after transfer to FW both cell and nuclear volumes were still increasing, the nucleoli were becoming more prominent, and the number of cytoplasmic hormone granules had greatly increased, especially by day 5. Morphometric analyses of the prolactin cells from both transfer and control fish at this time, revealed that by days 6 and 7 the difference in nuclear volume was no longer significantly different. Further, by this time the cells had about the same degree of cytoplasmic granulation (Figure 10-Days 6,7) and electrolyte balance was normalized (Figures 8, 9).

These quantitative cytological changes have ultrastructural bases that were examined at the EM level in figures 11 and 12. Figure 11 shows several PRL cells of a SW to FW transfer fish on the first day after the transfer. Of note are the cilia with the characteristic 9+2 microtubule doublet arrangement at the luminal surface of the cells. This photograph also illustrates the dark tight junctions between cells nearer the central lumen. In addition, figure 11 also shows the typical "control" (FW) fish with extensive cytoplasmic granulation, basally located nucleous, rough endoplasmic reticulum, and moderate numbers of mitochondria and Golgi.

The morphological correlates associated with secretory activity of the transfer PRL cells were considerably activated 24 hours after transfer to FW. Comparing the transfer fish cells of Figure 11 B with control cells of Figure 11 A reveals a higher magnification (19,500x) the electron luscent pre-secretory hormone granules of the SW to FW transfer cells not seen in the control cells.

The nucleoli of the transfer fish were more prominent than the control fish suggesting high ribosome synthesis necessary for protein translation (figure 11A-low mag.). In addition, quantitatively more mitochondria and more extensive Golgi systems were exhibited by transfer fish cells cytological features indicative of secretory activity, yet there were fewer cytoplasmic granules in these cells (figure 11A). Nagahama et al 1973, decribed a similar phenomena in G. mirabilis and suggested that at this time cell synthesis and secretion rates are higher than hormone storage rates. This type of cytoplasmic "degranulation" is not to be confused with the degranulation of dying cells.

On the final day of the study the cells of SW to FW transfer fish had advanced to a point where they exhibited extensive granulation (figure 12). The organelles accumulated earlier in order to possibly manufacture prolactin such as mitochondria, Golgi systems, and rough endoplasmic reticulum, were still in abundance (figure 12). As a result, the cell had employed large numbers of both primary lysosomes involved in the crynophagy of the excess organelles and secretory products, and secondary lysosomes, (heterogeneous in electron density) also involved in hormone degradation (figure 12). In addition, the nuclei of the cells of SW to FW fish had been reduced in size and the nucleoli are less prominent.

Figure 12 shows the typical FW control PRL cells on the seventh day of the study demonstrating the typically extensive granulation, and prominent nucleus and nucleolus. Very few lysosomes were present as these cells exhibited homeostasis not yet achieved by the PRL cells of SW to FW transfer fish that had presumably been synthesizing and secreting PRL at a great rate.

Figure 8

Effect of SW to FW transfer on uni-directional calcium influx plasma total calcium and PRL cell nuclear volumes in SW and FW acclimated American eels, Anguilla rostrata, at 1,2,3,4,5,6, and 7 days following transfer. Means (bars, circles) and S.E.M. (lines) shown. N=7 in all cases. * indicates a significant difference ($P \leq 0.05$) relative to control groups. Control groups (FW to FW) open bars and circles, experimental groups (SW to FW) solid bars and circles.

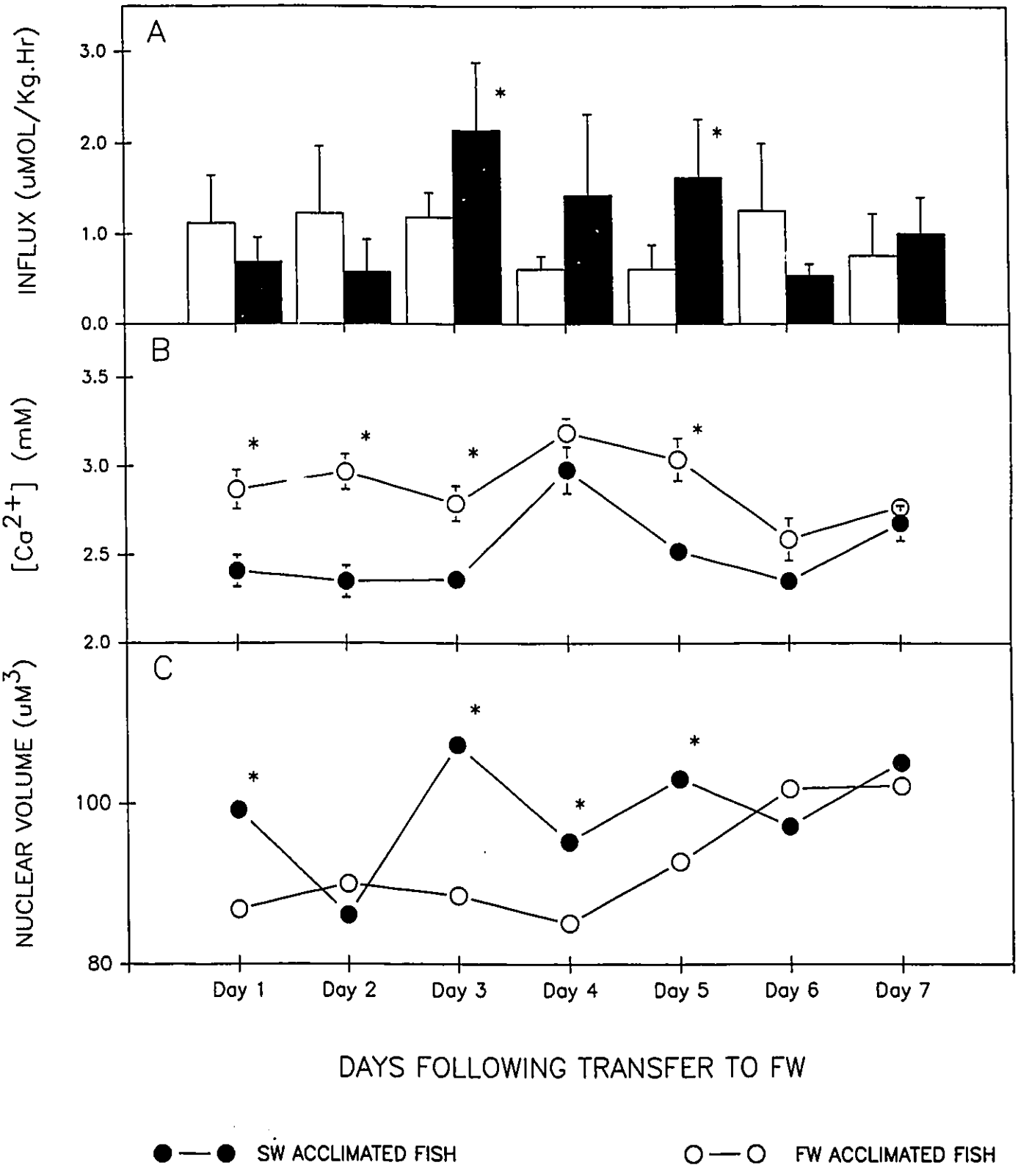

A
B
C
INFLUX (uMOL/Kg.Hr)
3.0
2.0
1.0
0.0
[Ca2+] (mM)
3.5
3.0
2.5
2.0
NUCLEAR VOLUME (uM3)
100
80
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
DAYS FOLLOWING TRANSFER TO FW
SW ACCLIMATED FISH
FW ACCLIMATED FISH

Figure 9

Effect of SW to FW transfer on various plasma parameters including, blood osmolarity (A), plasma sodium (B), plasma potassium (C), and plasma total magnesium (D) in SW and FW adapted American eels, Anguilla rostrata, at 1,2,3,4,5,6, and 7 days following transfer. Means (circles) and S.E.M. (lines) are shown N=7 in all cases. * indicates a significant difference ($P \leq 0.05$) relative to control groups. Control groups (FW to FW) open circles, experimental groups (SW to FW) solid circles.

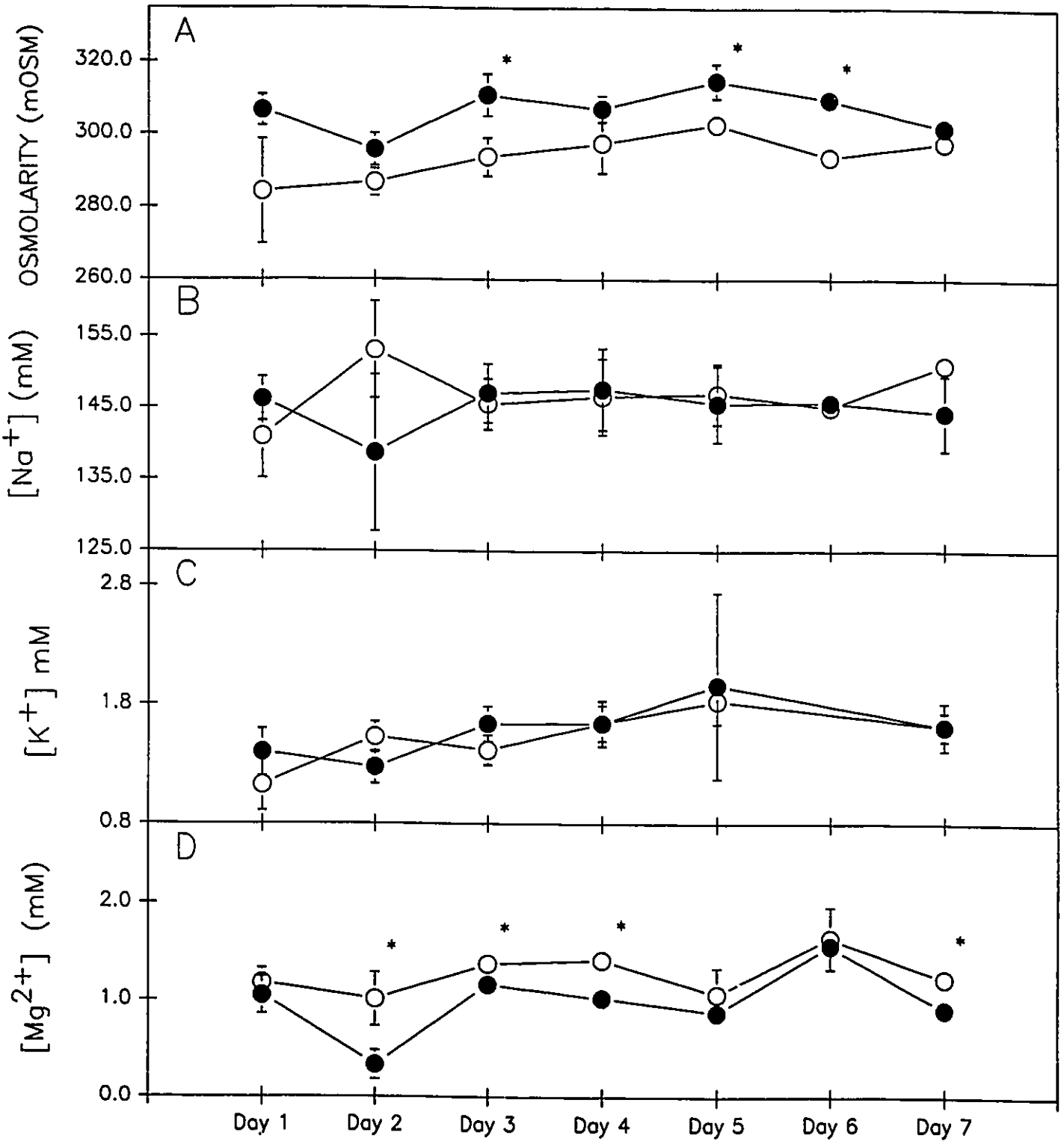
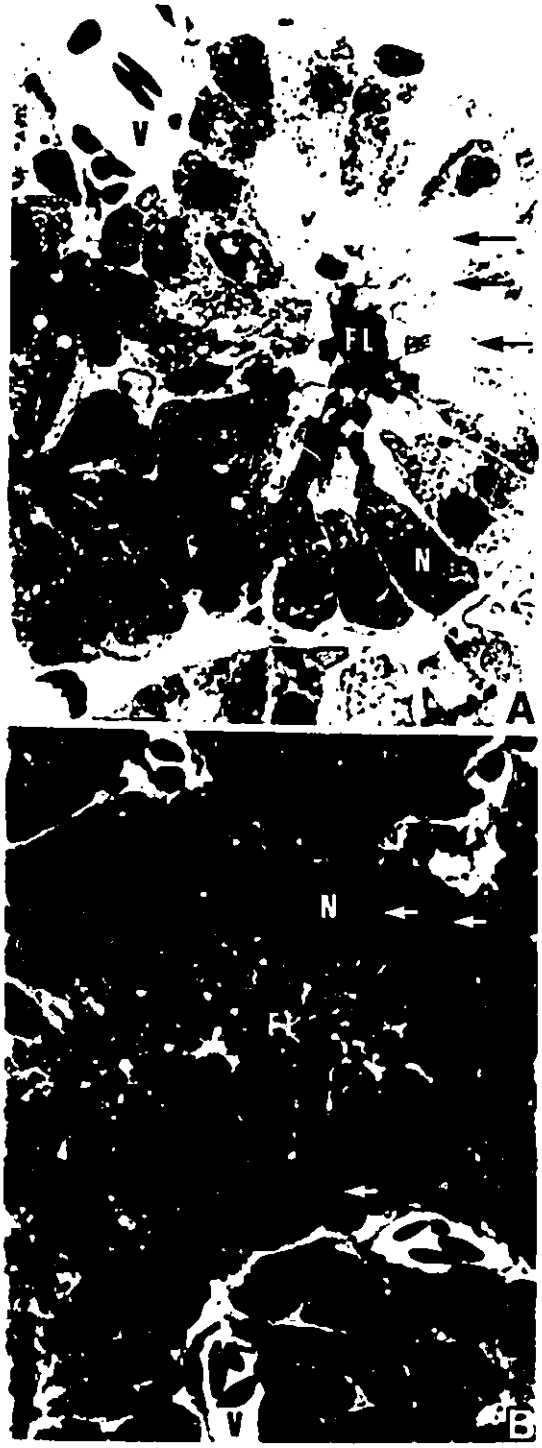

A
B
C
D
OSMOLARITY (mOSM)
320.0
300.0
280.0
260.0
[Na+] (mM)
155.0
145.0
135.0
125.0
[K+] mM
2.8
1.8
0.8
[Mg2+] (mM)
2.0
1.0
0.0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
DAYS FOLLOWING TRANSFER TO FW
FW ACCLIMATED FISH
SW ACCLIMATED FISH

