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The Effect of Calcitonin and Oesophageal Ligation on Total Animal Calcium Accumulation and Plasma Ion Levels in the American Eel, *Anguilla rostrata* LeSueur

written by

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A Thesis presented to the
University of Ottawa in partial fulfillment
of the requirements for the degree of
Master of Science
in the Department of Biology

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D.L. Kingsbury

A C K N O W L E D G E M E N T S

I owe an unpayable debt of gratitude to my supervisor, Dr. James C. Fenwick, a man who listens, advises and cares.

Thanks also go Steve Clarke for productive bull sessions and especially for help with the drudgery of weekend and weekday experiments. My computer will miss your voice.

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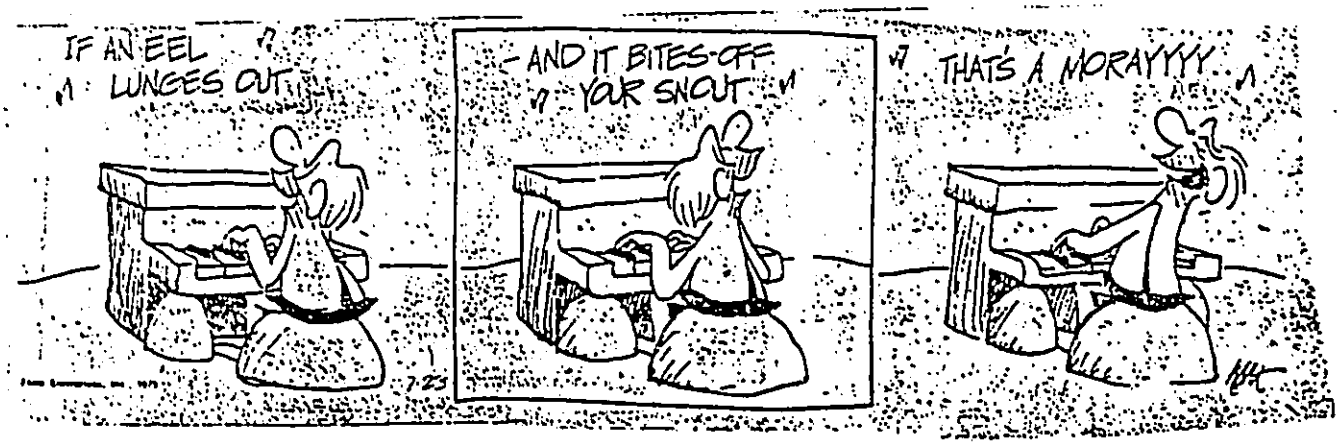

A B S T R A C T

The effect of calcitonin (CT) and oesophageal ligation on total body calcium accumulation and plasma ion levels were studied in the American eel, Anguilla rostrata.

Calcitonin reduced total body calcium accumulation in freshwater eels rapidly transferred to seawater in a manner which does not involve the modification of the drinking response.

Plasma total calcium, sodium, potassium and magnesium concentrations varied sporadically and did not always correspond with changes in calcium accumulation.

Light microscopy of ultimobranchial tissue from freshwater fish acutely transferred to seawater showed the presence of large, ovoid, membrane bound structures in each cell.

Overall, the conclusions of this study are:

1. Calcitonin caused a reduction in total animal calcium accumulation in intact fish.
2. This reduction is not a result of decreased ingestion of the ambient medium.
3. Transfer from freshwater to 70% seawater incurs morphological changes in the ultimobranchial tissue of eels.
4. Plasma total calcium changes are a poor index of overall calcium regulation in eels.

R É S U M É

L'effet de la calcitonine (CT) et la ligature d'oesophage sur l'accumulation de calcium dans le corps totale et les concentrations des ions de la plasma a été étudié dans l'anguille d'Amerique, Anguilla rostrata L.

La calcitonine est réduit l'accumulation de la calcium dans le corps totale dans les anguilles d'eau douce a transféré rapidement à l'eau de mer dans un façon ce ne fait pas impliqué une modification de la réponse de boire.

Les concentrations de la plasma des calcium, des sodium, des potassium et des magnesium sont variés mais ils ne s'assortissent pas les changes dans l'accumulation de la calcium.

La microscopique du tissu ultimobranchial des poisson d'eau douce a transféré rapidement à l'eau de mer a indiqué la presence des grandes structures dans cettes cellules.

Les conclusions de cettes étude sont:

1. La calcitonine a causé une reduction dans l'accumulation de la calcium dans la corps totale dans les anguille.
2. Cette reduction n'est pas un résultat d'un modification de la réponse de boire.
3. La déplacement des anguilles d'eau douce à l'eau de mer a résulté dans les changes dans les cellules de la corps ultimobranchial.

4. Les changements du calcium totale de plasma ne sont pas un bon indicateur de la regulation totale de la calcium dans les anguilles.

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I N T R O D U C T I O N

Fish maintain very precise homeostatic control over their plasma calcium concentration (Chan and Chester Jones, 1968; Fenwick and Forster, 1972; Dacke, 1979), but how this control is effected is not clear. Unlike land based vertebrates which rely on the exchange of plasma calcium with internal calcium stores accumulated while feeding for the maintenance of plasma calcium levels (Fenwick and Wendelaar Bonga, 1982), fish use the virtually limitless supply of calcium dissolved in the aquatic media as both a source and sink for this ion (Taylor, 1985). It is well known that fish can accumulate calcium from the environment (Mashiko and Jozuka, 1964; Berg, 1970; Simmons, 1971) by actively absorbing this ion across their gills (Simmons, 1971; So and Fenwick, 1977; Mugiya and Ichii, 1981; Perry and Wood, 1985). This mechanism is so powerful that in some fish the calcium taken up through the gills can supply up to 70% of the calcium required for growth (Flik et al., 1986) and is even used in preference to ingested calcium (Berg, 1970).

The uptake of calcium appears to be enhanced, or at least supported, by two hormones, cortisol (Flik and Perry, 1988) and prolactin (Fenwick, 1982). Cortisol, the primary adrenosteroid in fish (Hadley, 1984), increases chloride cell number and the size of immature chloride cells (Foskett et al., 1983). Further, under conditions of low environmental [Ca^{+2}], plasma cortisol

concentrations, calcium influx rate and levels of high affinity Ca^{+2}-ATPase all increase (Perry and Wood, 1985).

Prolactin, produced in the pituitary, affects reproduction and osmoregulation of fish (Hadley, 1984). Hypophysectomy results in a hypocalcemic state related to the degree of calcium deficiency in the surrounding water (Fenwick and Wendelaar Bonga, 1982) but this response lags several days behind the surgery and is transient in nature (Pang et al., 1978). Injections of prolactin or pituitary extracts produce a log-dose related hypercalcemia and hypernatremia (Pang et al., 1978; Pang et al., 1986), actions which indirectly promote bone mineralization (Flik et al., 1986). The mechanism by which prolactin causes changes in plasma calcium is thought to be related to alterations in gill permeability and actions on the integument (Fenwick and Wendelaar Bonga, 1982).

Two hormones may be involved in reducing calcium uptake. One of these, the most rigorously established calcitropic hormone in fish, is variously referred to as hypocalcin (Pang and Pang, 1986), teleocalcin (Ma and Copp, 1978), parathyrine of the corpuscles of Stannius (Milet, 1980) or simply the protein of the corpuscles of Stannius (Lafeber et al., 1987). Removal of the corpuscles results in increases in plasma calcium and ^{45}Ca uptake leading to the conclusion that hypocalcin exerts an antihypercalcemic action by reducing branchial calcium uptake (Fenwick and So, 1974; Pang et al., 1980; Fenwick and Wendelaar Bonga, 1982).

The other possible hypocalcemic calcitropic hormone is calcitonin (CT). Numerous studies have determined that calcitonin is produced in the ultimobranchial tissue of "lower" jawed vertebrates and in the homologous parafollicular C-cells of the thyroid gland in mammals (Copp et al., 1967; Pang et al., 1971b; Orimo et al., 1972; Otani et al., 1976; Dacke, 1979; Sasayama et al., 1984). In fish, ultimobranchial glands (UBBs) are found only in the gnathostomes (Robertson, 1986) and consist of two types of cells (Yamane, 1978; Robertson, 1986), granulated, which immuno-react with antisera to calcitonin (McMillan et al., 1976) and non-granulated, which probably function as support cells (Robertson, 1986).

All natural calcitonins isolated to date have 32 amino acids (Queener and Bell, 1975; Gorbman et al., 1983) and a 1-7 disulfide bridge (O'Dor et al., 1969; Noda and Narita, 1976; Homma et al., 1986). While the nucleotide sequence is not always consistent (Queener and Bell, 1975), six of the first nine amino acids within the bridge are highly conserved (Orlowski et al., 1987). However, no specific active sequence is known (Hadley, 1984), rather the ability of the molecule to flex and form helices is now thought to be essential for activity (Epand et al., 1986).

Two types of calcitonin specific receptors have been described in fish gills (Fouchereau-Peron et al., 1981), a high affinity-low capacity receptor and a low affinity-high capacity receptor. Binding at both of these receptors is time and

temperature dependent. A high affinity-low capacity receptor has also been demonstrated on trout bone (Warshawsky et al., 1980; Arlot-Bonnemains et al., 1983). These studies indicate that the effect of the hormone on calcium homeostasis may be exerted on one or both of these tissues.

Spawning migrations of fish can encompass long distances and, in the cases of catadromy and anadromy, a complete change in external ionic composition and osmotic concentration. Anadromy, the migration from freshwater to seawater, is performed by many species, including the American eel, Anguilla rostrata. Within a few hours of transfer from freshwater to seawater there is an overall increase in the concentration of body fluids (Bath and Eddy, 1979) and the calcitonin content of both the ultimobranchial body (UBB) and plasma increase (Fouchereau-Peron et al., 1986). Within the same time frame, no changes in plasma [Ca^{+2}] occur (Fouchereau-Peron et al., 1986). On the one hand, this might indicate that the synthesis and release of calcitonin may be maintaining body calcium concentrations. But the data does not rule out the possibility that the UBB was responding to the other changes.

Further evidence, however, favors the conclusion that CT effects calcium homeostasis. That is, following partial ultimobranchialectomy, goldfish transferred from freshwater to 30% seawater showed increased plasma calcium compared to those normally associated with transfer of intact fish (Fenwick, 1975). In addition, ultimobranchialectomized European eels (Anguilla

anguilla), maintained in Ca^{+2} rich water, exhibited higher serum calcium concentrations than control eels (Lopez et al., 1976). Thus, it appears that calcitonin in fish acts to prevent calcium loading during a hypercalcemic stress.

However, studies involving the injection or infusion of various exogenous calcitonins in different species of fish have produced inconsistent results on plasma calcium concentrations (Table 1) casting some doubt on the role and importance of CT in teleosts. For example, data has been published which concluded that calcitonin can be hypocalcemic (Chan et al., 1968; Mathur, 1979; Glowacki, 1985), hypercalcemic (Wales, 1984; Glowacki, 1985; Fouchereau-Peron, 1987) or have no effect (Pang, 1971b; Hirano et al., 1981; Fouchereau-Peron, 1986) on plasma calcium concentrations. Another study showed that delivery of CT via an intra arterial cannula was ineffective in producing hypocalcemia in either freshwater or seawater acclimated eels (Hirano et al., 1981). Injection into the ventral venous vessels of trout and eels has proven more likely to generate a response (Wales, 1984; Fouchereau-Peron et al., 1987). Perhaps the best example of the ambiguous effect of CT in fish is that whereas Chan et al. (1968) found a clear hypocalcemic response to 50 MRC mU[1] of porcine

[1] An MRC unit is a Medical Research Council milli-unit and is equivalent to the biological activity of 5 nanograms of porcine calcitonin as established by the Division of Biological Standards of the National Institute of Medical Research, Mill Hill, England. [Copp, D.H., 1970. Endocrine Regulation of Calcium Metabolism. Ann. Rev. Physiol. 32:61-86.]

Table 1: Different responses to calcitonin on blood calcium concentrations in fish and sharks.

Species	Type of CT	Effect on Blood Ca^{+2}	Reference
Ictalurus melas (catfish)	porcine	hypocalcemia	Louw et al., 1967
Anguilla anguilla (European eel)	porcine	hypocalcemia	Chan et al., 1968
Anguilla anguilla (European eel)	salmon	hypocalcemia	Lopez et al., 1976
Channa punctatus (Indian murrel)	porcine	hypocalcemia	Mathur, 1979
Anguilla anguilla (European eel)	salmon	hypocalcemia	Wales, 1984
Anguilla anguilla (European eel)	salmon	hypocalcemia	Wales and Barrett, 1983
Carassius auratus (goldfish)	salmon	hypocalcemia	Wales and Barrett, 1983
Paralabrax clathratus (kelp bass)	salmon	hypocalcemia	Glowacki et al., 1985
Anguilla rostrata-FW (N.A. eel)	salmon	hypocalcemia	Pang, 1971a
Periophthalmodon schlosseri (mudskipper)	eel	anti-hypercal-cemic	Fenwick and Lam, 1988
Fundulus heteroclitus (killifish)	porcine	none	Pang and Pickford, 1967

Table 1 continued

Species	Type of CT	Effect on Blood Ca^{+2}	Reference
Paroderma africanum(G.) (lazy shark)	porcine	none	Louw, 1969
Galeorhinus guleus(L.) (school shark)	porcine	none	Louw, 1969
Squalus acanthias (elasmobranch)	salmon	none	Hayslett et al., 1972
Lepidosiren paradoxa (South American lungfish)	salmon	none	Pang and Sawyer, 1975
Anguilla japonica (Japanese eel)	eel	none	Yamauchi et al., 1978
Gadus morhua L. (Atlantic cod)	salmon	none	Bjornsson and Deftos, 1985
Anguilla japonica (Japanese eel)	eel	none	Hirano et al., 1981
Salmo gairdneri (rainbow trout)	salmon	none	Fouchereau-Peron et al., 1986
Heteropneustes fossilis (catfish)	mammal	none	Suryawanki and Mahajan, 1976
Gasterosteus aculeatus (sticklebacks)	salmon	none	Wendelaar Bonga, 1980

Table 1 continued

Species	Type of CT	Effect on Blood Ca^{+2}	Reference
Fundulus heteroclitus (killifish)	porcine, salmon, cod	none	Pang, 1971b
Ictalurus punctatus (catfish)	porcine, salmon	none	
Oncorhynchus kisutch (salmon)	salmon	none	
Anguilla rostrata-SW (N.A. eel)	salmon	none	
Anguilla anguilla (European eel)	salmon	hypercalcemia	Wales, 1984
Triakis semifasciata (leopard shark)	salmon	hypercalcemia	Glowacki et al., 1985
Salmo gairneri (rainbow trout)	salmon	hypercalcemia	Fouchereau-Peron et al., 1987
Chelon labrosus (grey mullet)	salmon	hypercalcemia	Fouchereau-Peron et al., 1987
Anguilla anguilla (European eel)	salmon	hypercalcemic	Wales, 1984

calcitonin per 100 grams in freshwater acclimated European eels, Dacke (1972) could not duplicate the results even though he worked in the same laboratory with the same species of fish and hormone, even when he increased the dose to 200 MRC mU 100 g^{-1} of eel. In the present study, administration of calcitonin was accomplished by intraperitoneal injection, a method which has previously proven successful (Fenwick and Lam,1988).

Other explanations besides diverse delivery methods rationalize the discrepancy in results. Glowacki (1985) postulates that the dissimilar results are due to the various types of calcitonins used, as well as the different durations of treatment and various acclimation conditions employed. The observation that sharks respond to exogenous CT by becoming hypercalcemic, and not hypocalcemic as expected, led him to conclude that there may be multiple types of calcitonin receptors in fish, each responding differently to CT injections (Glowacki, 1985).

Variations in the dose given to precipitate certain responses suggests that, in seawater acclimated trout, the high affinity calcitonin receptors may respond at low, physiological, doses of CT to produce hypercalcemia and that the low affinity receptors are stimulated at high, pharmacological, doses to produce hypocalcemia (Fouchereau-Peron, 1987). However, in seawater acclimated eels, the opposite dose effect was obtained by Wales and Barrett (1983) and Wales (1984) suggesting that species differences may account for some of the variable responses. Changing the concentration of Ca^{+2} in the external

medium and the resultant endogenous CT levels (Fouchereau-Peron et al., 1986) may also be responsible for differing degrees of receptor saturation and sensitivity to exogenous calcitonin (Wales, 1984).

In both the Wales (1984) and Fouchereau-Peron (1987) studies the calcitonin employed was sequenced from salmon. Salmon calcitonin (sCT), at least in mammals, is the most biologically potent form of the hormone known (Otani et al., 1976) probably because it has a longer half life in vivo due to less susceptibility to tissue and plasma enzyme degradation than other CTs (Hadley, 1984). While injection of sCT into many species of fish has produced hypo- and hypercalcemia as well as no effect (see Table 1), the administration of homologous calcitonin in eels has consistently produced little or no effect on their plasma calcium concentration (Yamauchi et al., 1978; Hirano, et al., 1981). Nevertheless, to increase the physiological nature of the experiments in the present study, and because a new parameter was studied, synthetic eel sequence calcitonin was used.

The myriad of results obtained from research measuring plasma calcium changes after treatment with calcitonin indicates that this parameter is a poor index of calcium regulation in teleosts. For this reason, and because the major component of calcium uptake occurs through the gills, the present study measured in vivo calcium accumulation changes to explore the function of calcitonin. This approach seemed eminently reasonable given the fact that isolated gill perfusion studies done to

assess the effect of CT (Milhaud et al., 1977; Peignoux-Deville et al., 1978; Milet et al., 1979) showed consistently reduced in vitro calcium influx. Milet et al., (1979) reported that gill calcium influx in eels, Anguilla anguilla, was inhibited 39% in sham operated controls after sCT infusion, while it decreased only 26% in ultimobranchialectomized fish. Conversely, efflux increased 47% in the shams and 80% in the UBX eels after sCT infusion, leading the authors to conclude that calcitonin works primarily on the efflux, and not influx, mechanisms to produce hypocalcemia. However, this study involved isolated gill preparations, a model from which conclusions relevant to the intact fish are tenuous at best.

Thus, despite the various methods of exogenous CT administration and isolated gill experiments that have been used to measure the effects of calcitonin in fish, none has proven adequate to correlate changes in gill calcium flux and plasma calcium concentrations with the administration of calcitonin. Experimental emphasis in fish endocrinology has recently begun to shift towards more physiological whole-animal flux studies which have proven valuable in studies involving the measurement of calcium influx as well as in experiments involving cortisol and prolactin. Whole body calcium influx has been determined in eels (Fenwick and Leung, 1981) and tilapia, Oreochromis mossambicus, (Flik et al., 1985) and has been used as a reliable bioassay for the effect of teleocalcin in trout (Wagner et al., 1986; Wagner et al., 1988) and American eels (Wagner et al., 1989). Branchial

calcium uptake after administration of cortisol in intact rainbow trout, Salmo gairdneri (Perry and Wood, 1985; Flik and Perry, 1988) and the effects of administration of exogenous ovine prolactin (PRL) in intact tilapia resulting in data outlining changes in [Ca^{+2}] in several body compartments (Flik et al., 1985) has allowed the drawing of broad physiological conclusions as to the function of the hormones being investigated.