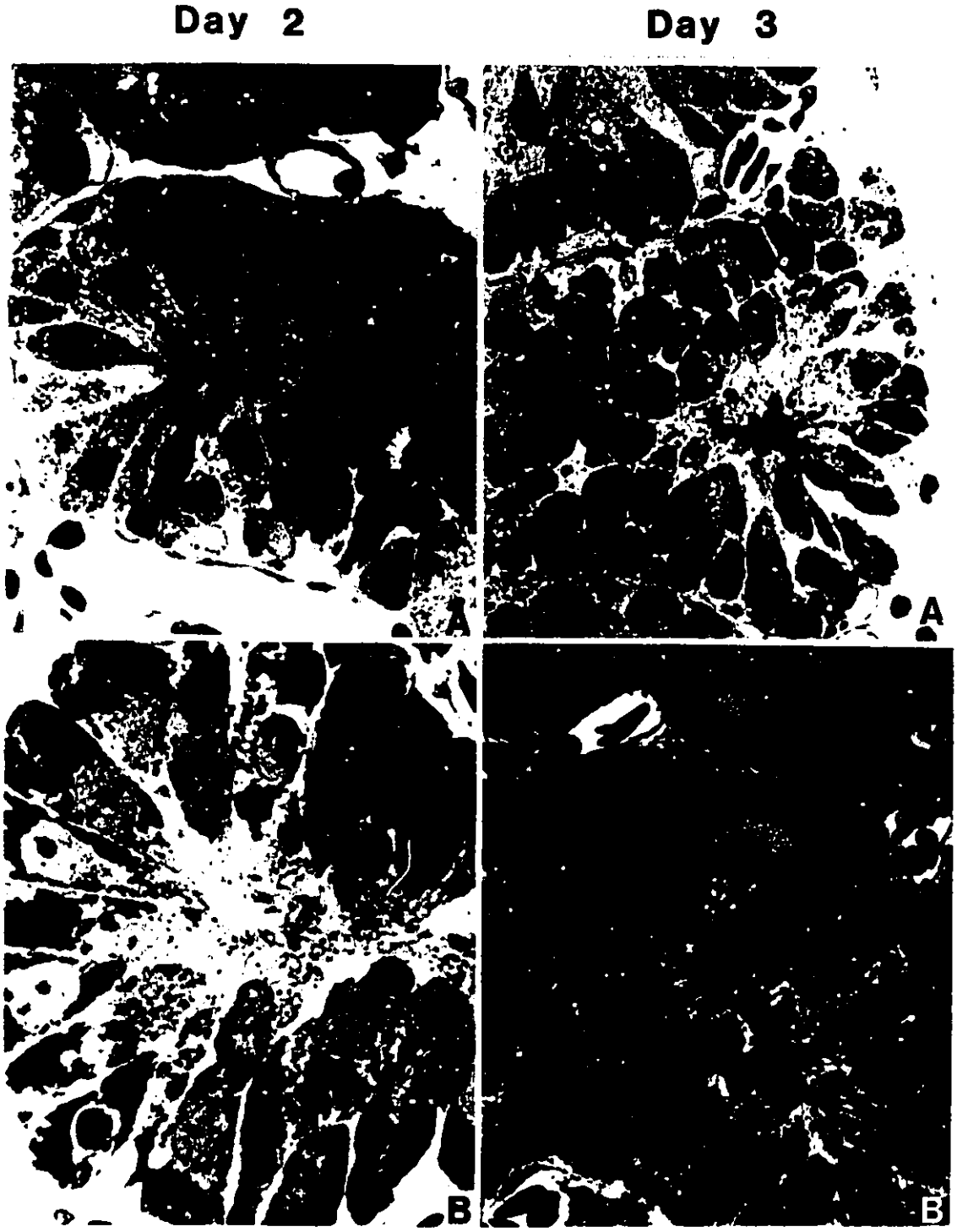
Figure 10

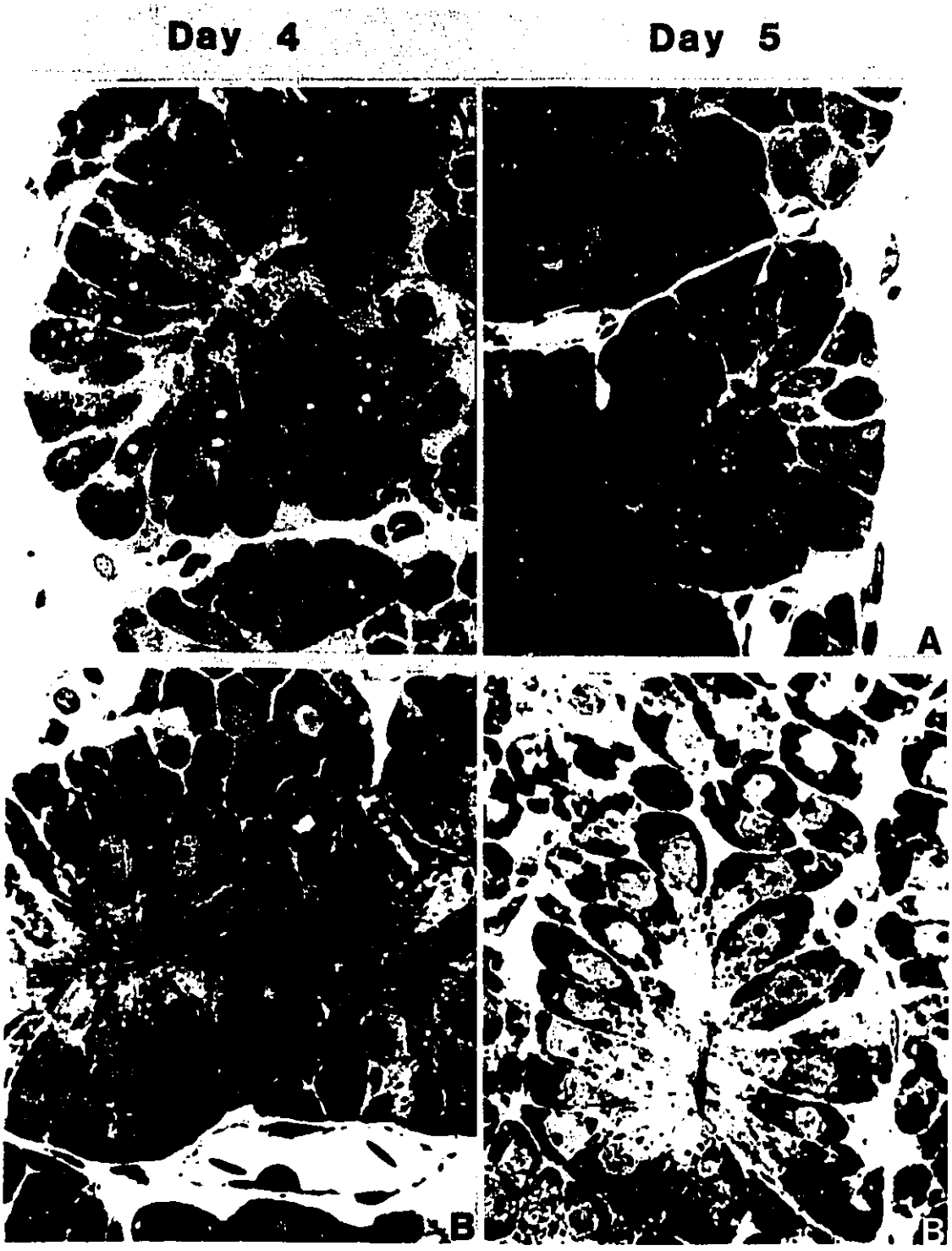
Series of light micrographs illustrating the progressive changes in the cytology of prolactin cells of SW adapted A rostrata over a one week period following direct transfer to FW. Representative follicles are shown from each day with SW to FW (transfer) fish shown in "A" plates and FW to FW (controls) shown in "B" plates. In general, as the week progressed transfer fish (A) showed increases in cell, and nuclear (N) volumes (see also Figure 10) and subsequently increased cytoplasmic granulation. Days 6 and 7 indicate that at the light microscope level prolactin cells from transfer fish resemble those of control fish.

(all micrographs x 2950)

Day 1




Day 2
Day 3
A
A
B
B


Day 4
Day 5
A
B
B

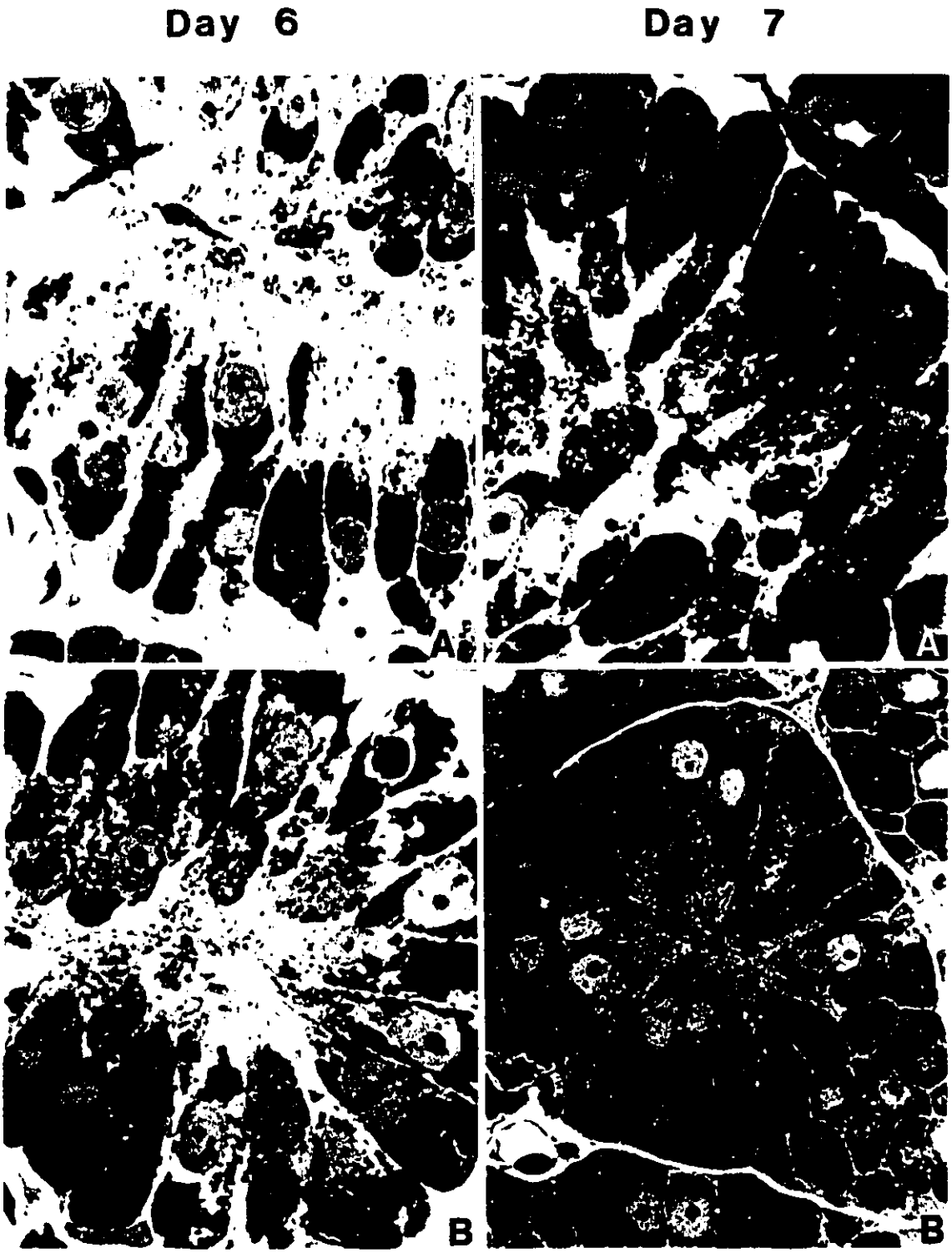

Day 6
Day 7
A
A
B
B

Figure 11

Electron micrograph of prolactin cells of A. rostrata transferred from SW to FW (transfer) and FW to FW (controls) on the first day after transfer. Transfer fish exhibit large nuclei (N) with prominent nucleoi (nu), extensive Golgi; apparatus systems (Ga), and prevalent mitochondria (M); all accepted as morphological correlates associated with high secretory activity. At higher magnification are are the electron luscent pre-secretory hormone granules (arrows-plate B). Note also the follicular lumen containing cilia with characteristic 9 + 2 m.t. doublet arrangement. Control fish display quantitatively fewer numbers of mitochondria, and Golgi systems, ovoid pre-secretory hormone granules, and large nuclei with nucleoli typical of the steady state activity levels of the cells of FW control fish (A plates x 6100, B plates x 19,500).