Theoretical analysis of a hypocalcemic and calcium accumulation reducing role for calcitonin suggests that this hormone would be useful when fish travel to more hyperosmotic and hypercalcemic environments as occurs during anadromous migration. Preliminary research done as a fourth year honors research project (Kingsbury and Fenwick, to be published elsewhere) involved the investigation of exogenous eel calcitonin administration on whole body calcium accumulation and plasma total calcium, sodium, potassium and magnesium concentrations in American eels, Anguilla rostrata. Whereas calcitonin had no effect in freshwater acclimated eels, it reduced calcium influx by 50% in seawater acclimated eels and by an average of 74% in freshwater eels transferred to 70% seawater (see Appendix A). Plasma calcium concentrations were unchanged following CT injection in both the transfer and seawater acclimated fish (see Appendix A). The lowest dose (0.02 μg CT/fish) in freshwater eels elicited a hypercalcemic response, a finding which concurs with those of Fouchereau-Peron et al., (1987).

This apparent reduction in calcium accumulation in transfer

and seawater acclimated fish following treatment with CT, which was not matched with any change in plasma calcium, was either due to the action of calcitonin on branchial calcium uptake or perhaps on calcium ingestion, as even a small reduction in drinking would greatly affect accumulation. Salmon calcitonin is known to affect feeding patterns in rats (Levine et al., 1981; Fargeas et al., 1985) and suppress gastric emptying in humans (Jonderko et al., 1987). As well, both bolus injections and constant infusion of calcitonin were found to stimulate intestinal absorption of calcium in rats by increasing the circulating levels of 1,25 $(OH)_2$ Vitamin D (Jaegar et al., 1986). In dogs, salmon CT decreased the amount of water and sodium absorbed in the gut (Primi and Bueno, 1986), but, in stripped non-everted eel intestines, CT had no effect on calcium or water absorption (Nakamura and Hirano, 1986).

Acclimation of euryhaline fish to seawater begins with the immediate commencement of drinking (Hirano, 1974) to replace water lost due to the osmotic gradient present in seawater, a behaviour which is thought to be triggered by an increase in plasma osmolality (Sharratt et al., 1964; Maetz and Skadhauge, 1968), Cl^- ions (Hirano, 1974) or local dehydration (Kirsch and Mayer-Gostan, 1973; Hirano et al., 1978). The maximum drinking rates in acclimating eels are seen 4-7 days after transfer from freshwater to seawater (Hirano, 1974) with the peak of water ingestion occurring 5 days post-transfer (Oide and Utida, 1967). Body weights decrease by 15% by day 2 but recover by day 7 post-

transfer (Oide and Utida, 1967) and, once stabilization of drinking rates occurs after 2-3 weeks (Hirano, 1974), 75% of the water drunk by a seawater fish is absorbed in the intestine (Oide and Utida, 1968).

Drinking is one of the behaviours known to be hormonally activated in fish (Takei et al., 1979; Malvin et al., 1980) and mammals (Epstein, 1980). Angiotensin II (AII) is a specific and potent activator of drinking in fish (Hirano et al., 1978; Kobayashi et al., 1983; Hirano and Hasegawa, 1984). The ingestion of water upon encountering a more hypertonic medium, in response to AII, occurs in two stages, an initial acute phase proportional to the [Cl^{-1}] followed by an adjustment phase dependent on the volume regulatory ability of the fish (Hirano et al., 1978). The effect of angiotensin on drinking lasts only as long as the pressor effect of the drug on the ventral aorta indicating that baroreceptors or volume receptors on the afferent side of the branchial artery may be involved in the control of AII stimulated drinking in fish (Hirano and Hasegawa, 1984). This mechanism may also be important as an emergency reaction to dehydration stress (Kobayashi et al., 1983).

Only Hirano and Hasegawa (1974) addressed the impact of calcitonin on drinking rates in fish and found that 50-100 MRC mU of eel CT had no effect on drinking rates or blood pressure in freshwater eels, a not unexpected result as freshwater fish do not normally ingest water (Kobayashi, et al., 1983). However, the effect of calcitonin on calcium ingestion in fish acclimated

to different conditions of salinity has not been studied.

The general purpose of the present study then was to investigate the effect of eel calcitonin on calcium regulation in American eels, Anguilla rostrata, and to investigate specifically its possible inhibitory effect on drinking in freshwater acclimated, seawater acclimated and freshwater acclimated eels transferred to hyperosmotic seawater. The experimental approach taken involved testing to determine if oesophageal ligation could remove the inhibitory action of calcitonin on calcium accumulation. If it could, the injection of eel CT would mimic the effects of ligation in transfer and seawater acclimated eels. If not, then calcitonin does not reduce apparent calcium influx by causing a reduction in ingested calcium and most probably affects calcium influx by causing changes in the general body surface and the gills. Additionally, bone and muscle sites were tested for changes in ^{45}Ca specific activity following injection with CT as calcitonin specific receptors have been identified in these tissues in other teleosts (Warshawsky et al., 1980; Arlot-Bonnemains et al., 1983).

Histological evidence indicates that the ultimobranchial bodies are more active in female salmon in seawater than freshwater but alterations in serum calcium levels do not correlate with the morphological changes (Yamane and Yamada, 1977). In the present study the ultimobranchial tissue from freshwater and transfer eels was examined to determine if observable changes

occur within the four hour time frame of the experiments.

M A T E R I A L S and M E T H O D S

EXPERIMENTAL ANIMALS

Immature American eels, Anguilla rostrata LeSueur, 134 ± 3 (S.E.) grams, were collected between July and September, 1987 from an eel ladder located at the Saunders Hydroelectric Dam in Cornwall, Ontario. The fish were transported to the University of Ottawa, Ottawa, Ontario, in moist trays stacked in boxes cooled with ice.

Immediately upon arrival at the laboratory, the fish were transferred to two 4m X 1m X 1m grey fiberglass tanks. Both tanks were supplied with well aerated, running, dechlorinated City of Ottawa tap water (5-15°C; $[Ca^{+2}]$ = 0.4 mM). The fish were not fed and were exposed to a constant photoperiod of 12 hours light alternating with 12 hours darkness. All eels were held under these conditions for a minimum of two weeks before being used in an experiment. The study was conducted between May, 1987 and April, 1988 (see tables for specific dates of experiments).

Experimental fish, removed from the stock population, were transferred to 200L white plastic holding tanks supplied with the same water as described above. For saltwater experiments, fish were transferred to 45L aquariums situated in a 4°C cold room where they were held under constant light conditions for fourteen days in 46-96% SW. A portion of the saltwater was changed as

required. Constant light was required to prevent the eels from escaping from the tanks as they become very active in the dark (Tesch, 1977). Appropriate control fish were exposed to the same conditions.

Seawater was prepared by adding Instant Ocean (Aquarium Systems, Eastlake, Ohio) to City of Ottawa dechlorinated tap water. The calcium concentration was measured using a Varian SpectraAA-10 atomic absorption spectrophotometer.

EXPERIMENTAL REGIMENS

A) Measurement of ^{45}Calcium Influx:

Eels were placed individually into opaque plexiglass flux boxes (7cm H X 8cm W X 31cm L) twenty-four hours before each experiment. Each box was supplied with running dechlorinated water which was not aerated. Immediately prior to the start of an experiment, the boxes were drained and the eels removed one at a time with a net. The eels were then injected intraperitoneally, without anaesthetic, with either calcitonin (experimental eels), carrier buffer only (carrier injected eels) or nothing (control eels). The injected volume never exceeded 0.2ml and was held constant within any given experiment. The eels were returned to their boxes to which were added 500ml of either freshwater or seawater.

Sufficient ^{45}calcium (Amersham International or ICN Biomedicals, Inc.), diluted in distilled water, was added to the boxes to give about 25,000 DPM/ml in freshwater and 500,000 DPM/ml in seawater. Water samples were taken 10 minutes after the start of the experiment and at the end of the four hour flux period in order to measure the radioactivity and total calcium concentration for the calculation of the specific activity of the ambient medium.

After four hours, the eels were rapidly anaesthetized in 3g/L of MS-222 (3-aminobenzoic acid ethyl ester methanesulfonate salt, Sigma) which was made up to 10mM Ca^{+2} by adding $CaCl_2$ to

help displace surface bound ^{45}calcium. The fish were then rinsed under running water, weighed and terminal blood samples taken directly from the ventral aorta into ammonium heparinized 1ml syringes fitted with 1"-23 gauge needles. The blood was immediately centrifuged at 5000 X g for 2 minutes and the plasma was stored at -20°C for later analysis. When necessary, the intestines were removed, placed in test-tubes and refrigerated at 4°C. As not all fish could be fluxed for exactly four hours the actual flux time, in minutes, for each fish was recorded. The dead eels were frozen, in plastic bags, at -20 °C until homogenized.

Unidirectional calcium influx (J_{in}) was calculated by the following formula:

$$J_{in} = \frac{WBC \times 1000}{SA \times T \times W} \qquad [1]$$

$$= \mu mol\ kg^{-1}\ hr^{-1}$$

Where WBC = whole body ^{45}Ca counts; SA = specific activity of the ambient medium at the end of four hours; T = duration of the flux in hours; W = weight of the animal in grams.

Separate body (B_{in}), gut (G_{in}) and total animal (T_{in}) calcium influxes were calculated by the following formulas:

$$B_{in} = \frac{BC \times 1000}{SA \times T \times W} \quad [2]$$

$$= \mu mol\ kg^{-1}\ hr^{-1}$$

Where BC = Body (no stomach or intestines) ^{45}Ca counts; SA = specific activity of the ambient medium at the end of four hours; T = duration of the flux in hours; W = weight of the animal in grams.

$$G_{in} = \frac{GC \times 1000}{SA \times T \times W} \quad [3]$$

$$= \mu mol\ kg^{-1}\ hr^{-1}$$

Where GC = gut ^{45}Ca counts; SA = specific activity of the ambient medium at the end of four hours; T = duration of the flux in hours; W = weight of the animal in grams.

$$T_{in} = B_{in} + G_{in} \quad [4]$$

$$= \mu mol\ kg^{-1}\ hr^{-1}$$

Where B_{in} = body calcium accumulation; G_{in} = gut calcium accumulation.

The ability of this technique to consistently measure changes in total animal calcium accumulation following treatment with calcitonin was demonstrated in similar experiments involving

the axolotl, Ambystoma mexicanum, a neotonous salamander (see Appendix B). This same study also shows that the CT used was bioactive and that the dose and route of administration could invoke effects in at least one other species of aquatic vertebrate.

B) Measurement of ^{45}Calcium Efflux:

For ^{45}Ca efflux measurements the eels were injected, intraperitoneally, with 2 μCi of ^{45}calcium in 0.1 ml of distilled water seventy-two hours before the experiment. With this regimen the specific activity of the plasma did not change significantly over the four hour flux period.

After loading, the eels were held in a 200L white plastic tank equipped with running dechlorinated water ($[Ca^{+2}]$ = 0.4 mM/L) and bubbled air.

The influx protocol was then followed except that the ambient medium was not spiked with ^{45}calcium and the bodies were disposed of immediately after blood sampling as no tissue homogenization was necessary.

Whole body ^{45}calcium efflux (J_{out}) was calculated by the following formula:

$$J_{out} = \frac{\text{DPM in 1ml } H_2O \text{ over 4 hours}}{SA \times T \times W} \qquad [5]$$

$$= \mu\text{mol } kg^{-1} \text{ } hr^{-1}$$

Where SA = specific activity of the plasma at the end of four hours; T = duration of the flux in hours; W = weight of the animal in grams.

SURGICAL PROCEDURES

A) Anaesthetization:

Before surgery or blood sampling, the eels were anaesthetized for approximately 4 minutes in $NaHCO_3$ buffered (pH 7.4) MS-222 (3g/L for surgical anaesthetic and 4g/L for terminal blood sampling anaesthetic) and then rinsed under running tapwater. Excess surface water was removed by blotting the eels with a Kim-Tuff paper towel.

B) Oesophageal Ligation:

After anaesthetization in surgical anaesthetic a lateral incision, starting about 3cm posterior to the opercular opening and extending about 1.5cm, was made halfway between the ventral median and the lateral line. This exposed the oesophagus at its entry point into the stomach near to the posterior end of the liver. The oesophagus was then pulled up with a pair of dull forceps to expose its most medial edge. A ligature consisting of a double strand of 00 silk was held at the folded end by pointed curved forceps, looped around the oesophagus and then tied tightly with a surgeon's knot. Three-0 silk was used to stitch the wound. Care was taken to ensure that the prominent blood vessel along the median edge of the oesophagus was not ripped. If rupture did occur pressure was applied until the bleeding stopped.

The fish were allowed to recover from surgery in a 200L

white plastic tank for three to four days prior to the experiment. The sham operated fish, identified either by a wound clip on the dorsal fin or by clipped pectoral fins, underwent the same procedure except that no ligature was installed.

C) Injections of Carrier Buffer and Calcitonin:

Eels were injected intraperitoneally (I.P.) 2cm anterior to the vent, halfway between the lateral line and the ventral median line. Injections were carried out with 1 c.c. tuberculin syringes fitted with 1"-23G needles filled with the appropriate solution.

Two one milligram vials of synthetic eel sequence Thyrocalcitonin Acetate Salt (5000 MRC units per milligram) were purchased from Sigma Chemical Co., St. Louis, MO. in August, 1986 (Lot #53F-0428 and 116F-05571). One milligram was then dissolved in 2 ml of buffer solution (1% sodium acetate and 0.1% glycine in distilled water, pH = 4.3) to a concentration of 0.5 mg/ml. Twenty 100µl aliquots were lyophilized in a Labconco Freeze Dryer-3 overnight (50 µg/vial) and were stored at -90°C.

Before each experiment, one aliquot was warmed to room temperature and redissolved in 2ml of buffer (25 µg/ml). One milliliter was immediately refrozen at -90°C and the remaining 1ml was diluted with a further 2.5ml of buffer to a working concentration of 10µg/ml. The chosen dose of eel calcitonin was 2µg/fish in all experiments.

SAMPLING TECHNIQUES

A) Blood:

After the eels were anaesthetized in terminal anaesthetic a longitudinal incision was made on the ventral surface of the eel's head between the heart and lower mandible. The ventral aorta was exposed and freed from its connective tissue with a dull probe. An open curved hemostat was used to pull the aorta taut so that a heparinized syringe fitted with a 1"-23G needle could be inserted. With the hemostat clamped around the needle, between 0.6 and 1.0 milliliter of whole blood was taken from each fish and placed in an Eppendorf centrifugation tube. Plasma was separated by centrifugation (2 minutes, 5000 x g), transferred to a clean centrifugation tube and stored at -20°C.

B) Gut Removal:

A longitudinal incision extending from the posterior end of the ligation incision (or, in the case of controls, from the left opercular opening) to the vent was made to expose the stomach and the intestine. If the eel was not previously ligated, the oesophagus, just anterior to the stomach, was clamped with a curved hemostat. The posterior end of the intestine was clamped just anterior to the vent. Scissors were then used to free the clamped gut from its connective tissue. Five milliliters of distilled water were flushed through each gut to rinse out the contents. The excised stomach and intestine were stored in

parafilm sealed test-tubes and refrigerated at 4°C until homogenized.

C) Muscle and Bone Samples:

A square incision was made on the left side of the fish 1cm posterior to the midpoint of the body. The skin was carefully removed with a scalpel fitted with a #11 blade. The exposed muscle was removed with forceps and scissors and placed in a pre-weighed numbered ceramic crucible.

The underlying vertebrae (5-7 in number) were taken from the spinal column by a scalpel fitted with a #20 blade and were transferred to a pre-weighed numbered ceramic crucible.

TISSUE HOMOGENIZATION

Frozen eels were cooked in a Sharp Carousel Microwave Oven on high for three minutes. The microwaved fish were then homogenized in 200ml of tap water in a Waring Commercial blender. One sample (10-15 ml) of the homogenate was taken per fish.

Refrigerated weighed eel intestines were mixed with 5ml of distilled water in a test-tube and were homogenized using a Brinkmann Kinematica PCU-2 homogenizer. The homogenate was poured into a weighed ceramic crucible and the test-tube rinsed with 1ml of distilled water. This rinse water was added to the crucible contents.

ANALYTICAL PROCEDURES

A) Plasma ^{45}Ca and Total Plasma Calcium, Sodium, Potassium, Magnesium, Osmolarity and Chloride:

Plasma samples (50μl), mixed with 5ml of distilled water and 10ml of ACS II, were analyzed for their radiocalcium content in a LKB Wallac 1215 Rackbeta Liquid Scintillation Counter with a built-in DPM program.

Plasma calcium, sodium, potassium, and magnesium concentrations were measured using a Varian SpectraAA-10 atomic absorption spectrophotometer.

Osmotic pressure was determined with a Wescor, Inc. 5100C Vapor Pressure Osmometer.

Chloride concentrations were determined using a Buchler-Cotlove Chlorideometer automatic titrator (Buchler Instruments Inc., Fort Lee, N.J., U.S.A.).

B) Body, Gut, Bone and Muscle Sample ^{45}Ca:

Body tissue homogenate aliquots, as well as gut, bone and muscle samples were weighed, dried overnight at 75°C and then ashed in a Fisher Isotemp muffle furnace, Model 184A, for five to seven hours at 500-600°C. Ash samples were dissolved overnight in 5ml of 1N HCl and the resulting solution neutralized with 833μl of 6N NaOH. Three 1ml samples of this solution, to which were added 4ml of distilled H_2O and 10ml of Aqueous Counting Scintillant (ACS II, Amersham Canada, Ltd., Oakville, Ontario), were counted for their ^{45}Ca content in the same scintillation counter as above.

EXPERIMENTAL DESIGNS

A) Effects of ligation plus calcitonin on body, gut, and total animal calcium influx in freshwater (FW) and seawater acclimated (SWA) eels and in freshwater eels rapidly transferred to seawater (SWT).

There were three groups of eels per experiment, sham ligated, ligated/injected with buffer, and ligated/injected with calcitonin (2μg/animal).

Four days prior to the FW and SWT experiments, two batches of 18 fish were separated from the general population and placed in separate 200L white plastic tanks. The eels of the first batch, designated for the transfer experiment, were anaesthetized

in surgery anaesthetic and operated on to produce N = 6 fish for each of the three groups listed above. The second batch, for the freshwater experiment, were similarly handled.

Eighteen eels were transferred to an aquarium containing seawater ($[Ca^{+2}]$ = 7.5 mM/L) and maintained in a 4°C coldroom under constant light. After 15 days of acclimation thirteen eels (125 ± 8 grams) survived. Surgery was performed four days prior to the experiment and resulted in N = 3, 4, 6 respectively for the three SWA experimental groups.

On the days of the experiments, the influx procedure was followed. In the freshwater experiment, sufficient ^{45}calcium was added to the boxes to produce 6.0 x 10^4 DPM/mL. In the freshwater to seawater ($[Ca^{+2}]$ = 7.4 mM/L) transfer and seawater acclimated ($[Ca^{+2}]$ = 7.5 mM/L) fluxes, 3.2 x 10^5 DPM/mL were used.

The intestine and stomach of each eel were removed for further analysis.

B) The effect of calcitonin compared to ligation on total animal calcium accumulation in SWT and SWA eels.

In the batch of eels rapidly transferred from freshwater to seawater ($[Ca^{+2}]$ = 7.8 mM) there were 7 sham ligated, 6 ligated/buffer injected, and 7 sham ligated/calcitonin injected eels. Fifteen seawater acclimated ($[Ca^{+2}]$ = 7.9mM) eels were evenly divided into the same three groups as the transferred fish.

The amount of radiocalcium pipetted into the boxes produced

counts of 5.0×10^5 DPM/ml in the SWT experiment and 6×10^5 DPM/ml in the SWA experiment.

The influx protocol was followed except that neither the stomach nor the intestines were removed from the eels.

C) The effect of sham ligation, ligation and combinations of surgery and injection of carrier buffer and calcitonin on SWA and SWT eels.

The influx protocol was followed except that the stomachs and intestines were not removed.

Table 2: Summary of groups and water calcium concentrations in experiments to determine the effect of sham ligation, ligation and combinations of surgery and injection in SWA and SWT eels.