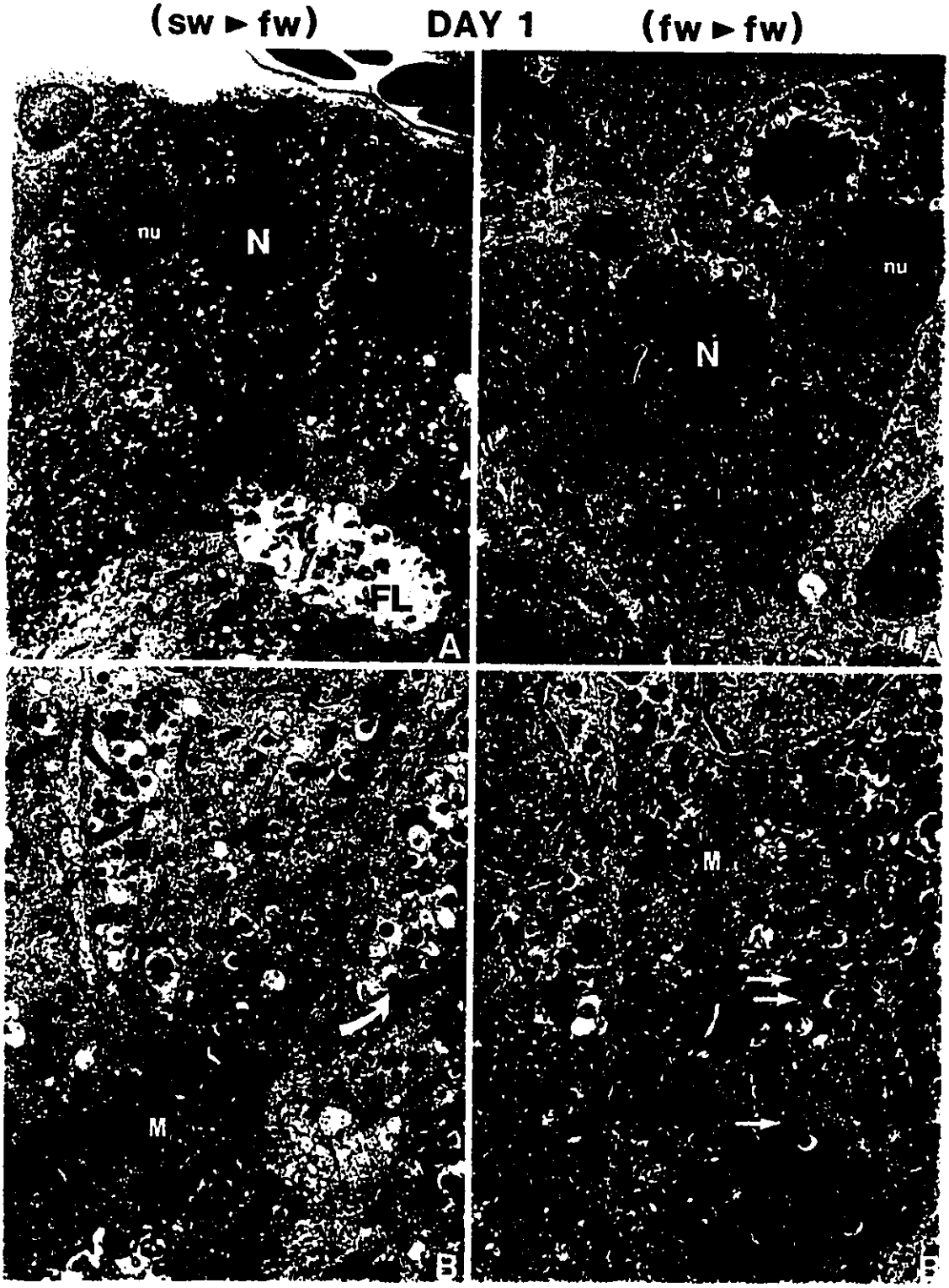

(sw ► fw)
DAY 1
(fw ► fw)
nu
N
FL
A
nu
N
A
M
B
M
B

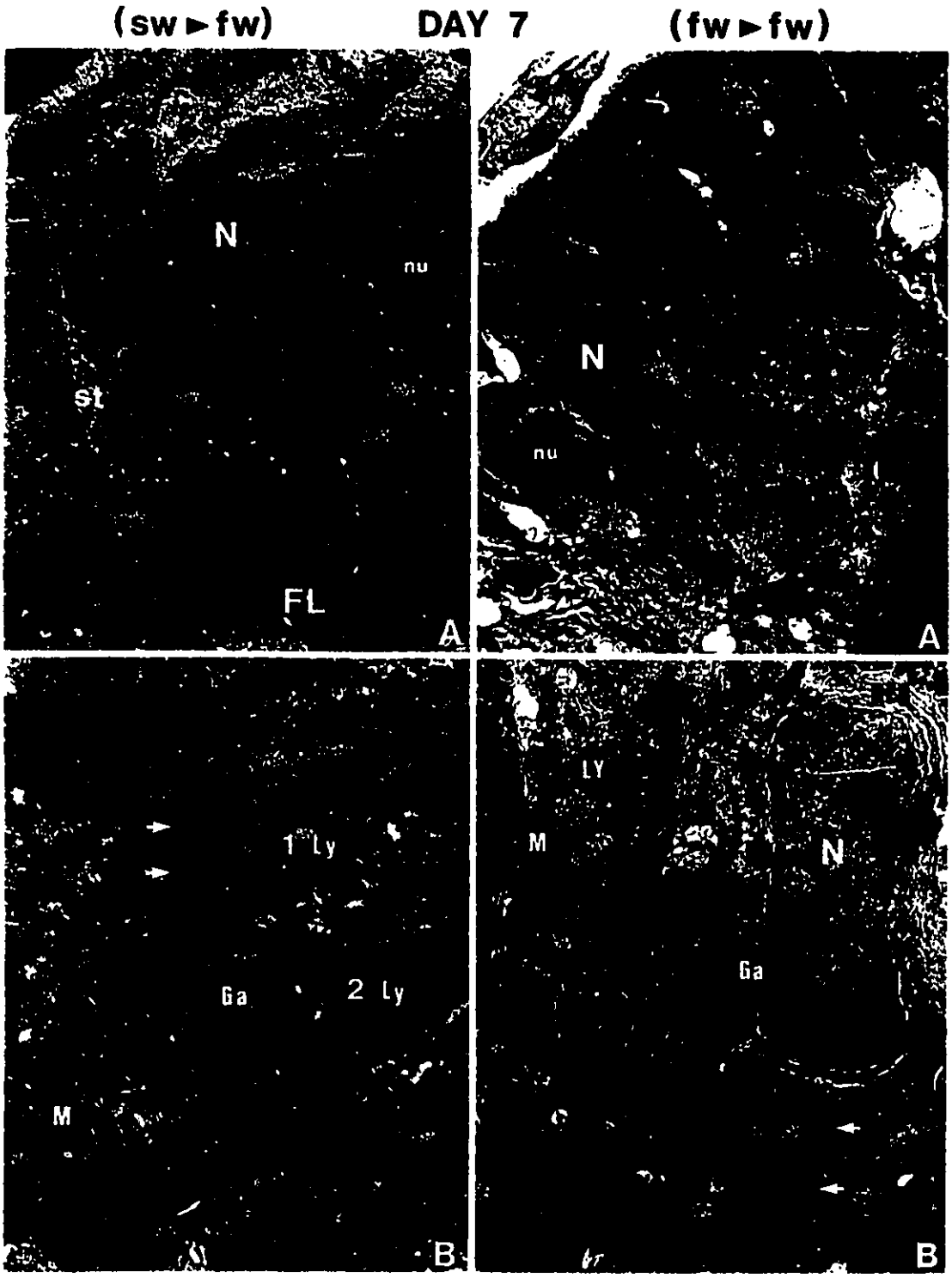
Figure 12

Electron micrographs of the prolactin cells of transfer and control fish 7 days after transfer to FW. The PRL cells of transfer fish possess hormone granulation equivalent to controls suggesting a normalization of PRL secretion. Transfer fish show primary and secondary lysosomes employed by the cell to eliminate the excess organelles involved in the earlier high prolactin synthesis rates (B plate). PRL control cells of fish exhibit extensive basally located rough endoplasmic reticulum (RER). Primary lysosome (1°Ly), Secondary (2°Ly).

A x 6,100

B x 19,500

Of note are the nonsecretory stellate cells occasionally seen in the follicles (SW to FW cell -12A). The stellate cell processes extend between the prolactin cells to the follicular lumen, forming a framework of support for the cells of the follicle.


(sw►fw)
DAY 7
(fw►fw)
N
nu
St
FL
A
N
nu
A
1 Ly
Ga
2 Ly
M
B
LY
M
N
Ga
B

Effect of Stanniectomy, hypophysectomy, prolactin lobe grafts and, continuous ovine PRL infusion in intact fish on calcium efflux rates, and various plasma parameters; and effect of PRL infusion on radiocalcium deposition in various calcium pools.

Figure 13 and Table 12 show the low calcium efflux rates typical of the eel. Stanniectomy had no effect on whole body efflux rates Figure 13A in spite of showing the typical hypercalcemia (Figure 13B) and low PRL cell activity (Figure 13C) of stanniectomized fish. In addition, there were significant, albeit spurious changes, in other parameters (Figure 14). Hypophysectomy likewise did not effect calcium efflux rates (Table 12) or any of the other parameters measured (Tables 12 and 13). However, both PRL lobe homografts and ovine PRL infusion signficantly reduced calcium efflux rates (Table 12), and both treatments induced hypercalcemia (Table 12).

Ovine PRL infusion, simultaneous with ^{45}calcium radioisotope infusion demonstrated that compared to solvent infused fish, ovine PRL induced a shift in radiocalcium distribution such that relatively more calcium was deposited in bone than in soft tissue (consisting of skin, muscle and organs) and mucous (Table 14). In terms of the actual amounts of calcium deposited, both soft tissue and bone radiocalcium contents increased significantly relative to solvent treated fish, however the greatest increase was exhibited in the bone fraction (Table 14).

Table 12 Changes in whole body Ca^{2+} efflux rates in response to Hypophysectomy, PRL lobe implants, and ovine PRL infusion in FW fish [a]

Treatment	Group	N	Date	Whole Body Efflux rates [b]	Plasma $[Ca^{2+}]$ [c]
Hypophysectomy (5 days)	Sham	6	Jan. 29/87	0.047 ± .0013	2.619 ± .164
	HYPEX	6		0.032 ± .0012	2.891 ± .037
2 PRL lobe grafts (10 days)	Sham	7	Feb. 12/87	0.046 ± .0008	2.371 ± .067
	HYPEX	7		0.019 ± .0004[d]	2.999 ± .059[d]
Ovine PRL infusion (10 days)	Sham	5	June 8/87	0.028 ± .0008	2.629 ± .065
	HYPEX	5		0.013 ± .0002[d]	3.051 ± .032[d]

* Each of these 2 groups initially consisted of 10 eels, 5 of which were dissected in order to examine various Ca^{2+} pools (see table 15)

[a] Values are $\bar{X}$ ± S.E.M.

[b] Flux rates $umole.kg^{-1}.hr^{-1}$

[c] Calcium levels (mM)

[d] Significantly diff. (p .05)

Table 13 Changes in various plasma parameters in response to Hypophysectomy, PRL lobe implants, and ovine PRL infusion in FW fish[a].

Treatment	Group	N	Osmolarity [b]	Plasma $[Na^+]^c$	$[k^+]^c$	$[Mg^{2+}]^c$
Hypophysectomy (5 days)	Sh	6	275.69 ± 15.98	151.23 ± 8.26	2.040 ± .085	2.671 ± .107
	HYPEX	6	282.71 ± 10.67	147.89 ±12.41	2.146 ± .148	2.790 ± .080
2 PRL lobe grafts (10 days)	Sh	7	n.m.	155.95 ± 3.57	2.132 ± .812	2.223 ± .855
	HYPEX	7	n.m.	156.78 ± 4.12	2.256 ± .610	2.418 ± .610
Ovine PRL infusion (10 days)	Sh	5	n.m.	185.77 ± 3.08	1.298 ± .126	2.341 ± .651
	HYPEX	5	n.m.	183.86 ± 1.38	1.150 ± .150	2.379 ± .427

a) Values are $\bar{X} \pm$ S.E.M.

b) Osmolarity (mOsm)

c) Values are mM/litre

n.m., not measured

Table 14 Effect of Ovine PRL on ^{45}Ca content and relative ^{45}Ca content of various calcium pools[a]

Pool	^{45} Calcium deposited[d]		Relative ^{45}Ca Content	
	SHAMS	PRL	SHAMS	PRL
Whole body[b]	1.409 ± .121	3.631 ± .312[e]	100%	100%
Soft tissue (blood, skin, mucous, muscle, organs)	0.397 ± .096	0.638 ± .100	28.23 + 2.23	17.56 ± 1.99[e]
Bone	1.012 ± .135	2.994 ± .221[e]	71.77 + 3.26	82.44 ± 2.99[e]
Mucous[c]	0.46 ± .019	.076 ± .025[e]	1.88 + .22	2.10 ± .19

[a] Values are X S.E.M., n = 5 for all groups.

[b] Whole body accumulation was calculated by summing values for soft tissue and bone

[c] Mucous values also included in "soft tissue" pool

[d] Values are in umoles and are calculated from isotope specifications, Amersham, Intl.

[e] Significantly diff. (p .05)

Figure 13

Effect of Stanniectomy on whole body calcium efflux (A), plasma total calcium (B) and PRL cell nuclear volumes (C), in FW acclimated American eels, Anguilla rostrata, at 5,10,14, 21 and 56 days post Stanniectomy N-6 in all cases. * indicates a significant difference ($p \leq 0.05$) relative to control groups. Control group (sham operated) open bars and circles, experimental groups (stanniectomized) solid bars and circles.

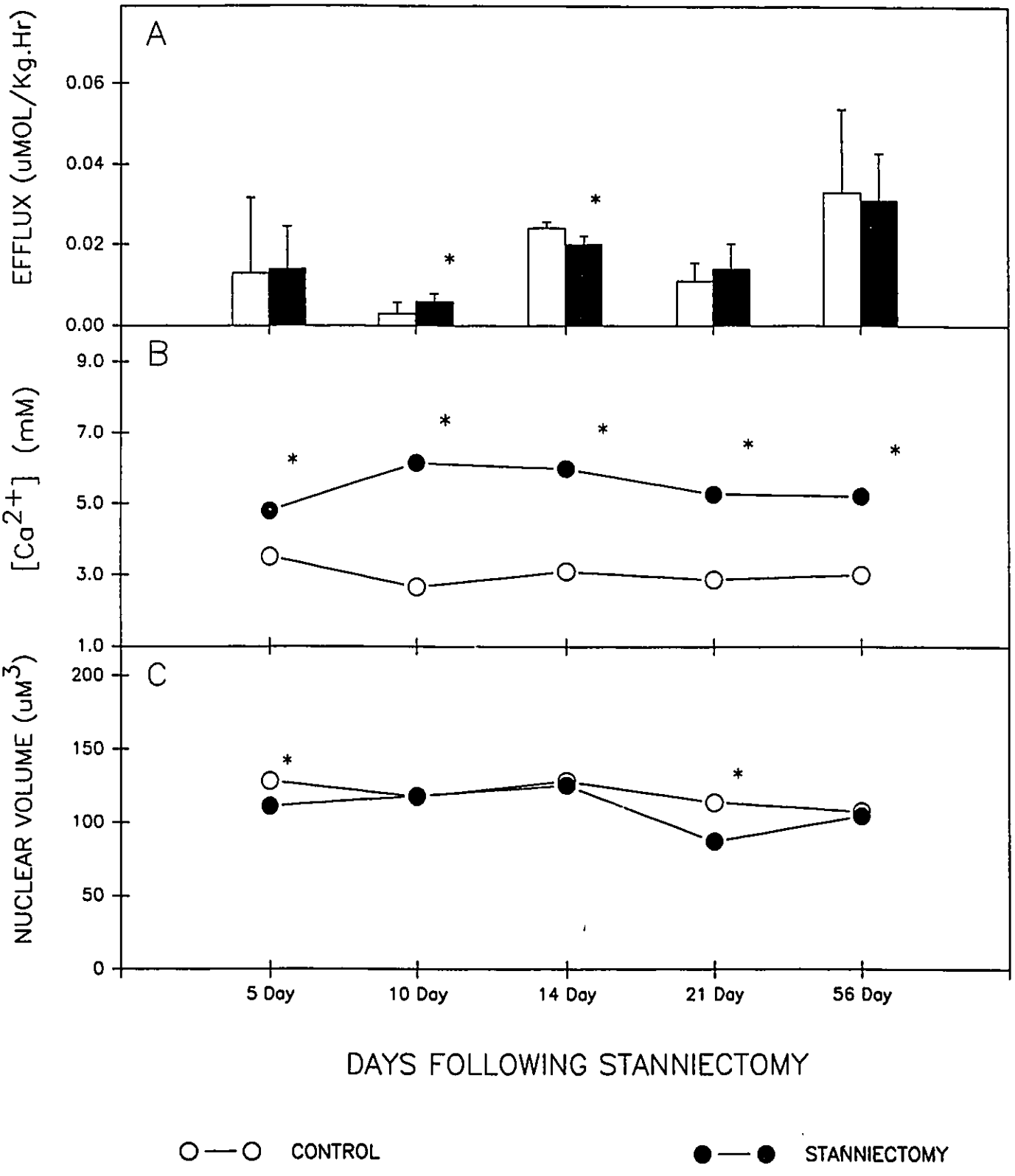
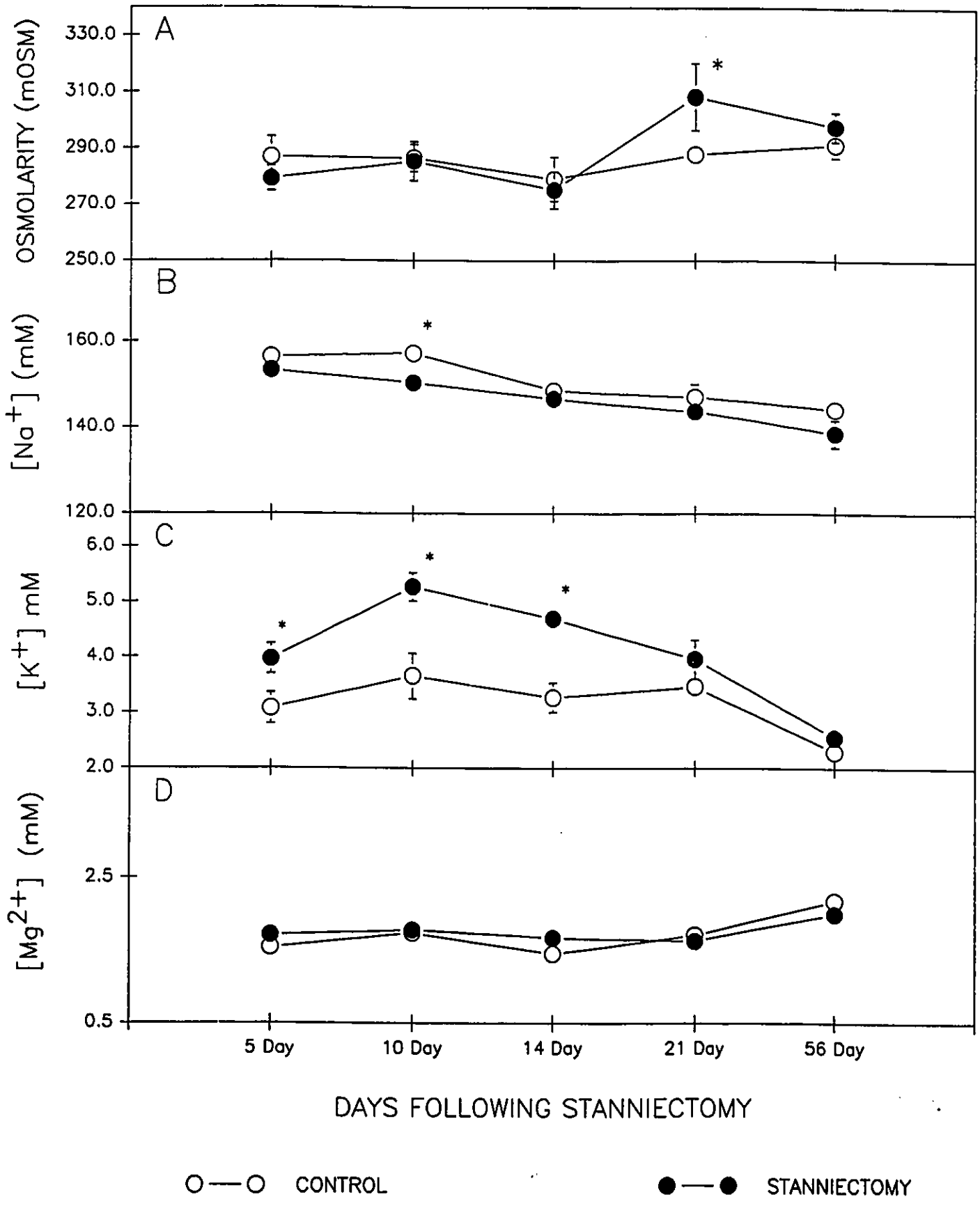

A
EFFLUX (uMOL/Kg.Hr)
0.06
0.04
0.02
0.00
B
[Ca2+] (mM)
9.0
7.0
5.0
3.0
1.0
C
NUCLEAR VOLUME (uM3)
200
150
100
50
0
5 Day
10 Day
14 Day
21 Day
56 Day
DAYS FOLLOWING STANNIECTOMY
CONTROL
STANNIECTOMY

Figure 14

Effect of Stanniectomy on various plasma parameters including blood osmolarity (A), plasma sodium (B), plasma potassium (C) and plasma magnesium (D), in FW acclimated American eels, Anguilla rostrata, at 5, 10, 14, 21 and 56 days after stanniectomy. N=6 in all cases and * indicates a significant difference ($p \leq 0.05$) relative to control groups. Control groups (sham operated) open bars and circles experiment groups (stanniectomized) solid bars and circles).


A
B
C
D
OSMOLARITY (mOSM)
330.0
310.0
290.0
270.0
250.0
[Na+] (mM)
160.0
140.0
120.0
[K+] mM
6.0
5.0
4.0
3.0
2.0
[Mg2+] (mM)
2.5
0.5
*
5 Day
10 Day
14 Day
21 Day
56 Day
DAYS FOLLOWING STANNIECTOMY
CONTROL
STANNIECTOMY

Calcium influx rates and various plasma parameters in control fish over a 16 month period.

Whole body influx rates were relatively constant from September, 1986 until May 1987, but increased significantly in June (Figure 15). In July a new population of fish was brought into the lab so that the data shown at that time may reflect the different population. However, as influx rates were already increasing in June (when the initial population of eels was being used), the increase between May and July may represent a real rhythm. Fluctuations in plasma sodium and potassium also occurred in June (Figure 16). The high plasma total calcium levels in July, in the new fish, accompanied the high flux rates of this group upon introduction to the lab. These alternate, however, to match the levels of the 1st population. Blood magnesium remained fairly stable throughout except for a drop from December to April which coincided with a general decrease in ion levels.

Figure 15

Record of uni-directional calcium influx rates and plasma (A) total calcium levels (B) in control eels, Anguilla rostrata over a 16 month period from September 1986 to December, 1987. N=at least 12 in all cases.

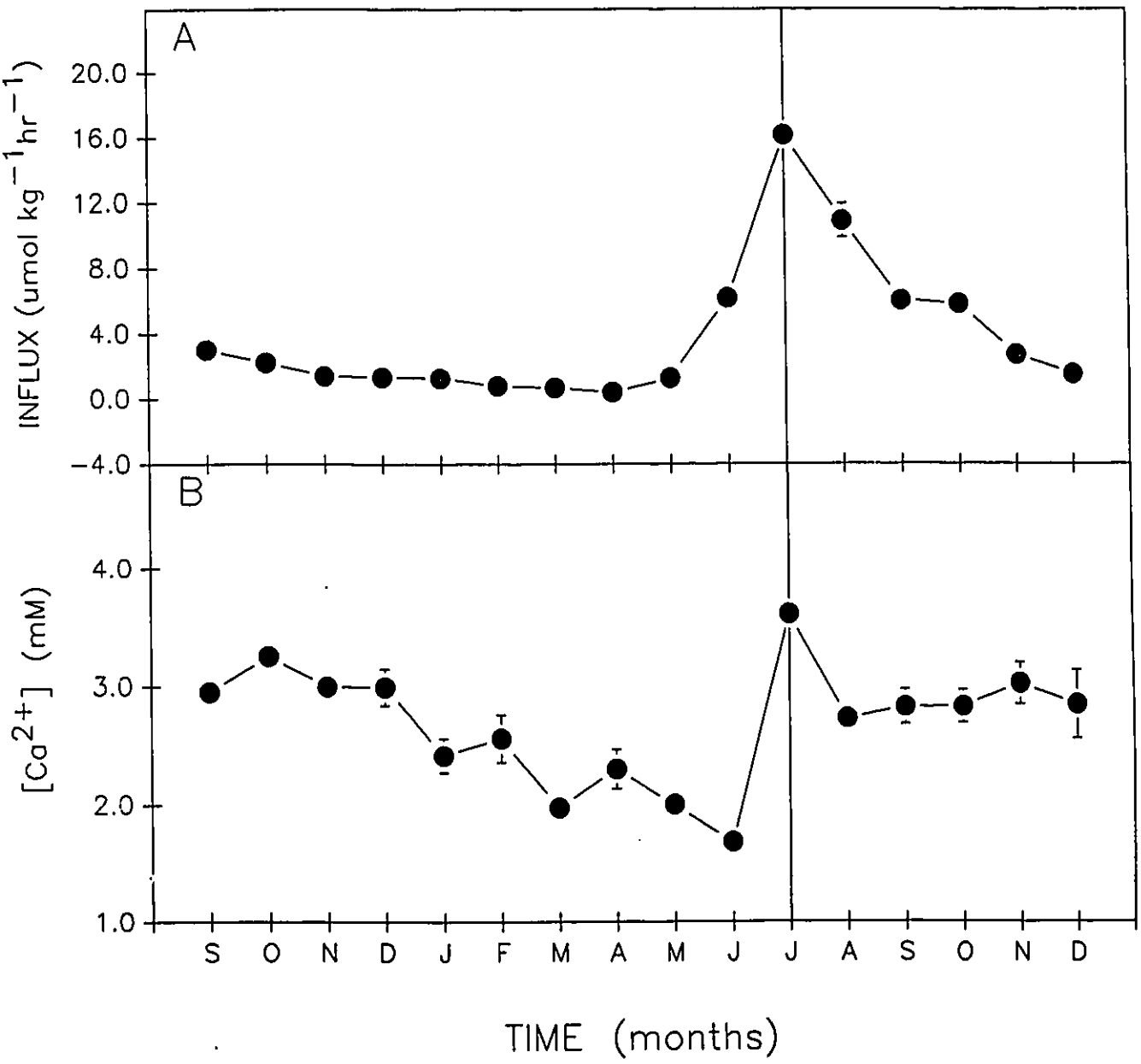

A
B
INFLUX (umol kg^{-1}hr^{-1})
20.0
16.0
12.0
8.0
4.0
0.0
−4.0
[Ca^{2+}] (mM)
4.0
3.0
2.0
1.0
S O N D J F M A M J J A S O N D
TIME (months)

Figure 16

Record of various plasma parameters including, plasma sodium (A), plasma potassium (B), and plasma total magnesium (C), in control eels, Anguilla rostrata over a 16 month period from September 1986 to December 1987. N=at least 12 in all cases.

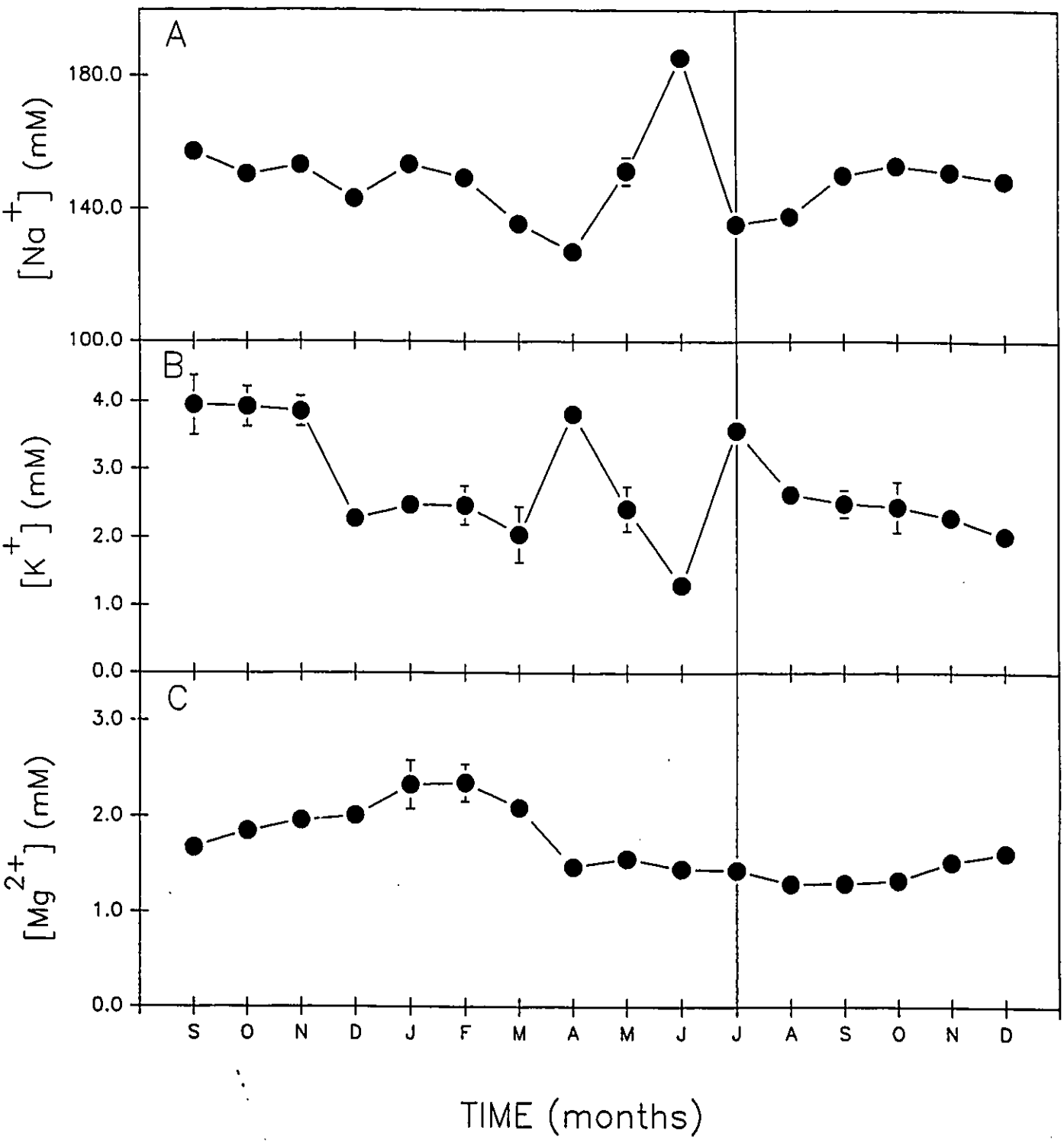
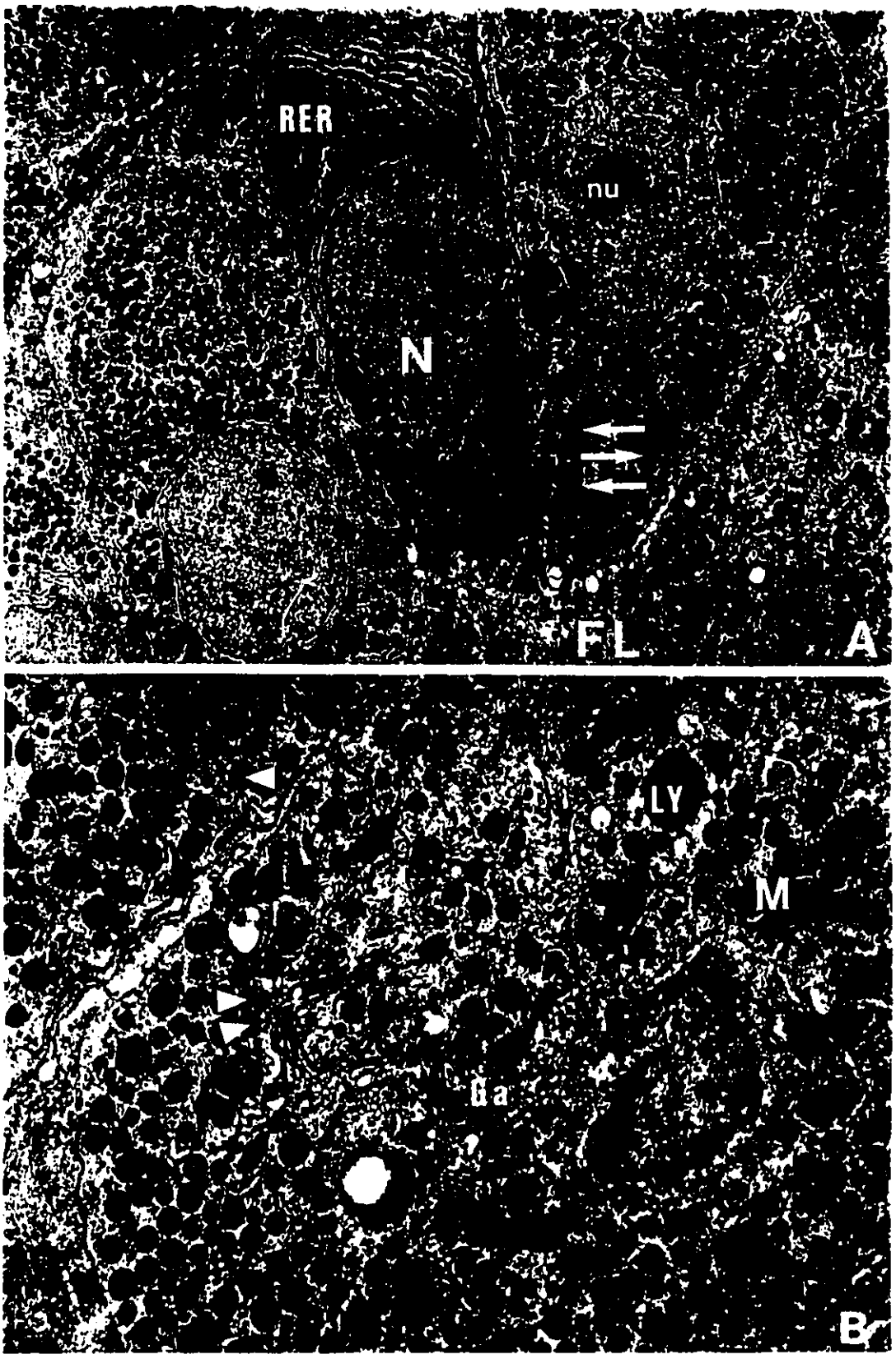