Acclimation State[a]	N	Groups	Seawater [Ca+2][b]	Water 45Ca content[c]
SWT	6	intact	5.5	1 X 105
	5	sham lig.[d]		
	3	ligated		
SWT2	5	intact	7.2	4.5 X 105
	5	sham lig.		
	5	ligated		
SWA	5	intact	7.0	4 X 105
	5	sham lig.		
	5	ligated		
SWT	5	sham lig.	7.0	4 X 105
	5	sham + B.[e]		
	5	sham + CT[f]		
SWA	3	sham lig.	9.6	4 X 105
	5	sham + B.		
	5	sham + CT		
SWT	5	ligated	7.5	4.5 X 105
	5	lig. + B.		
	5	lig. + CT.		
SWA	4	ligated	4.6	3 X 105
	5	lig. + B.		
	5	lig. + CT		

a SWT = freshwater to seawater transfer
SWA = seawater acclimated.
b units are mM/L
c units are DPM/ml
d lig. = oesophageal ligation
e B. = carrier buffer injected
f CT = calcitonin; dose is 2µg/eel

D) Effect of calcitonin on bone and muscle specific activity following transfer from freshwater to 66% seawater.

Eighteen fish were divided into three groups of six. The first group were intact controls; fish in the second group were injected with buffer and those in the third were injected with 2μg/eel of calcitonin.

The influx protocol was followed with radiocalcium levels of 5.3×10^5 DPM/mL. After blood samples were taken, samples of bone and muscle were removed. The intestines were not removed.

E) Effect of calcitonin on calcium efflux on SWT eels.

Fourteen fish were divided into two groups of seven. The first group were controls injected with carrier buffer and the second were experimental eels injected with calcitonin.

An efflux experiment was performed following the I.P. injection of 2μg/eel of calcitonin and transfer from freshwater to seawater ($[Ca^{+2}]$ = 7.0 mMol/L).

HISTOLOGY

Ten FW and ten SWT fish were anaesthetized in 3g/L of MS-222. A 3cm longitudinal incision was made directly behind the left operculum to expose the heart, transverse septum, liver and ventral oesophagus. The entire area was fixed *in situ* with paraformaldehyde-gluteraldehyde fixative (see below). A small piece of ventral oesophagus containing the ultimobranchial tissue was removed from a point near to the transverse septum and was fixed in 1ml of paraformaldehyde-gluteraldehyde fixative for one hour after which it was replaced with fresh fixative and the tissue left overnight. The fixative was then replaced with cacodylate buffer containing 7% dextrose and the tissue stored at 4°C.

The ultimobranchial tissue was post-fixed in osmium for two hours after which it was washed in distilled water. An eight step dehydration procedure involved 10 minutes each in 50%, 75%, and 95% ethanol followed by three washes of ten minutes each in 100% ethanol and two washes of 15 minutes each in pure propylene oxide (McMillan *et al*, 1976).

The tissues was then infiltrated overnight in the infiltration solution (see below). Twenty-four hours later, the infiltration solution was replaced with pure infiltration solution not containing propylene oxide and the tissues allowed to sit for four hours (J.N. Fryer, personal communication).

Fresh pure infiltration solution (without the propylene

oxide) was then mixed, centrifuged (Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge; 2000 X g, 10 minutes) and pipetted into the embedding molds. Each ultimobranchial body was transferred from its one dram vial to a gully in the embedding mold. Orientation of the tissues was performed under a dissecting microscope. The embedding molds were placed in a Thelco Precision Scientific Co. Dry Oven maintained at 60°C for 36 hours for polymerization.

Each block was then trimmed, using a single edged razor blade, under a Wild Hferbrugg dissecting microscope illuminated by an Intralux 4000 fibre-optic light source.

Thick (1μm) serial sections were cut on a Sorvall Porter-Blum MT2-B Ultra Microtome using glass knives made on an LKB Knife Maker, Type 7801B. Sections were placed on specimen slides, dried on a Corning PC-35 Hot Plate and then heat stained with methylene blue -azure II stain for one minute. Verification of cell type was done using a Bausch and Lomb light microscope.

Thin (70nm) sections were cut using fresh glass knives. Sections were transferred to clean copper electron microscopy grids using copper pick-up grids and were dried on Wattman No.1 filter paper. Thin sections were stained for 7 minutes each with uranyl acetate and lead citrate. Blotting and drying occurred on filter paper.

Electron micrographs were obtained using a Phillips 201C electron microscope. Light micrographs were obtained using a Zeiss Light Microscope equipped with a 16X Phase II objective

lens and a Planapo 63X/1.4 oil immersion lens.

Solutions:

A) Cacodylate Buffer:

4.28 grams of sodium cacodylate (BDH Chemicals) and 0.10 grams of calcium chloride (BDH Chemicals) were added to 200ml of distilled water. The pH was adjusted to 7.4 and the solution stored at 4°C.

B) Paraformaldehyde-Gluteraldehyde Fixative:

3.0 grams of paraformaldehyde (J.B.EM Services Inc., Dorval, P.Q.) was added to 100ml of cacodylate buffer. This solution was heated to 60-70°C on a magnetic stirrer. Once clear, the mixture was cooled in a water bath and then refrigerated at 4°C. Immediately before being required, 2ml of 70% gluteraldehyde (J.B.EM Services Inc., Dorval, P.Q.) was added to 70ml of the above paraformaldehyde-cacodylate buffer solution. The complete paraformaldehyde-gluteraldehyde fixative was stored on ice, in a beaker covered with parafilm, throughout the experiment.

C) Dextrose Buffer:

7.0 grams of dextrose (BDH Chemicals) was added to 100ml of cacodylate buffer.

D) Methylene Blue-Azure II Stain:

1g of sodium borate(BDH Chemicals) and 1g of methylene blue were added to 100ml of distilled water and stored at 4°C. 1g of azure II was added to 100ml of distilled water and stored at 4°C. The solutions were mixed 1:1, in a small bottle, just prior to use.

E) Lead Citrate Stain:

1.33 grams of $Pb(NO_3)_2$ (BDH Chemicals) and 1.76 grams Na_3-$(C_6H_5O_7).2H_2O$ (BDH Chemicals) were mixed with 30 ml of distilled water. This solution was shaken vigorously for 1 minute and then allowed to stand for 30 minutes (with intermittent shaking) after which 8.0ml of 1N NaOH (BDH Chemicals) was added. The suspension was further diluted to 50ml with distilled water. pH = 12.

F) Osmium:

One half gram of osmium (J.B.EM Services) was dissolved in 25ml of distilled water. This solution was mixed 1:1 with a solution of 20ml of cacodylate buffer and 2.8 grams of dextrose (BDH Chemicals).

G) Infiltration Solution:

20 grams of Araldite 502 Resin (J.B.EM Services) was mixed with 13.6 grams of Dodecyl Succinic Anhydride (DDSA, J.B.EM Services) and 0.46 ml of Tri(dimethylaminomethyl)phenol (DMP30, J.B.EM Services). This mixture was stirred until homogeneous in

color and then mixed 1:1 with propylene oxide (J.B.EM Services).

STATISTICAL ANALYSIS

All data were analyzed using either Student's T Tests or One Way Analyses of Variance (ANOVA). $P < 0.05$ was taken as the limit of significance.

R E S U L T S

Effect of oesophageal ligation on the response to calcitonin in freshwater (FW), seawater (SWA) and during transfer from freshwater to seawater (SWT)

Freshwater acclimated eels:

Table 3 shows that there was no significant ($P < 0.05$) effect of ligation or ligation plus calcitonin on body, gut or total animal calcium accumulation in FW eels. Gut accumulation was very low and as it was not affected by ligation the data indicate that the eels did not drink during the experimental period.

Further, neither of the ligation treatments significantly ($P < 0.05$) altered plasma total calcium, sodium, or potassium (Table 4). Ligated eels, whether injected with calcitonin or the solvent alone, showed significantly ($P < 0.05$) higher plasma Mg^{+2} levels than did the sham ligated eels.

Seawater acclimated eels:

Acclimation to seawater significantly ($P < 0.05$) increased body, gut and total animal calcium accumulation (Table 3) and this was associated with a general increase in plasma total calcium, sodium, potassium and magnesium (Table 4). On the basis of gut accumulation (Table 3), the unligated SWA eels showed

Table 3: Effect of oesophageal ligation on the response to calcitonin in FW, SW and SWT[a]

Acclimation State	Groups	N	Body Ca^{+2} Accumulation	Gut Ca^{+2} Accumulation	Total Animal Ca^{+2} Accumulation
FW[b]	sham ligated	6	2.39 ± 0.87	0.04 ± 0.02	2.43 ± 0.88
	ligated + carrier	6	1.50 ± 0.88	0.05 ± 0.03	1.54 ± 0.91
	ligated + CT	6	1.47 ± 0.89	0.05 ± 0.03	1.51 ± 0.19
FW-SW[b]	sham ligated	6	3.13 ± 1.05[d]	0.36 ± 0.18[d]	3.49 ± 0.94[d]
	ligated + carrier	6	5.32 ± 1.29[c,d]	0.24 ± 0.08[f]	5.57 ± 1.34[c,d]
	ligated + CT	6	4.33 ± 2.13[d]	0.16 ± 0.06[c,d]	4.49 ± 2.16[d]
SWA[b]	sham ligated	3	16.64 ± 5.42[e]	5.61 ± 6.13[e]	22.26 ± 8.53[e]
	ligated + carrier	4	13.86 ± 3.49[e]	0.30 ± 0.11[c,e]	14.17 ± 3.60[e]
	ligated + CT	6	21.15 ± 12.00[e]	0.35 ± 0.10[c,e]	21.50 ± 12.09[e]

Table 3 continued.