A
180.0
140.0
100.0
[Na+] (mM)
B
4.0
3.0
2.0
1.0
0.0
[K+] (mM)
C
3.0
2.0
1.0
0.0
[Mg2+] (mM)
S O N D J F M A M J J A S O N D
TIME (months)

Figure 17

Electron micrographs of the PRL cells of a typical untreated fish kept in running dechlorinated water in the laboratory. Large nuclei, with prominent nucleoli are displayed, as are extensive RER basal to the nucleus, extensive cytoplasmic granulation, and cytoplasm well populated with Golgi apparatus systems (Ga) and mitochondria.

A x 6,100

B x 19,500


RER
nu
N
FL
A
LY
M
Ga
B

DISCUSSION

Overall the evidence accumulated during this study implicates prolactin as hypercalcemic hormone in the American eel. In general, exposure to exogenously administered prolactins, or presumably higher circulating endogenous prolactin, is followed by a positive shift in the calcium balance of the fish. The resultant elevation in plasma total calcium appeared to result from enhanced whole body calcium uptake from the water, and, to a lesser extent, from decreased whole body efflux of calcium into the water. Conversely, simulated decreases in endogenous prolactin, or depressed lactotroph (prolactin cell) activity, were variously associated with hypocalcemia, decreased calcium influx rates, or both.

It is evident that fishes regulate their serum calcium efficiently (Fenwick & Wendelaar Bonga, 1982), and that the endocrine systems involved are different from those in tetrapods (Pang, 1973). A functional parathyroid gland has not been demonstrated in fishes, and the majority of evidence indicates calcitonin has little or no effect on fish calcium regulation (Fouchereau-Peron *et al*, 1986, 1987). Instead, the pituitary gland and the corpuscles of Stannius are the two main endocrine systems involved in fish plasma calcium regulation, (Pang & Pang, 1986).

Prolactin from the pituitary, appears to function as a hypercalcemic hormone in teleost fish. Administration of mammalian prolactins induces hypercalcemia in various teleosts including; the European eel, *Anguilla anguilla* (Olivereau & Olivereau, 1978), tilapia, *Oreochromis mossambicus* (Wendelaar Bonga & Flik, 1982, Flik

et al, 1986b), goldfish Carassius auratus (Wendelaar Bonga, Flik & Fenwick, 1984a) and the killifish Fundulus heteroclitus (Pang, 1981a). In addition, hypophysectomized killifish become hypocalcemic in Ca^{2+} deficient seawater; and this is overcome either by supplying Ca^{2+} to the water or by administering exogenous prolactin to the fish (Pang, 1981b). Further, the treatment of tilapia with homologous prolactin lobe homografts induces hypercalcemia (Wendelaar Bonga and Flik, 1982), indicating an effectiveness of endogenous prolactin. The physiological relevance of the hypercalcemic action of prolactin was demonstrated in tilapia, Oreochromis mossambicus (Wendelaar Bonga et al, 1983, 1985), and the stickleback, Gasterosteus aculeatus (Wendelaar Bonga & Van Der Meij, 1980, 1981), where an inverse relationship was found between levels of environmental Ca^{2+} and prolactin cell activity. Whereas these observations indicate a hypercalcemic role for prolactin in most teleosts, similar criteria have not been tested in the American eel. In addition, the mechanisms whereby the hypercalcemia is produced are not fully understood.

The majority of the findings in this study are consistent and do indeed suggest a hypercalcemic role for prolactin in the American eel. The treatment of eels with homologous prolactin (PRL) lobe homografts (Table 8) or with ovine prolactin either by infusion (Table 10) or injection (Table 11), induced hypercalcemia, presumably supported by enhanced calcium uptake rates (Tables 8, 9, 11) and, to a lesser extent by depressed calcium efflux rates (Table 12).

The effects of ovine prolactin infusion in teleosts have not been well documented. The data reported here (Table 10) indicate that 10

days of ovine prolactin infusion induced hypercalcemia. This hypercalcemic action of infused prolactin agrees with the results of Flik et al (1984) on the American eel, A rostrata and those of Wendelaar Bonga & Flik (1982) on tilapia, Oreochromis mossambicus. In general, the hypercalcemia following prolactin therapy in teleosts is well documented (Pang, 1981a, Pang et al, 1973e, 1978, Flik et al, 1986b), yet the mechanisms whereby this hormone achieves hypercalcemia are poorly understood. For this reason the effects of prolactin infusion on whole body calcium uptake were also tested (after Flik et al, 1986).

The validity or the importance of whole body calcium uptake (influx) rates rests on a number of necessary assumptions including; that the unfed fish used in these experiments rely entirely on environmental calcium (Rodgers, 1984), that the major component of calcium uptake occurs through the gills (Flik et al, 1985a), that the whole body flux technique employed reflects branchial uptake (Flik et al, 1985a), and that the gill is the target organ for calciotropic hormones (Payan et al, 1981, Foskett et al, 1983, Perry & Wood, 1985, Flik et al, 1986b, Fenwick, in press).

The data of table 10 suggest that the prolactin infusion induced hypercalcemia was associated with enhanced rates of whole body calcium uptake. Although not previously reported, such prolactin stimulation of calcium uptake in the American eel has recently been observed by Flik et al (in press). In addition to these effects, prolactin infusion also induced plasma sodium retention, which is the best

established effect of prolactin treatment in fish (Foskett et al, 1983, see also reviews by Nicoll, 1981, Loretz & Bern, 1982, Bern, 1983, Hirano, 1986). Hyperkalemia and hypermagnesemia were also observed following prolactin treatment (Tables 10 & 11). Unfortunately, similar results for plasma potassium have not previously been documented, and little is known about the metabolism of magnesium in fish.

Ovine prolactin injection therapy also stimulated whole body calcium uptake rates (Table 10); and resulted in plasma sodium retention (Table 11). To date, this is the first report of the effects of ovine PRL injections on unidirectional calcium influx rates in the American eel, but Flik et al (1986b) have reported similar results for Tilapia. Although the hypercalcemic action of injected ovine prolactin has been reported for various teleosts including; goldfish, Carassius auratus (Wendelaar Bonga, Flik & Fenwick, 1984a), tilapia, Oreochromis mossambicus (Wendelaar Bonga et al, 1985), the sailfin molly Poecilia latipinna (Ensor & Ball, 1972), killifish, Fundulus heteroclitus (Pang, 1981a), and sticklebacks Cichlosoma nigrofasciata (Wendelaar Bonga & Flik, 1985), hypercalcemia was not observed following prolactin injection in the present study. Reasons for this are unclear. Several authors have speculated that calcium metabolism studies can become complicated due to various factors such as cyclical fluctuations in plasma calcium (Fleming, Brehe & Hansan, 1973) or shifts in the relative distribution of calcium between various fractions (Dacke, 1979). Additionally, it is known that a bolus injection of PRL remains in the blood stream for only 3-7 hours (Blum, 1968) and, this time frame may be insufficient for inducing hypercalcemia.

As these fish were held in water with Ca^{2+} concentrations of of [0.4mM], and, as it is known that endogenous prolactin production is below maximum under these conditions (Wendelaar Bonga et al, 1985), in all likelihood the observed effects of prolactin therapy are physiological rather than pharmacological. One could argue that the doses of ovine PRL used for infusion or injection (0.15 IU/g/fish/day^{-1}) are excessive, however, others (Flik et al, 1986b, in press) have indicated that this is a requirement for fish. In addition, Hasegawara et al (1986) recently reported that chum salmon prolactin was one hundred times more potent than were mammalian prolactins in the Fundulus sodium retention test (Grau et al, 1984). With this in mind, it was felt that homologous eel prolactin therapy would provide a more physiologically relevant approach to studying the calciotropic actions of PRL. In addition, the possibility that the ovine prolactin doses were in fact pharmacological could be avoided.

It is known that the eel rostral pars distalis contains TSH, ACTH, and PRL cells, the greater portion of which is comprised of PRL cells (Holmes & Ball, 1974, Peter & Fryer, 1983). In addition, upon explantation only the PRL cells survive and become activated (Olivereau & Ball, 1966, Ball & Baker, 1969); and such auto transplantation of pituitaries were successfully performed in several teleosts, Gillichthys mirabilis (Nagahama, Nishioka & Bern, 1974, Olivereau & Dimovska, 1969), Anguilla anguilla, Fundulus heteroclitus (Pang et al, 1978), and Anguilla rostrata (Flik, Fenwick & Wendelaar Bonga, in press).

For these experiments pituitary glands were carefully dissected so that only the "PRL lobes" were installed. However, when using pituitary glandular implants one must keep in mind that in addition to the possible actions of PRL cells and ACTH cells (via cortisol) there exist the PAS positive cells of the pars intermedia (PIPAS cells), sources of the putative fast acting hypercalcin (Parsons et al, 1978, Pang, 1981a). If, by chance, any PRL lobe was contaminated with PIPAS cells, and, if this hormone is important to calcium regulation (Hirano, 1986), then any effects resulting from PRL lobe therapy might have been wrongly attributed to the presence of PRL cells. Intact fish were thus treated with "residual pituitary" implants consisting of only the pars intermedia, and compared with shammed implants. The results (Tables 8 & 9) showed no effect on any parameter measured save for a decrease in plasma osmolarity. Although Pang et al (1973c) reported hypercalcemia in killifish treated with PIPAS cells, this effect occurred within hours, whereas the present experiment lasted five days. Quite possibly, Pang's implants contained some acute residual hypercalcemic activity which would not be observed after five days. This is supported by the observation that upon transplantation the PAS positive cells of the pars intermedia atrophy and become inactive (Holmes & Ball, 1974).

The present study is the first to show that the hypercalcemic effect of prolactin lobe implants is associated with enhanced unidirectional calcium uptake. The data in Tables 8 & 9 show that intact fish with 2 supplementary PRL lobes exhibited hypercalcemia,

along with high calcium uptake, and, in situ (pituitary) PRL cell activites were similar in PRL and sham treated fish. This suggests that the stimulation of calcium uptake may be responsible for at least part of the observed hypercalcemia. This conclusion is supported by the observations of Flik, Fenwick & Wendelaar Bonga (in press) who recently showed that homografts in eels stimulated the branchial high affinity calcium ATPase by increasing the maximal velocity of the pump, thereby enhancing overall calcium uptake rates.

Tables 8 and 9 show that eels with four extra prolactin lobes exhibited hypercalcemia, but curiously, increased calcium influx was not observed and, PRL cellular activity was depressed in these fish. Further, plasma sodium was higher in the PRL lobe treated fish relative to shams and plasma magnesium was lower. It seems reasonable to assume that the presence of the four extra lobes, and presumably very high levels of circulating PRL could be responsible for the inactivation of the in situ PRL cells. Indeed, Olivereau et al (1971) reported that 10-15 days after pituitary homograft implantation in intact eels the in situ PRL cells became inactive. Further, Nagahama et al (1975) showed that 40 days after grafts were installed in Gillichthys mirabilis, the PRL cells of the in situ pituitary became inactivated. Evidently, here too the PRL secreted by the implants greatly reduces the PRL released from the in situ gland (Olivereau & Dimovska, 1969, Nagahama et al, 1974). However, this solution becomes problematic when we make the claim that the high prolactin levels inhibit the in situ PRL cells, yet did not stimulate influx rates, at

least not at the time of measurement. Nevertheless, at some point before the two week measurement interval, influx rates must have been high enough to induce the observed hypercalcemia. It could be that in this case hypocalcemic compensation by either hypocalcin or calcitonin is substantial enough to negate the effects of the high circulating PRL levels on calcium uptake mechanism. Even more speculative is the possibility that the grafts that were installed have negatively inhibited each other in much the same manner as circulating PRL typically inhibits the in situ gland (Nagahama et al, 1974). Finally, the observed increase in plasma sodium but not magnesium is consistent with the known sodium retentive effect of prolactin therapy (Grau et al, 1984).