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a Values are mean μmol kg^{-1} hr^{-1} $\pm$ S.D.; CT dose = 2.0 μg/eel

b FW = freshwater ($[Ca^{+2}]$ = 0.4 mMol/L) 6 Oct 1987
FW-SW = freshwater to seawater transfer ($[Ca^{+2}]$ = 7.4 mMol/L) 22 Nov 1987
SWA = seawater acclimated ($[Ca^{+2}]$ = 7.5 mMol/L) 29 Sept 1987

c significantly ($P < 0.05$) different relative to the sham ligated group of the same acclimation state

d significantly ($P < 0.05$) different than the corresponding values from the FW and SWA acclimation states

e significantly ($P < 0.05$) different than the corresponding values from the FW and SWT acclimation states

f significantly ($P < 0.05$) different than the corresponding value from the FW acclimation state

Table 4: Effect of oesophageal ligation on the response of plasma total ions to calcitonin[a]

Acclimation State	Groups	N	Plasma [ions] mMol L^{-1} Ca^{+2}	Na^{+}	K^{+}	Mg^{+2}
FW[b]	sham ligated	6	2.31 ± 0.27	141.9 ± 3.4	2.80 ± 0.79	1.1 ± 0.1
	ligated + carrier	6	2.58 ± 0.19	141.9 ± 5.3	3.01 ± 0.73	1.3 ± 0.1[c]
	ligated + CT	6	2.45 ± 0.36	139.2 ± 11.7	3.44 ± 0.67	1.4 ± 0.2[c]
FW-SW[b]	sham ligated	6	3.55 ± 0.30	146.7 ± 6.6	3.10 ± 0.48	1.6 ± 0.2
	ligated + carrier	6	3.92 ± 0.34	153.1 ± 10.3	2.75 ± 0.12	1.7 ± 0.2
	ligated + CT	6	3.73 ± 0.27	152.0 ± 10.6	3.24 ± 0.75	1.9 ± 0.2[c]
SWA[b]	sham ligated	3	3.10 ± 0.31	153.5 ± 11.6	3.62 ± 0.98	1.4 ± 0.3
	ligated + carrier	4	3.23 ± 0.29	158.7 ± 14.5	4.15 ± 0.56	1.6 ± 0.2
	ligated + CT	6	3.72 ± 0.26[c,d]	175.9 ± 10.3[c,d]	4.41 ± 0.57	2.3 ± 0.9

Table 4 continued.

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a Values are mean ± S.D.; CT dose = 2.0 μg/eel

b FW = freshwater ($[Ca^{+2}]$ = 0.4 mMol/L) 6 Oct 1987
FW-SW = freshwater to seawater transfer ($[Ca^{+2}]$ = 7.4 mMol/L) 22 Nov 1987
SWA = seawater acclimated ($[Ca^{+2}]$ = 7.5 mMol/L) 29 Sept 1987

c significantly ($P < 0.05$) different relative to the sham ligated group of the same acclimation state

d significantly ($P < 0.05$) different than both other groups of the same acclimation state

significantly ($P < 0.001$) higher drinking rates than the unligated FW fish.

Ligated eels in seawater also had significantly ($P < 0.05$) higher gut calcium accumulation than did the FW eels but the rate of accumulation was significantly ($P < 0.05$) less than in the unligated animals. The low values recorded for gut calcium accumulation in SWA ligated eels were taken as firm evidence that the ligation procedure did prevent drinking. The calcium which was found in the guts of ligated eels probably entered through the blood.

A calcitonin injection 10 minutes before the start of the four hour flux experiment did not effect body, gut or whole animal calcium accumulation in ligated eels when compared to ligated eels injected with the carrier buffer only (Table 3). This suggested that the CT might have reduced drinking in seawater as its effect on calcium accumulation was abolished by ligation.

Calcitonin induced significant ($P < 0.05$) increases in both plasma calcium and sodium compared to the sham ligated or ligated/carrier injected control fish, but had no effect on plasma potassium or magnesium (Table 4).

Freshwater acclimated eels acutely transferred to 74% seawater:

Eels rapidly transferred from freshwater to 74% seawater exhibited significantly ($P < 0.05$) higher body, gut and total animal calcium accumulation values than did FW eels and sig-

nificantly ($P < 0.05$) lower than that of SWA eels (Table 3). Plasma total calcium, sodium, potassium and magnesium concentrations were similarly intermediate relative to those seen in FW and SWA eels.

Ligated eels, injected with carrier alone, showed significantly ($P < 0.05$) higher body and total animal calcium accumulation values compared to sham ligated fish. This significance was eliminated by injection with calcitonin (Table 3).

Treatment with calcitonin resulted in significantly ($P < 0.05$) lower gut calcium accumulation in ligated eels, relative to the sham ligated fish but not the carrier injected ligated fish, but did not decrease total animal accumulation.

Additionally, eels injected with CT showed significantly higher plasma magnesium concentrations but no change in plasma total calcium, sodium, or potassium.

Comparison of the effect of oesophageal ligation and injection with calcitonin on total animal calcium accumulation and plasma ions in SWA and SWT eels

In this pair of experiments (Tables 5 and 6), neither ligation nor treatment with calcitonin had any effect on total animal calcium accumulation. Table 6, however, shows that the ligation plus carrier treatment causes significant hemoconcentration which is reflected in an increase in all of the ions measured except chloride. Although the CT treated eels retained significantly ($P < 0.05$) higher plasma calcium levels than the sham ligated group, the plasma total calcium concentration was significantly ($P < 0.05$) reduced in the CT treated eels relative to the ligated eels. Therefore, calcitonin treatment is more effective at preventing hypercalcemia in seawater acclimated eels than is ligation.

Table 5: Effect of calcitonin compared to oesophageal ligation on total animal calcium accumulation

Acclimation State	Groups	N	Total Animal Ca^{+2} Accumulation[a]
FW-SWT[b]	sham ligated	7	5.68 ± 1.33
	ligated + carrier	6	8.19 ± 3.28
	sham ligated + CT	7	10.05 ± 7.78
SWA[b]	sham ligated	5	10.91 ± 3.79
	ligated + carrier	5	7.28 ± 1.87
	sham ligated + CT	5	9.58 ± 4.46

[a] Values are mean μmol kg^{-1} hr^{-1} ± S.D.; CT dose 2.0 μg/eel

[b] FW-SW = freshwater to seawater transfer ($[Ca^{+2}]$ = 7.8 mMol/L) 24 Nov 1987
SWA = seawater acclimated ($[Ca^{+2}]$ = 7.9 mMol/L) 17 Nov 1987

Table 6: Effect of calcitonin compared to oesophageal ligation on plasma parameters[a]

Acclimation State	Groups	N	Plasma [ions] mMol L^{-1}					
			Ca^{+2}	Na^{+}	K^{+}	Mg^{+2}	Osm[a]	Cl^{-}
FW-SW[b]	sham ligated	7	3.05 ± 0.28	155.8 ± 3.5	2.1 ± 0.6	1.8 ± 0.1	299.0 ± 7.7	100.0 ± 5.8
	ligated + carrier	6	2.88 ± 0.20	159.4 ± 3.3[c]	2.1 ± 0.7	2.0 ± 0.3	312.5 ± 12.3[c]	105.2 ± 12.1
	sham ligated + CT	7	3.72 ± 1.10	167.7 ± 7.2[c,d]	2.5 ± 0.9	2.3 ± 0.6	320.1 ± 24.7[c]	108.9 ± 10.0
SWA[b]	sham ligated	5	2.32 ± 0.27	151.5 ± 3.0	2.6 ± 0.1	1.1 ± 0.1	327.2 ± 7.6	128.0 ± 1.3
	ligated + carrier	5	3.55 ± 0.43[c]	167.8 ± 3.3[c]	3.4 ± 0.1[c]	1.9 ± 0.1[c]	375.6 ± 9.0[c]	122.0 ± 3.7
	sham ligated + CT	5	2.97 ± 0.36[c,•]	158.9 ± 2.4	2.5 ± 0.1[•]	1.5 ± 0.2	333.4 ± 4.4[c,•]	123.4 ± 2.6

[a] Ca^{+2}, Na^{+}, K^{+}, Mg^{+2} and Cl^{-1} values are mean ± S.D., Osm = osmolarity in mOsm; CT dose = 2.0 µg/eel

[b] FW-SW = freshwater to seawater transfer ([Ca^{+2}] = 7.8 mMol/L) 24 Nov 1987
SWA = seawater acclimated ([Ca^{+2}] = 7.9 mMol/L) 17 Nov 1987

[c] significantly ($P < 0.05$) different relative to the sham ligated group of the same acclimation state

[d] significantly ($P < 0.05$) different than both other groups of the same acclimation state

[•] significantly ($P < 0.05$) different than the value for the ligated + carrier

Effect of sham oesophageal ligation and oesophageal ligation surgery on total animal calcium accumulation and plasma ions in SWA and SWT eels

Seawater acclimated eels:

Both surgical procedures elicited hypercalcemia and hypermagnasemia in seawater acclimated eels, whereas total animal calcium accumulation, sodium and potassium levels remained unchanged (Table 7).

Unlike previous data (Table 3), the calcium accumulation values in this experiment were not statistically different from those seen in the transfer fish (Table 7). Plasma total calcium and magnesium levels remained the same in SWA fish as in the transfer fish while the levels of sodium and potassium increased significantly ($P < 0.05$) compared to the transfer eels (Table 7).

Freshwater eels rapidly transferred to seawater:

Sham oesophageal ligation in SWT eels produced a significant ($P < 0.05$) increase in total body calcium accumulation compared to the intact controls (Table 7). This increase was eliminated when the fish were ligated indicating that the surgery itself caused an increase in drinking, an effect which could not be seen in the ligated eels. However, this effect was not seen in a repeat of this experiment in seawater with 17% higher [Ca^{+2}].

In both transfer experiments, the sham oesophageal ligation and ligation surgery elicited hypernatremia but no change in

Table 7: Effect of oesophageal ligation on total animal calcium accumulation and plasma ion levels

Acclimation State	Group	N	Total Animal Ca^{+2} Accumulation[a]	Plasma [ions] mMol L^{-1} Ca^{+2}	Na^{+}	K^{+}	Mg^{+2}
FW-SW (1)[b]	intact	6	4.10 ± 1.65	3.22 ± 0.43	158.1 ± 2.9	1.8 ± 0.1	1.6 ± 0.2
	sham ligated	5	6.70 ± 1.43[c]	3.35 ± 0.10	163.4 ± 2.3[c]	1.9 ± 0.4	1.7 ± 0.1
	ligated	3	5.32 ± 1.29	3.19 ± 0.41	163.1 ± 2.1	1.9 ± 0.2	1.9 ± 0.2
FW-SW (2)[b]	intact	5	6.04 ± 2.78	3.33 ± 0.43	187.4 ± 3.0	2.2 ± 0.5	1.8 ± 2.2
	sham ligated	5	8.47 ± 3.87	3.36 ± 0.26	181.9 ± 1.7[c]	1.8 ± 0.2	1.8 ± 0.2
	ligated	5	4.96 ± 2.04	3.03 ± 0.28	177.7 ± 6.1[c]	2.3 ± 0.9	1.8 ± 0.1
SWA[b]	intact	5	4.51 ± 1.78	2.49 ± 0.33	172.2 ± 14.6	2.6 ± 0.3	1.2 ± 0.2
	sham ligated	5	8.67 ± 6.25	3.06 ± 0.37[c]	184.2 ± 10.3	2.8 ± 0.3	1.9 ± 0.6[c]
	ligated	5	5.76 ± 2.61	3.48 ± 0.30[c]	178.6 ± 5.2	2.8 ± 0.2	1.8 ± 0.2[c]

[a] values are μmol kg^{-1} hr^{-1} ± S.D.; CT dose = 2.0 μg/eel

[b] FW-SW (1) = freshwater to seawater transfer ([Ca^{+2}] = 5.5 mMol/L) 07 Jan 1988
FW-SW = freshwater to seawater transfer ([Ca^{+2}] = 7.2 mMol/L) 28 Jan 1988
SWA = seawater acclimated ([Ca^{+2}] = 7.0 mMol/L) 25 Jan 1988

[c] significantly ($P < 0.05$) different relative to the intact group of the same acclimation state

plasma total calcium, potassium or magnesium.

Effect of calcitonin on total animal calcium accumulation and plasma ions in SWA and SWT sham oesophageal ligated eels

Seawater acclimated eels:

Table 8 shows that calcitonin in sham oesophageal ligated SWA eels induces no significant change in total animal calcium accumulation compared to both the carrier injected fish and the controls. Treatment with CT did produce hypermagnasemia relative to the other groups, while plasma total calcium, sodium and potassium concentrations remained unaltered.

Freshwater eels rapidly transferred to 70% seawater:

As in the SWA eels, calcitonin was ineffective in eliciting any changes in total animal calcium accumulation or in plasma total potassium (Table 8). However, the injection of carrier buffer or calcitonin produced significant ($P < 0.05$) hypercalcemia and hypernatremia compared to the uninjected control fish (Table 8). In addition, CT caused a significant ($P < 0.05$) increase in plasma sodium compared to the carrier injected eels. Plasma magnesium concentrations were unaffected by the injections.

Table 8: Effect of calcitonin on total animal calcium accumulation and plasma ion levels in sham oesophageal ligated eels

Acclimation State	Group	N	Total Animal Ca^{+2} Accumulation[a]	Plasma [ions] mMol L^{-1} Ca^{+2}	Na^{+}	K^{+}	Mg^{+2}
FW-SW[b]	sham ligated	5	5.02 ± 1.59	2.42 ± 0.19	156.6 ± 6.3	1.9 ± 0.2	1.5 ± 0.1
	sham ligated + carrier	5	5.52 ± 2.67	2.73 ± 0.09[c]	167.8 ± 3.7[c]	1.9 ± 0.2	1.6 ± 0.3
	sham ligated + CT	5	5.34 ± 3.19	2.99 ± 0.39[c]	175.9 ± 5.6[c,d]	1.9 ± 0.3	1.6 ± 0.3
SWA[b]	sham ligated	5	4.98 ± 6.17	2.70 ± 0.44	153.3 ± 23.4	3.3 ± 0.4	1.9 ± 0.3
	sham ligated + carrier	5	8.31 ± 7.29	2.92 ± 0.59	169.3 ± 18.7	3.5 ± 0.5	2.3 ± 0.3
	sham ligated + CT	5	14.27 ± 12.48	2.71 ± 0.52	164.7 ± 2.5	3.0 ± 0.2	2.4 ± 0.3[c]

[a] values are μmol kg^{-1} hr^{-1} ± S.D.; CT dose = 2.0 μg/eel

[b] FW-SW = freshwater to seawater ($[Ca^{+2}]$ = 7.0 mMol/L) transfer 21 Jan 1988
SWA = seawater acclimated ($[Ca^{+2}]$ = 8.4 mMol/L) 21 Mar 1988

[c] significantly ($P < 0.05$) different relative to the intact group of the same acclimation state

[d] significantly ($P < 0.05$) different relative to both other groups of the same acclimation state

Effect of calcitonin on total animal calcium accumulation and plasma ions in SWA and SWT oesophageal ligated eels

Calcitonin significantly ($P < 0.05$) reduced total calcium accumulation in transfer ligated eels relative to control ligated eels but not ligated/carrier injected fish and had no effect on calcium accumulation in seawater acclimated eels (Table 9).

Carrier buffer injection resulted in a significant ($P < 0.05$) decrease in plasma total potassium levels in the transfer fish but not in the SWA eels (Table 9). Treatment with calcitonin produced significant ($P < 0.05$) hypercalcemia in SWA ligated eels relative to the control oesophageal ligated fish and significantly ($P < 0.05$) increased plasma total magnesium concentrations compared to both the carrier injected and uninjected fish (Table 9).

Table 9: Effect of calcitonin on total animal calcium accumulation and plasma ion levels in oesophageal ligated eels

Acclimation State	Group	N	Total Animal Ca^{+2} Accumulation[a]	Plasma [ions] mMol L^{-1} Ca^{+2}	Na^{+}	K^{+}	Mg^{+2}
FW-SW[b]	ligated	5	13.10 ± 7.89	3.13 ± 0.62	170.1 ± 13.2	2.0 ± 0.3	1.9 ± 0.5
	ligated + carrier	5	5.61 ± 1.88	3.13 ± 0.22	173.8 ± 6.8	1.6 ± 0.1[c]	1.8 ± 0.3
	ligated + CT	5	4.62 ± 1.31[c]	2.99 ± 0.15	168.5 ± 5.3	1.7 ± 0.4	1.7 ± 0.5
SWA[b]	ligated	5	13.11 ± 2.92	3.00 ± 0.20	171.2 ± 8.2	3.9 ± 0.6	2.5 ± 0.2
	ligated + carrier	5	10.49 ± 3.46	2.83 ± 0.11	171.9 ± 5.2	3.3 ± 0.3	2.4 ± 0.4
	ligated + CT	5	13.45 ± 4.99	3.79 ± 0.22[c]	188.5 ± 17.1	3.7 ± 0.6	3.0 ± 0.2[c,d]

[a] values are μmol kg^{-1} hr^{-1} ± S.D.; CT dose = 2.0 μg/eel

[b] FW-SW = freshwater to seawater transfer ([Ca^{+2}] = 7.5 mMol/L) 12 Jan 1988
SWA = seawater acclimated ([Ca^{+2}] = 4.5 mMol/L) 22 Mar 1988

[c] significantly ($P < 0.05$) different relative to the ligated group of the same acclimation state

[d] significantly ($P < 0.05$) different relative to both other groups of the same acclimation state

Effect of calcitonin on total animal calcium accumulation, plasma ions and bone and muscle specific activity in freshwater eels rapidly transferred to seawater

Calcitonin produced no significant change in total animal calcium accumulation in intact fish (Table 10). In one experiment injection with CT elicited significant hypocalcemia compared to both carrier injected and control fish (Table 10) and significant ($P < 0.05$) hypernatremia relative to the intact eels, but in the other, treatment with calcitonin resulted in no changes in any of the ions measured. Injection with carrier buffer produced a significant ($P < 0.05$) increase in magnesium in one instance but otherwise had no effect (Table 10). Throughout both experiments the chloride concentration and plasma osmolarity remained unchanged.

Bone and muscle specific activity, per gram wet weight and per gram ash, were also unchanged in the CT treated group relative to the carrier injected and uninjected fish (Table 11).

Table 10: Effect of calcitonin on total animal calcium accumulation and plasma ion levels in freshwater eels rapidly transferred to seawater[a]

Acclimation State	Group	N	Total Animal Ca^{+2} Accumulation[a]	Plasma [ions] mMol L^{-1} Ca^{+2}	Na^{+}	K^{+}	Mg^{+2}	Osm[a]
Expt. I[b]	intact	6	5.30 ± 3.01	2.35 ± 0.41	150.6 ± 11.3	1.8 ± 0.3	1.3 ± 0.3	292.8 ± 22.7
	intact + carrier	6	7.99 ± 4.13	2.79 ± 0.31	150.9 ± 11.0	1.9 ± 0.8	1.8 ± 0.3[c]	304.3 ± 16.0
	intact + CT	6	9.36 ± 8.32	2.62 ± 0.33	138.7 ± 18.8	1.6 ± 0.2	1.6 ± 0.2	296.3 ± 22.2
Expt. II[b]	intact	6	2.96 ± 1.52	2.78 ± 0.26	154.8 ± 4.7	2.1 ± 0.3	1.4 ± 0.3	303.8 ± 14.4
	intact + carrier	6	3.00 ± 1.52	2.91 ± 0.28	159.8 ± 6.5	2.0 ± 0.2	1.6 ± 0.2	306.2 ± 11.9
	intact + CT	6	2.57 ± 0.67	2.46 ± 0.23[c,d]	162.6 ± 6.7[c]	2.1 ± 0.3	1.6 ± 0.2	311.8 ± 15.4

[a] values are μmol kg^{-1} hr^{-1} ± S.D.; Osm = osmolarity in mOsm; CT dose = 2.0 μg/eel