A final means of simulating increases in prolactin involved the treatment of eels with prolactin releasing factors (PRF's). This method would allow observation of the effects of endogenously released homologous prolactin without the use of surgical intervention. The secretion of prolactin from the pituitary is under tonic inhibitory regulation from the hypothalamus (see review by Schally, 1978, Krieger, 1980). Mammalian studies indicate that major prolactin release inhibiting factor (PIF) is dopamine (Hill-Samli & MacLeod, 1974, Clemens et al, 1974, Swennen and Denef, 1982, Schrey, Clark & Franks, 1986, Blask & Orstead, 1986), which is released into the hypophysial portal blood (Gibbs & Neill, 1977, Macleod, 1976, Lafond & Collu, 1986), and probably acts on receptors on the PRL cells

(Cronin, Roberts & Weiner, 1978, Calabro & Macleod, 1977).

Although the inhibitory role of dopamine is thought to account for the usually low prolactin levels in mammalian blood (Haisenleder et al, 1986a), a decrease in DA in hypophysial portal blood cannot explain increases in plasma PRL (de Greef & Neill, 1979). This implies that a PRL releasing factor (PRF) may also be secreted into the hypophysial portal system. Evidence for a PRF has been provided during suckling in rats (Grosvenor & Mena, 1980, de Greef & Visser, 1981), and two hypothalamic peptides, thyrotropin releasing hormone (TRH) and vasoactive intestinal peptide, are currently under serious consideration as PRF's (Collu et al, 1984), as is the dopamine antagonist haloperidol (Arita & Kimura, 1986). Additionally, in rats, estradiol treatment decreased the number of anterior DA receptors in the anterior pituitary, and this was associated with increased plasma prolactin levels (Di Paolo & Falardeau, 1986, Haisenleder et al 1986 b). Further although TRH has consistently been described as a potent PRF in mammals (Hill-Samli & Macleod, 1974, Fagin & Neill, 1981, Collu et al, 1984, Frawley & Clark, 1986, Hoeffler & Frawley, 1987) a similar role in fish has yet to be confirmed. The possible participation of TRH in this matter, which stimulates prolactin release in several mammals (Hil Samli & Macleod, 1974), and also the

participation of some serotonergic pathway in fish which may intervene at some level to stimulate PRL secretion (Olivereau & Olivereau, 1979, Ball, 1981) must be considered. TRH was shown by various immunological techniques to occur in teleost nervous systems (Peter & McKeown, 1973, Vale et al, 1976), and, high affinity TRH receptors have been found on goldfish pituitary glands (Burt & Ajah, 1984). Furthermore, the distribution pattern of TRH binding sites in the fish brain resembles that in birds and mammals (Thompson et al, 1981, Taylor & Burt, 1982). There is also some physiological evidence that a PRF, possibly TRH, acts to promote PRL synthesis and release in fish (Ball et al, 1972, Olivereau, 1975, Hall & Chadwick, 1978, Ball, 1981).

The present experiments were based on the prediction that stimulation of in situ PRL cell activity through PRF treatment could enhance unidirectional calcium uptake. As relatively little is known concerning the control of teleost prolactin secretion, the drugs chosen for these experiments were based on the knowledge of mammalian prolactin secretion (see reviews by Schally, 1978, Clemens & Schaar, 1979, Krieger, 1980).

The results show that neither TRH or haloperidol affected any changes in PRL cell activity, unidirectional calcium influx rates, plasma total calcium or other plasma parameters (Tables 4 and 5) suggesting that in the American eel haloperidol and TRH do not promote prolactin cell activity. To our knowledge no other studies have reported the effects of any PRF on the activity of eel prolactin cells in vivo. Likewise, no reports of unidirectional calcium

influx rates or plasma total calcium following PRF treatment are available for comparison. However several studies have assessed the effects of PRF treatments on PRL release *in vitro* in fish and such studies have been contradictory. For example, TRH *in vitro* at 2.77×10^{-7}M inhibited PRL release from the RPD of *Tilapia mossambicus* (Wigham *et al*, 1977) but stimulated release from the pituitary of the sailfin molly, *Poecilia latipinna* (Wigham & Batten, 1984). On the basis of the present data, a comparable dose of TRH *in vivo* seemingly neither inhibited or promoted PRL cell activity. This discrepancy between species casts doubt on the role of TRH as a universal regulator of PRL in fish. Indeed, Jackson (1981) argues that TRH regulation of PRL is a late evolutionary development appearing only in birds and mammals. Certainly the present data would support this.

If TRH is to be established as a hypophysiotropic hormone in the eel, TRH receptors must be be shown on eel PRL cell membranes, and it must be shown that TRH is actually delivered to the PRL cells. In mammals, priming with estradiol enhances the response of PRL cells to TRH stimulation (Labrie *et al*, 1980) by increasing the number of TRH receptors on PRL cells (Gershengorn *et al*, 1979, Di Paolo & Falardeau, 1985). Moreover, 17b estradiol can act directly to stimulate PRL secretion in mammals (Nicoll & Meites, 1962, 1964) and fish (Nagahama *et al* 1975, Pang & Balbontin, 1978). In addition, Barry & Grau (1986) reported that pre-incubation with estradiol *in vitro* of the tilapia anterior pituitary permitted dose-related TRH stimulation of the PRL cells. Finally, since TRH seems to function only after PRL cells have

been previously exposed to estradiol, a steroid whose levels rise with the onset of gonadal recrudescense in female teleosts (Fostier et al, 1985), and, as its effects on the PRL cells of sexually immature eels (Tesch, 1977) used in this study were negligible, this suggests that the control of the mechanisms of PRL secretion may change with variations in the reproductive state of the animal (Barry & Grau, 1986).

Another possible explanation for the lack of effect following PRL treatments is that at the calcium concentration used in this study (0.4mM), PRL secretion may be maximal already. Indeed, several authors report extreme PRL cell activity in waters containing calcium concentrations of 0.6mM in Killifish, (Pang & Sawyer, 1981), 0.8mM in Tilapia (Wendelaar Bonga et al, 1985), and 2.0mM in eels (Olivereau & Olivereau, 1983).

Thirdly, the lack of potency of various PRF's lies in the fact that their effect on prolactin cells undergoes circadian (Nagayama, Takagi & Takahashi, 1987) and seasonal changes (Tyndale - Biscoe & Hinds, 1984). The fact that all experimental procedures using PRF's in the present study were performed at the same time on any given day and the same calendar month could account for the lack of effect after treatment with these drugs.

In summary, the data from PRL treated eels suggests that the observed hypercalcemia occurs as a result of the accompanying increases in calcium uptake from the water. Others, (Flik et al 1986b) have reported that in Tilapia the hypercalcemia resulting from PRL treatment was due to a combination of high calcium uptake rates and decreased efflux rates. The data reported here

(Table 12) for the eel concur in that both ovine PRL infusion for 10 days and RPD implants for 10 days suppressed efflux rates by over 50%. But these rates are 100 times lower than typical efflux rates reported for other intact species such as, Salmo gairdneri (Hobe et al, 1984b, Perry et al, 1988, Perry and Flik, 1988) and Oreochromis mossamibicus (Flik et al, 1985a). This is the first report of the effects of heterologous or homologous PRL on whole body calcium efflux rates in the eel, and it seems that the eels used in this study had inherently low efflux rates.

The reduced whole body efflux data following prolactin therapy reported here for the eel (Table 12) and elsewhere for Tilapia (Flik et al, 1986b) possibly reflect lower gill permeability. Indeed prolactin was shown to reduce branchial permeability to water and ions in the European eel, Anguilla anguilla (Maetz et al, 1967a), killifish Fundulus heteroclitus (Maetz et al, 1967b), molly Poecilia latipinna (Ensor and Ball, 1968) tilapia (Dharmamba & Maetz, 1972) and the Japanese eel Anguilla, japonica (Ogawa, 1974, 1977). Further, prolactin has been shown to tighten the tight junctions of kidney proximal tubules in the stickleback (Wendelaar Bonga & Veenhius, 1974). Together, these facts have led some to suggest that the control of integumental permeability is a key part of the action of prolactin on hydromineral regulation (Wendelaar Bonga & Van Der Meij, 1981, Flik et al, 1986b).

Clearly then, in the American eel both homologous and ovine prolactin stimulated unidirectional calcium uptake, and decreased whole body efflux rates, producing hypercalcemia, and these results are in direct agreement with similar data for male tilapia (Flik et al, 1986b).

In addition, Flik et al (1985a) discovered that the resultant hypercalcemia was also accompanied by increased bone deposition of calcium phosphate that raised the density of Tilapia bone. In order to test this possibility in eels, an experiment was performed in an attempt to observe the relative effects of PRL on radio-calcium deposition into bone, soft tissue, and epithelial-mucous "calcium pools". Table 14 shows the eels infused with a solution of both ovine PRL and ^{45}calcium for 10 days show a 296% increase in the actual amount of circulating radiocalcium deposited into the bone as compared to fish infused with a solution of solvent and ^{45}calcium. This suggests that prolactin somehow promoted the deposition of extracellularly available radiocalcium into the bone of these eels. This shift in radiocalcium deposition is fairly substantial when we consider that it resulted in an 11% decrease in the relative deposition of radiocalcium into soft tissue that translated into almost an 11% increase in the relative distribution of radiocalcium into bone.

Unlike terrestrial vertebrates, fish bone cells are of less importance in calcium homeostasis (Hermann-Erlee & Flik, 1988). The structure of the fish bone osteocyte is typical of that found in other vertebrate classes. Osteocytes are formed from mesenchyme cells, which, in an earlier phase are termed osteoblasts. Osteoblasts are involved in de novo bone accretion by secreting about themselves a thick matrix containing collagen into which calcium salts deposit, becoming osteocytes. Osteocytes do not divide but are replaced by constant recruitment from the mesenchyme (Dacke, 1979). Most teleost

fish bone may be divided into one or two types depending on whether the bone contains osteocytes (cellular bone) or not (acellular bone). Accellular bone is considered to contribute much less to calcium homeostasis than cellular bone (Simmons, 1971). Evidence suggests that accellular bone undergoes relatively little mineral exchange (Simkiss, 1974) whereas resorption of the calcium reservoirs in cellular bone occurs in even the most hypercalcic of environments (Dacke, 1979).

The present data (Table 14) provides no information as to how prolactin promotes calcium deposition in bone, although, in tilapia an acellularly boned fish, prolactin exerts its effects on bone independent of activation of osteoblastic activity (Wendelaar Bonga et al, 1984a). According to Hermann-Erlee & Flik (1988) this acellular bone acts as a storage site for minerals, and it appears that from these data that cellular eel bone can work in the same way (Fenwick, 1974). To date no studies have been published concerning the actions of PRL on cellular eel bone, although the present data suggest that prolactin treatment of American eels induced a substantial positive shift in calcium balance as a result of increased ^{45}calcium uptake and decreased whole body efflux of ^{45}calcium. The resultant hypercalcemia affected an increase in the ^{45}calcium activity of cellular eel bone, and presumably this reflects a comparable increase in total ^{40}calcium deposition (Hermann Erlee & Flik, 1988). However, as none of the other components of apatite were tested we can make no comment as to whether or not the excess deposited calcium in PRL treated eel bone is indicative of actual bone mineralization.

Following various attempts to simulate increases in prolactin, the second portion of the study involved attempts to simulate decreases in circulating prolactin and to observe the associated effects on calcium regulation. Experiments involving hypophysectomy, (pituitary removal) exposure to seawater, and prolactin releasing inhibiting factor (PIF) therapy were employed.

Mammalian studies have shown that the in vivo PIF is dopamine (Hill-Samli & MacLecd, 1974, Clemens et al, 1974, Gibbs & Neill, 1977, Swennen & Denef, 1982, Schrey, Clark & Franks, 1986, Ishibashi & Yamaji, 1985, Blask & Orstead, 1986, and Fernandez-Ruis et al 1986) released into the hypophysial portal blood (Gibbs & Neill, 1977, Macleod, 1976, Lafond & Collu, 1986). Probably dopamine is acting through a direct receptor-mediated effect on the anterior pituitary PRL cell, (Caron et al, 1978, Calabro & Macleod, 1977, Cronin, Roberts & Weiner, 1978), and its effects can be mimicked clinically by dopamine agonists such as ergocryptine (Caron et al, 1978) and bromocryptine (Ferrari et al, 1986, Montini et al, 1986, Johansen et al, 1986.

Evidence for a PIF in fish is suggested by increased activity of the prolactin cells of transplanted pituitaries (Ball & Baker, 1969, Ball et al, 1972, Peter, 1973, Peter & McKeown, 1974a). As in mammalian systems these effects can also be elicited by dopamine agonists such as ergocryptine (Caron et al, 1978), and bromocryptine (Ferrari et al, 1986, Montini et al, 1986, Johansen et al, 1986). In addition, dopamine, specifically the precursor L-dopa, effectively

inhibits prolactin cell activity and PRL release in various fish species, such as Gillichthys mirabilis and Tilapia mossambicus (Nagahama et al, 1975, Wigham et al, 1977, Peter & Fryer, 1983) as have injections of the drug 6-hydroxydopamine (Olivereau, 1969, Ball & Baker, 1969, Peter, 1973, Holmes & Ball, 1974), and injections of the ergot alkaloid 2-Br-ergocryptine, also a dopaminergic agonist, in Anguilla anguilla (Olivereau & Lemoine, 1973b, Olivereau, 1975) and Poecilia latipinna & Xiphophores hallerii (McKeown, 1972).

The results of this study showed that apomorphine and ergocryptine but not dopamine or bromocryptine inhibited unidirectional calcium influx rates but plasma total calcium was not affected (Table 6). Apomorphine and ergocryptine also reduced lactotroph (PRL cell) activity but bromocryptine did not (Table 7). To our knowledge, this is the first record of the use of ergot alkaloids that attempts showed depressed in situ PRL cell activity and attribute changes in calcium metabolism to the apparent depression. As such, we know of no other report in which eels were treated with apomorphine and ergocryptine and showed low PRL cell activity accompanied by significantly lower unidirectional calcium influx rates and lower plasma osmotic pressure.

The inhibition of PRL cell activity induced by both apomorphine & ergocryptine is in good agreement with various other studies using L-Dopa treatment in eels, Anguilla anguilla (Olivereau & Lemoine, 1973b) and Gillichthys mirabilis (Nagahama et al, 1975), SRIF (somatostatin inhibits somatrotropin (GH) secretion and has overlapping effects release-inhibiting factor) treatment in Poecilia latipinna (Wigham & Batten, 1984) and Tilapia, mudsucker & Killifish

(Grau et al, 1982), and bromocryptine administration in eels A. anguilla (Olivereau & Lemoine, 1973b). The data here (Tables 6 and 7) provide substantial evidence linking an apparent depression of lactotroph activity with a reduction in calcium uptake rates. Unexpectedly blood total calcium remained unchanged following PIF treatment (Table 6). Again, reasons for this are unclear although the possibility of shifts in the relative distribution of calcium from the ionized to the protein bound fraction seems plausible (Chan et al, 1967).

In a further attempt to simulate decreases in endogenous prolactin, several groups of fish were exposed to 66% seawater (SW), as it is well established that fish acclimated to SW show reduced in situ prolactin cell activity (Nagahama, Clarke & Hoar, 1977). When SW acclimated eels were transferred to FW, and compared with FW to FW transfer "controls", the SW to FW transfer fish showed a gradual increase in lactotroph activity concomitant with a gradual stimulation of calcium influx rates over the one week period (Figures 8, 9, 10). Further, in a seperate test, SW acclimated eels treated with a single ovine PRL injection showed a stimulation of calcium uptake rates within 20 hours, and subsequent hypercalcemia 48 hours post injection (Figures 6 & 7). Finally, multiple injections of PRL in SW acclimated eels elevated plasma total calcium, but, curiously, reduced both calcium influx rates, and PRL cell activity (Tables 10 & 11).

Evidence has been provided (Flik et al 1985a, 1986b, in press) that PRL increases whole body calcium accumulation through the stimulation of branchial calcium uptake in FW fish, and that PRL requires at least several days to a week to exert its effects (Bern et al, 1981b, Pang 1981 b, Foskett et al, 1983, Ogasawara & Hirano, 1984, Hasegawa et al, 1986b, Flik et al, 1986, Flik, 1989. It would appear that these data agree with this in the case of endogenously secreted prolactin but perhaps not for exogenously administered prolactin in SW fish, as observed by the responses of in situ PRL cell activity to the SW - FW transfer over the week. Others (Nagahama et al, 1973), have already demonstrated that as early as three hours after transfer the PRL cells of G. mirabilis demonstrate definite functional activation, exhibiting exocytosis of hormone granules together with development of rough endoplasmic reticulum (RER), Golgi systems and mitochondria; the cellular machinery required to manufacture prolactin. Similarly, the electron microscopic observations of Nagahama and Yomamoto (1971) and Holtzmann and Screibman (1971) show activation of the PRL cells of Oryzia latipes and Xiphorus maculatus within three and four hours respectively after transfer to FW. As a result, 24 hours was selected as the first measurement interval followed by 24 hour intervals thereafter throughout the week. Figure 10 (day 1) shows that at the light microscopic level, the PRL cells of SW-FW transfer fish (day 1-A) exhibited less granulation than the FW-FW control fish (day 1-B) 24 hours after transfer yet, the nucleoli were very prominent indicating high activation of the ribosomes. At the EM level (Figure 11), there existed proliferation of Golgi systems, and a general

increase in the number of mitochondria. Appreciable numbers of pre-secretory cytoplasmic hormone granules were not evident until day 6 or 7 (Figures 10, 11, 12). For comparison, Nagahama et al, (1973) reported secretory granule storage in Gillichthys 10 days after transfer to FW, and Ball (1969) reported that 72 hours after transfer, the PRL cells of Poecilia latipinna showed typical FW morphology. Here, the changes in the morphological correlates associated with secretory activity indicate that by day 7 of the transfer the PRL cells of SW-FW transfer fish had almost reached the steady state of the FW-FW control cells (Fryer, et al, 1988). Although there have not been any time course studies such as this on PRL cell activity in the American eel, these data are in good agreement with the works of others showing both at the light microscopic (LM) level (Ball & Baker, 1969, Olivereau, 1969, Sage & Bern, 1971) and by electron microscopy (EM) (Knowles & Vollrath, 1966a, Leatherland, 1970b, Nagahama & Yamamoto, 1971, and Holtzmann & Schreibman, 1971), that, in general, prolactin cells are more active in freshwater acclimated fish.

There is considerable debate as to whether or not the prolactin cells respond to external/internal osmotic pressures or external/internal calcium levels. Some authors report the activation of PRL cells in low calcium environments (Ball & Baker, 1963, Holtzman & Schreibman, 1971) and inactivation in high calcium or high magnesium environments in various species including, Tilapia mossambicus (Wendelaar Bonga & Van Der Meij, 1980, 1981, Wendelaar Bonga, Lowik & Van Der Meij, 1983, Wendelaar Bonga, Flik, Lowik & Van Eys, 1985), Fundulus heteroclitus (Pang, Pang & Sawyer, 1974), Anguilla anguilla

(Olivereau, Chambolle & Dubourg, 1981), and Anguilla japonica (Ogasawara & Hirano, 1984). The present observations show that the ultrastructual changes in PRL cells resulting from SW-FW transfer, were possibly in response to either external calcium or internal calcium, or both. In addition, figure 10 shows that up until day 6 prolactin cells were more active in transfer fish, about the time appreciable granulation was observed at the LM level (Figure 10). Further, influx rates were significantly higher in transfer fish on days 3 and 5 but not by day 6, along with the fact that by day 6 the plasma total calcium levels of transfer fish had increased to meet levels consistent with those of FW to FW controls. Additionally, SW-FW transfer fish experienced higher blood osmolarity 3,5, and 6 days after transfer, and, these fish were hypomagnesemic on days 2,3,4, and 7 after transfer. These data show that changes in PRL cell activity were accompanied by stimulated calcium influx rates after which the incoming calcium presumably accumulated sufficiently in the plasma to allow levels to rise to those of control fish (Figure 8). This sequence of events strongly implicates prolactin as a hypercalcemic hormone in the eel, and confirms that in the eel endogenous PRL requires at least several days to exert any effects on calcium metabolism (Bern et al, 1981).

A single injection of ovine PRL in SW eels either directly or indirectly induced an increase in calcium uptake rates (Figure 6), plasma osmolarity and plasma potassium (Figure 7) sometime between 9 and 24 hours post injection. Hypercalcemia was evident after 48 hours

(Figure 6). These data suggest that the increased calcium influx rates occurring 24 hours after ovine PRL injection were responsible for the elevated blood total calcium levels seen at 48 hours post-injections. This suggests that for these eels there appeared to be a lag time of about 24 hours before the high flux rates caused calcium to accumulate sufficiently in the plasma.

The effects of multiple ovine PRL injections on SW fish resulted in depressed PRL cellular activity and calcium influx rates, yet, at the time of measurement, plasma total calcium and sodium were significantly higher (Tables 10 and 11). Unfortunately, no similar reports similar are available in the literature. It may be that the exogenously administered PRL negatively influenced the _in situ_ PRL cells as was indicated by the morphometric measurements (Table 11) but that the injected PRL was metabolized quickly, such that, at the time of the flux measurement endogenous prolactin was also extremely low. If this were true, presumably neither exogenous or endogenous sources of PRL would have been present in sufficient amounts to support anything other than the low influx rates observed (Table 10). Indeed, ovine PRL injected into cichlid fish disappears from the plasma in 3-7 hours (Blum, 1969), and human PRL has a half life of only 15 minutes in the plasma (Miyai _et al_, 1974). In addition, in the FW eel, injections of ovine PRL for more than 20 days induced degranulation and atrophy of _in situ_ PRL cells and their nuclei suggesting that the increased circulating prolactin inhibits _in situ_ PRL cell activity

(Ball & Olivereau, 1964). Although it is assumed that the SW eel already has low endogenous circulating prolactin (Olivereau & Lemoine, 1973a, Olivereau, 1975), it could be that further depression of *in situ* PRL cell activity can account for the drop in calcium flux rates. At some point however, influx rates must have been higher in the PRL treated fish, as they did exhibit residual hypercalcemia at the time of measurement. The hypercalcemia witnessed in this experiment inspite of the absence of high influx rates does not seem implausible given that a single PRL injection induced hypercalcemia after 48 hours (Figure 6). For these multiple injection experiments the first pulse likely induced "up regulation" of receptors (Hadley, 1984) at the gill (Edery *et al*, 1984), so that subsequent injections were successful in creating hypercalcemia, and, these effects could conceivably have also remained for at least 48 hours after the final injectate had been catabolized.

d) Hypex

The final method for simulating decreases in endogenous prolactin was hypophysectomy. Hypophysectomy, removal of the pitutitary, and the source of prolactin, resulted in decreased calcium uptake rates after 10 days of hypophysectomy, (but not 5 days), but whole body calcium efflux rates were not affected (Table 2).

The success of hypophysectomy was tested by measuring plasma cortisol (Table 2) and by the observation of the pituitary itself during removal (see Materials and Methods, section 2-d). Several authors have reported significant plasma cortisol levels in *Fundulus*

grandis (Srivasta & Meier, 1972), and F. Kansae (Fleming, Brehe & Hanson, 1973), even after hypophysectomy, and it is thought cortisol plays a role in calcium uptake in trout, Salmo gairdniri (Perry & Wood, 1985, Flik & Perry, 1989). The data here (Table 2) show that in these eels plasma cortisol was essentially zero 10 days after hypophysectomy. Although this is the first report of whole body calcium influx and efflux rates for hypophysectomized eels, changes in the status of the plasma following hypophysectomy have been reported for a variety of teleosts including; killifish Fundulus kansae (Stanley & Fleming, 1966), Fundulus heteroclitus Pickford et al, 1966, Grau et al, 1984), Tilapia, Tilapia mossambicus (Dharmamba & Maetz, 1972), the eel, Anguilla japonica (Ogawa, 1977) and rainbow trout, Salmo gairdneri (Bjornsson & Hansson, 1983). Typically following hypophysectomy, FW fish experience hypocalcemia (Fontaine, 1956) and hyponatremia (Pickford et al, 1966). Whereas Table 2 illustrates that in this study, eels did not experience hypocalcemia or hyponatremia the results are still in agreement with earlier work on the European eel, A. anguilla (Fenwick & Forster 1972). Reasons for this can only be speculative at best, although it has been reported that hypophysectomy reduces urine flow rate (Clarke & Bern, 1980), with the reduction being primarily due to the loss of the diuretic effect of

PRL (Hirano, 1986). It would seem reasonable that less tubular ion reabsorption coupled with increased water retention could dilute the body fluids, but here total calcium levels were unaltered. Equally speculative is the possibility that bone calcium resorption took place, masking any hypocalcemic response that may have taken place (Moss, 1962a, Fleming, 1974). Nevertheless, the lack of change in total calcium seen here is in agreement with the work of Wendelaar Bonga (1981) on hypophysectomized Tilapia. In addition, Bjornsson & Hanson (1983) showed that in trout, hypophysectomy induced a marked drop in the ionizable fraction of calcium, while the amount of protein bound calcium rose, but total calcium remained stable. Wendelaar Bonga (1981) also reported that ionized calcium but not total calcium changed in hypophysectomized tilapia. Similarly, Srivasta and Pickford (1972) observed decreases in ionized calcium that were accompanied by such large increases in protein bound calcium that overall total calcium increased. In general, the changes in one fraction of calcium will be reflected in another, and there may be no change seen in total calcium at all (Dacke, 1979). Certainly these data would seem to support this for the American eel.

Subsequent to this, an experiment employing prolactin replacement therapy was performed whereby hypophysectomized eels received two RPD, prolactin lobe implants. Tables 8 and 9 clearly show that those eels treated with PRL replacement therapy became significantly hypercalcemic, and, plasma sodium retention was observed.

Unexpectedly, however, no significant changes in calcium uptake rates could be observed (Table 8).

In order to understand the latter result it may be of some significance that these influx rates were the lowest seen in this study. Figure 15 is a record of the variation in unidirectional calcium influx rates throughout the entire 17 months of the study, and, these particular experiments were performed at a time (March, 1987) when influx rates were, on average, very low. There is some evidence of seasonal variation in fish whole body calcium influx. Fleming, Brehe & Hanson (1973) reported variations in calcium uptake rates of Fundulus kansae and Wagner, Hampong & Copp (1985) reported an 11 day cycle for ^{45}calcium uptake in rainbow trout Salmo gairdneri although Flik et al (1985a) report no seasonal variation in calcium uptake for Tilapia. It seems reasonable to propose that the flux rates themselves were too low to detect significant changes, and that the limits of detection for this experimental design were reached.

Alternatively, the fact that PRL replacement therapy seemed to have had no effect on influx rates could be a significant result, as it is well documented that PRL cannot exert its effects on epithelial membranes, such as the gill, in the absence of cortisol (Chan et al, 1968a, Clarke & Bern, 1980, Perry & Flik, 1988, Flik et al in press). Indeed, we reported here that cortisol levels were effectively zero 10 after days hypophysectomy (Table 2), and it is known that in the eel ACTH cells degenerate upon explantation (Holmes & Ball, 1974). Furthermore most studies using fish prolactin replacement therapy actually administer pituitary "homogenates" and the effects reported

are observed within hours (Pang et al, 1973, Pang 1981). It is entirely possible that these pituitary "homogenates" contained ACTH cells with some residual activity.

The third section of this study looked briefly at the interrelationship between the pituitary and the Stannius corpuscles, as it is known that these are the two main endocrine systems involved in fish plasma calcium regulation (Pang & Pang, 1986). Hypercalcemia following Stanniectomy, removal of the corpuscles of Stannius, is one of the most consistent observations in fish endocrinology (Fenwick & Wendelaar Bonga, 1982). Attempts were made here to attribute this hypercalcemia to the action of some pituitary factor, presumably prolactin.

Stanniectomy resulted in frank hypercalcemia as early as five days following Stanniectomy and this persisted up to 56 days after Stanniectomy (Figures 2, 13). Hyponatremia and hyperkalemia were also observed, (Figures 3 and 14), and this is in agreement with the results of Chester Jones et al (1965), Fontaine (1967), and Fenwick & Forster (1972). Other authors (Chan, 1969, Chester Jones et al, 1969) report a return to normocalcemia between four and six weeks post-Stanniectomy. This was not tested in the present study (Figures 2 & 13).

Reports indicate that Stanniectomy results in hypertrophy of the chloride cells of the gills (Chartier et al, 1977) and stimulation of branchial calcium influx in isolated, perfused eel gills (Fenwick & So, 1974). The hypercalcemia following Stanniectomy observed in this

study was also accompanied by enhanced unidirectional calcium influx rates at all times post Stanniectomy, except 14 days, (Figure 2). The effects on whole body efflux rates were inconsistent (Figure 13). Unidirectional calcium influx and efflux rates following Stanniectomy in the American eel have not previously been reported.

Presumably, the hypercalcemia and increased flux rates observed after Stanniectomy are under the control of one or more of the calciotropic hormones, possibly prolactin. Figure 2 shows that significantly high influx rates are accompanied by generally low prolactin cell activity at five and twenty-one days post-Stanniectomy as indicated by morphometric analysis (Benjamin, 1974 a,b, Batten & Ball, 1976, Olivereau & Olivereau, 1983, Fryer, Valentine & Tikkala, 1988). This data agrees with a report on euryhaline sticklebacks, Gasterosteus aculeatus (Wendelaar Bonga & Greven, 1978) who also reported lower PRL cell activity, as well as decreased prolactin synthesis and release after 16 days Stanniectomy.

Figure 13 shows the effect of Stanniectomy on whole body efflux rates. Efflux rates in the eels were about 100 fold lower than influx rates (Figure 2), and Stanniectomy had no consistent effect on efflux. Very few studies have been performed determining the influx and efflux rates of fish, and as yet there are no published studies that report efflux values for the American eel. Values are available for rainbow trout and Tilapia (for review see Fenwick, in press), and they are comparable to influx rates of the eel. The method used here to

determine efflux rates in the eel has been previously validated for Oreochromis mossambicus (Flik et al, 1985a) and was recently employed in the eel (Flik, Fenwick, & Wendelaar Bonga, in press). In general, efflux rates in sham-stanniectomized and stanniectomized eels are approximately 100 times lower than reported efflux rates for other intact fish species; Salmo gairdneri (Hobe et al, 1984b, Perry et al, 1988, Perry and Flik, 1988) and Oreochromis mossambicus (Flik et al, 1985a).

In addition to these data, we reported earlier (Table 2) that 10 day hypophysectomized eels showed significant decreases in calcium uptake rates. Further, observations of hypophysectomized plus stanniectomized American eels showed these fish exhibited no changes in unidirectional calcium influx rates or changes in any plasma parameters measured (Table 3). Together these results provide several conclusions. Firstly, that the hypercalcemia and enhanced flux rates in stanniectomized eels could at times be accompanied by suppressed PRL cell activity. Secondly, that hypophysectomy reduced unidirectional calcium influx although it did not effect whole body efflux rates (Table 12) or plasma total calcium. Thirdly, although hypophysectomy plus Stanniectomy had no effect on any parameter measured. These results suggest that the increases in calcium influx and hypercalcemia following Stanniectomy are contingent upon the presence of an intact pituitary gland.

Whereas some pituitary factor, possibly prolactin, is necessary for post-Stanniectomy hypercalcemia and high influx rates, the data of Figure 2 and of others (Wendelaar Bonga & Greven, 1978) indicate that

following Stanniectomy PRL cell activity is depressed. This seems an indication that in the stickleback and possibly also in the American eel prolactin secretion or at least prolactin cell activity is inhibited by high levels of plasma total calcium.

Indeed, these same authors (Wendelaar Bonga & Greven, 1978) took sham stanniectomized and stanniectomized eels adapted to sea water and transferred them to lower calcium freshwater. In the sham-operated eels, plasma calcium levels were lowered slightly and PRL cell activity was high as in normal unoperated sticklebacks. In the stanniectomized fish, plasma calcium levels were high and yet the PRL cellular activity was also unexpectedly high. Despite high internal calcium concentration the prolactin cells appeared to react preferentially to the lower external calcium levels (Wendelaar Bonga & Greven 1978). These authors and others (Nagahama et al 1975) have proposed a dual control system of prolactin cell activity in teleosts involving both inhibiting nervous input and direct effects of plasma factors on these cells. Likely external calcium is the source for the hypercalcemia as seen in sticklebacks even in calcium poor media (Wendelaar Bonga & Greven, 1978). It is known that following Stanniectomy in eels there occurs hypertrophy of the chloride cells of the gills (Chartier et al, 1977) as well as stimulation of branchial calcium influx (Fenwick & So, 1974). It is also now known that prolactin is hypercalcemic in fishes (Pang et al, 1981b, Wendelaar Bonga & Flik, 1982), and that PRL stimulates branchial calcium uptake

from the water and the high affinity Ca^{2+}-ATPase enzyme (Flik et al, 1984).

The fact that Wendelaar Bonga and Greven (1978) measured low PRL cell activity in stanniectomized sticklebacks led them to question the contribution by prolactin to the hypercalcemia. However, this measurement was taken only at 16 days post-Stanniectomy whereas the data of figure 3 gives morphometric measurements of PRL cell activity at 5,10, 14, 21, and 56 days thereafter, and, in fact, PRL cell activity was not always significantly lower in Stanniectomized eels. It appears possible that in the present study prolactin was influenced by the so-called dual control mentioned by Nagahama et al,(1975) and may be responding to the lower external calcium.

The best interpretation of these data is that the post-Stanniectomy hypercalcemia and higher influx rates in FW adapted American eels can be accounted for by both the action of prolactin and the removal of hypocalcin (from the Stannius corpuscles) indicating a possible cooperative relationship between these two hormones in the minute to minute regulation of calcium metabolism in the American eel. It has been well documented that hypocalcin acts to prevent hypercalcemia (Fenwick, 1985). Indeed, it now appears that the primary target organ for hypocalcin is the gill (Fenwick, in press) but although it inhibits gill calcium uptake (Fontaine et al, 1972, Fenwick, 1974, Milet et al, 1975, Fenwick, 1982), it has not been shown to affect the high affinity Ca^{2+} ATPase (Fenwick, in press). Furthermore, the activity of the cells of the corpuscles of Stannius are directly related to the external calcium concentrations and hence

internal calcium levels (Cohen et al, 1975, Wendelaar Bonga et al, 1976, Olivereau & Olivereau, 1978). The fact that in FW PRL cells are extremely active (Ball & Ingleton, 1973, Nagahama et al, 1973, Wendelaar Bonga & Van Der Meij, 1983, Olivereau, Chambolle & Dubourg, 1981, Wendelaar Bonga, Lowik & Van Der Meij, 1980, Olivereau & Olivereau, 1983), and the cells of the Stannius corpuscles are less active (Pang & Pang, 1974, Cohen et al, 1975, Wendelaar Bonga, 1980) presumably indicates that the levels of both prolactin and hypocalcin in the blood and the antagonism between them gives stability to plasma total calcium. Therefore, removal of the corpuscles of Stannius creates a stituation in which calcium metabolism is regulated in the absence of the hypocalcemic control from hypocalcin. Meanwhile, although PRL cellular activity continues to be regulated by the hypothalamus (Peter & Fryer, 1983), as well as blood by prolactin levels (Nagahama et al, 1974), and ambient calcium levels (Wendelaar Bonga & Van Der Meij, 1980, 1981), the "system" lacks acute stringent hypocalcemic control in the absence of hypocalcin. It does not seem unreasonable to propose then, that the fact that PRL cell activity is not consistently lower at all intervals post-Stanniectomy is due to the so-called dual control of PRL cells whereby at times external calcium encourages prolactin cell activity, while at other times cellular activity is suppressed by the typical post-Stanniectomy hypercalcemia.

Argueably, some other hypercalcemic factor may be responsible for, or at least support, the post-Stanniectomy hypercalcemia. In this respect the most likely candidate is cortisol. Perry & Wood

(1985) report that cortisol exerts a hypercalcemic effect through differentiation and conversion of gill stem cells to chloride cells, a process requiring several days (Laurent et al, 1985). Indeed, Leloup-Hatey (1970b) showed that in stanniectomized eels plasma cortisol levels are very high one week post Stanniectomy and very low at six weeks. And, recently Flik & Perry (1989) demonstrated that in trout, cortisol stimulates branchial calcium uptake and induces hypercalcemia. On the other hand, Flik, Fenwick & Wendelaar Bonga (in press) observed that even though ovine PRL treatment in eels raised cortisol levels, prolactin, not cortisol, stimulated branchial calcium uptake and incuding hypercalcemia. Finally, there is another hypercalcemic factor in the fish pituitary first named hypercalcin (Parsons et al, 1978). However, it has shown only acute effects on fish plasma calcium levels (Pang & Yee, 1980) and its identity is not yet known (Parsons et al, 1978, Ball et al, 1982a).

It is unclear why there is no immediate hypocalcemic compensation post-Stanniectomy by the putative hypocalcemic factor calcitonin, although its exact physiological function in teleosts has yet to be uncovered, and data are contradictory (Fouchereau-Peron et al, 1986, 1987). Further, several authors have questioned its involvement at all in teleost hypocalcemic regulation (Wendelaar Bonga, 1980, Lafeber & Perry, 1988), and these present data seem to support this contention.

Lastly, one must keep in mind that the removal of the CS alters calcium exchange at sites other than the gills (Fleming 1967), so that some of the effects reported here might devolve from effects on the gut and kidney. However, these effects would probably be minimal as

the fish were not fed, and, as it was reported that the renal excretion of calcium is not increased following Stanniectomy (Fenwick, 1974).

Finally, following hypophysectomy, the fish is faced with having to regulate calcium in the absence of the source of prolactin. In contrast to the data following Stanniectomy, no changes in plasma total calcium were seen after hypophysectomy. This may suggest that the cells of the corpuscles of Stannius have more rigorous auto-regulation than do PRL cells and they may regulate calcium metabolism more acutely. As an added test, prolactin lobe replacement therapy of hypophysectomized plus stanniectomized eels resulted in no alterations in any parameter measured (Tables 8 and 9). As in the case of RPD homograft therapy of hypophysectomized eels, these experiments were performed at a time when flux rates were very low in general (Figure 15), possibly too low to detect significant changes.

An underlying theme during the study involved monitoring parameters measured regularly during the study (Figures 15 & 16). Month to month changes in the more frequently measured parameters were performed (influx rates and plasma ions) in order to avoid scheduling experiments during times when these parameters were possibly unstable. In keeping with this, all experimental methods include the dates that the work was done so that reproducability by other investigators is feasible.

These data (Figure 15) indicate that although there were variations in influx rates and calcium levels between certain months many of them were statistically insignificant (one way ANOVA).

Secondly, the peaks of influx and blood total calcium seen in July represent data from a new population of fish. Moreover, the calcium levels from September to December of 1986 are not significantly different from those during August to December of 1987. By June of 1987 this first population of eels had been in the lab and unfed for 11 months, and their calcium levels were significantly lower than those of 7 and 8 months earlier. Possibly the feeding state of these fish is responsible for the variations seen. Other ion data is presented in Figure 16.

It is suggested that the variations that occur in flux rates and plasma calcium are due to changes in both the feeding states of the eels and acclimation state of the fish to the laboratory conditions. Eels harvested from the wild certainly must have metabolic rates different from fish that had been kept unfed in the lab for almost one year (Tesch, 1977). In support of this, the data in Figure 15 shows that the group of flux rates from the first population of fish in September of 1986 are not significantly different from those of the second population during September 1987. It appears that after having been unfed for two months this second population of fish also exhibited depressed flux rates.

In addition, although the mean calcium levels in July were significantly higher than those from January to June of 1986 they were not significantly higher than those from September to December of 1986 nor were they different from those of August to December of 1987. Furthermore, the calcium levels from September to December of 1986 were not signficantly different from those during August to December of 1987. By June of 1987 this first population of eels had been unfed

for 11 months and their calcium levels were significantly lower than those of 7 or 8 months earlier. This too supports the likelihood that the feeding state may be responsible for any variations seen.

The fact that these parameters (Figures 15 and 16) underwent variations whether physiological, artifical or otherwise shows the importance of properly controlling all experiments. One must keep in mind that any physiological parameter can fluctuate due to circadian, seasonal or developmental influences. For example, several investigators reported variations in the whole body calcium influx rates of Fundulus kansae (Fleming, Brehe, and Hanson, 1973) and Salmo gairdneri (Wagner, Hampong & Copp, 1985) whereas no changes have been reported for Tilapia mossambicus (Flik et al, 1985a). Ion levels may also undergo seasonal changes; copper, zinc, magnesium and calcium levels accumulate in the gonads and liver of the female winter flounder (Pseudopleuroneites Americanus) in the summer, in preparation for winter gonadal development (Fletcher & King, 1978). In addition, Wagner & McKeown (1985) report a four week cyclical growth cycle in juvenile Salmo gairdneri.

Of significance to this study are the findings of various authors that plasma prolactin levels have been known to fluctuate in several teleosts. Diurnal variations in serum prolactin are reported for C. auratus (Leatherland & McKeown, 1973; McKeown & Peter, 1976), Onchorhynchis nerka (Kokanee Salmon - Leatherland, McKeown & John, 1974) and Poecilia latipinna, (Batten & Ball, 1976). Further, seasonal variation in the circadian rhythms of prolactin were reported in M. cephalus (Spieler et al, 1976), and, in Fundulus grandis changes

in the length of the daily photoperiod altered the onset of the serum prolactin peak (Spieler, 1975). Finally, annual cycles of PRL cell activity have been reported in G. aculeatus (Leatherland, 1970a), showing that long photoperiods stimulate prolactin secretion.

Evidence here and elsewhere point out the need to be aware of fluctuations in physiological parameters and in the endocrine factors that exert influence over these parameters. One cannot dispute the fact that keeping fish unfed more than likely hampers the typical physiology of the animal. Finally, although the fluctuations presented here may not be exhibited in the wild, they exist nonetheless and must be accounted for prior to any interpretation of results.

In conclusion, this study has provided considerable evidence implicating prolactin in the hypercalcemic control of calcium homeostasis in the American eel. Through various methods (Stanniectomy, PRF treatments, PRL lobe homografts, ovine PRL injections and infusion, and SW to FW transfer) attempts were made to simulate increases in endogenous PRL and to expose eels to exogenous sources of PRL. Conversely, other means were utilized (hypophysectomy, PIF treatments, and adaptation to SW) in order to simulate decreases in endogenous PRL. In addition, several accepted physiological correlates associated with the calciotropic actions of fish calcium regulating hormones including unidirectional calcium influx and efflux rates, blood total calcium, plasma osmolarity, sodium, potassium and total magnesium, PRL cell cytology and morphology, and the monitoring of internal calcium pools were measured

in an attempt to quantify the calciotropic actions of prolactin in eels.

On more than one occasion data has been presented in which changes in PRL cell activity (and presumably the presence of prolactin in the blood), and cytology, were associated with appropriate changes in either calcium influx rates and/or plasma total calcium. It is now possible to state quite categorically that these data point to the importance of prolactin in calcium homeostasis in the eel, and this must be considered in light of the role it has played throughout vertebrate evolution.

It does not therefore seem to imprudent to suggest that prolactin is a hypercalcemic hormone in the American eel; and that post-Stanniectomy hypercalcemia and the attending high calcium uptake are partially supported by prolactin, or are at least contingent upon the presence of the pituitary. A presumed decrease in endogenous prolactin following hypophysectomy or PIF treatment resulted in low calcium influx rates, and, PIF treatment was associated with low prolactin cell activity. Exposure of SW acclimated eels to the lower calcium FW environment showed that these fish exhibited gradually increased PRL cell cytology (at the light and electron microscopic level) and activity (as measured morphometrically), and as well, previously low calcium influx rates concomitantly rose to meet those of control fish over a one week test period. Exogenous, homologous, or heterolous PRL whether administered by infusion, injection or through the installation of RPD implants, elevated plasma total calcium and calcium influx rates, and profoundly decreased whole body efflux rates. In SW fish, a single PRL injection affected an increase

in calcium uptake within 24 hours, subsequently producing hypercalcemia at 48 hours post-injection. Finally, evidence here (Table 14) and elsewhere (Flik et al, 1986) suggest that fish cellular and acellular bone are amongst the target organs for the calciotropic action for prolactin. Indeed, it seems possible that fish bone is a more important source of readily mobilizeable calcium fraction than has previously been suggested.

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