[b] Expt I. = intact fish minus a sample of muscle and bone ($[Ca^{+2}]$ = 5.7 mMol/L) 28 Dec 1987
Expt. II. = intact fish ($[Ca^{+2}]$ = 5.5 mMol/L) 05 Jan 1988

[c] significantly ($P < 0.05$) different relative to the intact group of the same experiment

[d] significantly ($P < 0.05$) different relative to both other groups of the same experiment

Table 11: Effect of calcitonin on bone and muscle ^{45}Ca specific activity following transfer from freshwater to seawater[a]

Groups	N	Bone Specific Activity		Muscle Specific Activity	
		per gram wet wt.[b]	per gram ash[c]	per gram wet wt.[b]	per gram ash[c]
intact	6	363.4 ± 230.3	26.6 ± 25.7	44589.5 ± 43831.4	424.1 ± 362.5
intact + carrier	6	389.3 ± 162.1	27.8 ± 11.1	16982.2 ± 13844.8	137.3 ± 75.8
intact + CT	6	369.4 ± 241.8	27.9 ± 14.7	9013.2 ± 7504.0	123.3 ± 117.7

[a] values are means ± S.D.; CT dose = 2 μg/eel; 28 Dec 1987
[b] units are DPM μmol^{-1} g^{-1} wet weight
[c] units are DPM μmol^{-1} g^{-1} ash

Effect of calcitonin on total animal calcium efflux in freshwater acclimated eels rapidly transferred to 70% seawater

Total body calcium efflux levels, as well as plasma total calcium, sodium, potassium and magnesium concentrations were unaffected in SWT eels treated with calcitonin (Table 12).

Table 12: Effect of calcitonin on total animal calcium efflux and plasma ions following transfer from freshwater to seawater[a]

Group	N	Total Animal Ca^{+2} Efflux[a]	Plasma [ions] mMol L^{-1}			
			Ca^{+2}	Na^{+}	K^{+}	Mg^{+2}
intact + carrier	7	0.003 ± 0.002	2.64 ± 0.42	156.7 ± 9.7	2.0 ± 0.2	1.7 ± 0.4
intact + CT	7	0.003 ± 0.001	2.99 ± 0.32	163.1 ± 3.2	2.2 ± 0.5	1.9 ± 0.1

[a] values are mean μmol kg^{-1} hr^{-1} ± S.D.; CT dose = 2 μg/eel
$[Ca^{+2}]$ = 7.0 mMol/L 01 Jan 1988

Effect of rapid transfer from freshwater into 70% seawater on the overall morphology of the ultimobranchial bodies of the eel

The ultimobranchial bodies isolated from the transverse septi of freshwater eels were follicular in structure with a large central lumen (Figure 2). The parenchyma appeared to be normal with lightly staining nuclei and densely staining nucleoli. The surrounding tissue was well vascularized (Figure 3).

The ultimobranchial tissue removed from eels which had been acutely transferred to seawater four hours earlier exhibited distinctly different morphology. The width of the parenchyma was increased (Figure 4) compared to the freshwater UBBs (Figures 2 and 3) and most of both the SWT secretory and support cells possessed large ovoid bodies which stained dark around the edges with methylene blue/azure II stain but which did not stain in the interior (Figure 4). Electron micrographs of this tissue revealed that these ovoid bodies were full of unidentified, small, regular clusters of darkly staining objects (Figure 8). These stressed cells also contained large normal nuclei with many aggragates of chromatin (Figure 5).

The luminal surface of the cells contained many groups of thin cytoplasmic processes (Figures 6 and 7). These structures were larger than microvilli and the lack of microfilaments suggests that these processes may be lamellapodia.

Figure 2: Ultimobranchial body from a freshwater fish showing a narrow epithelial structure and a wide lumen (L).
Magnification is 1230 X.
(Planapo 63/1.4 oil immersion lens; red filter on condenser)

Figure 3: Ultimobranchial body from a freshwater eel showing extensive vascularization at the basal side (arrow).
Magnification is 1230 X.
(Planapo 63/1.4 oil immersion lens; red filter on condenser)


L
2
L
1
3

Figure 4: Ultimobranchial body from a freshwater eel after four hours of exposure to 70% seawater.
Note the expanded epithelial layer (arrow) and the large ovoid bodies in each cell (B).
L = lumen
Magnification is 1230 X.
(Planapo 63/1.4 oil immersion lens; red filter on condenser)

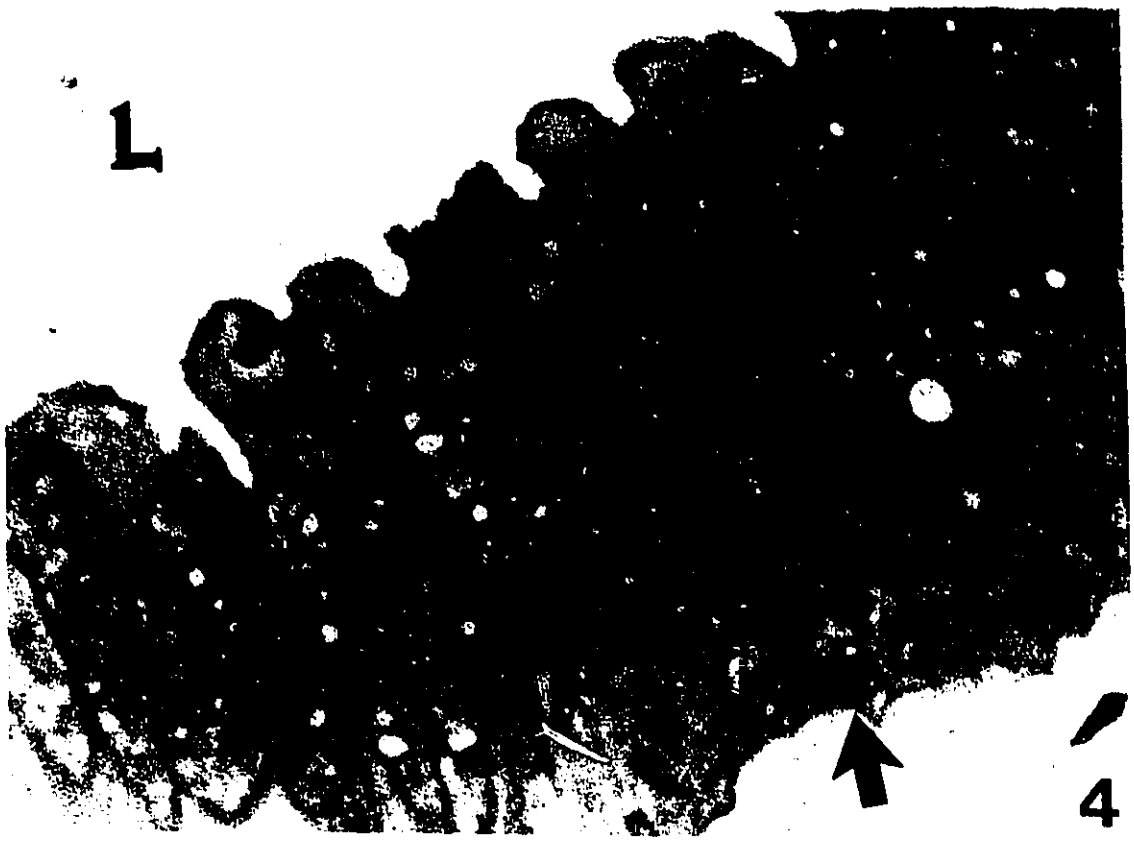
L
4

Figure 5: Electron micrograph of a nucleus from a freshwater eel after four hours of exposure to 70% seawater. Magnification is 30,000 X.
arrow = aggregated chromatin
N = nucleolus
G = golgi apparatus
H = hormone granules
M = nuclear membrane
S = supporting cell
R = ribosomes


N
R

Figures 6 and 7: Electron micrographs of the luminal membrane lamellapodia of an ultimobranchial body cell from a freshwater eel after four hours of exposure to 70% seawater.
Magnification on figure 6 is 34,500 X.
Magnification on figure 7 is 45,000 X.
D = cellular debris (pieces of discarded membrane)


D
6
7

Figure 8: Electron micrograph of the large, ovoid, membrane bound body within the secretory cell of an ultimobranchial body of a freshwater fish after four hours of exposure to 70% seawater. Note the regular darker staining objects (arrows). Magnification is 67,500 X.

D I S C U S S I O N

Overall, the calcium accumulation and plasma ion data obtained from this study are ambiguous and difficult to interpret. One clear conclusion, however, is that the occasional reduction in total body calcium accumulation seen following treatment with calcitonin is not a result of decreased drinking and if this apparent reduction is a physiological reality to the fish then the CT must be operating at a site other than the gut. A second general conclusion is that the plasma calcium concentration is not useful as an indicator of calcium regulation in eels. Histological results show that morphological changes in the ultimobranchial bodies occur within four hours after rapid transfer to a more hypertonic medium indicating that calcitonin, or at least the ultimobranchial tissue, might, in some way, be involved in calcium regulation in the American eel, Anguilla rostrata.

The life cycle of the American eel, Anguilla rostrata, involves a lengthy migration from the spawning grounds of the Sargasso Sea near Bermuda to the inland freshwater rivers of the east coast of the United States and Canada and then back again. Only the females extensively invade freshwater with the males tending to remain in the brackish water of the coastal region. During this migration, the eels encounter large fluctuations in

external ionic composition and, in order to survive, adjustments to homeostatic mechanisms are required. Hormonal interaction in these processes must be both extensive and complex. The discovery of the mammalian hypocalcemic hormone, calcitonin (Copp et al., 1962), led to the measurement of teleost plasma Ca^{+2} following CT injection, research which initially reported a hypocalcemic function for calcitonin in eels (Chan et al., 1968), catfish (Louw et al., 1967) and rainbow trout (Lopez et al., 1971).

However, some early reports presented data that calcitonin failed to produce any response in fish and sharks (Pang and Pickford, 1967; Louw, 1969). The isolation of the most biologically potent calcitonin from salmon (Otani et al., 1976) resulted in more research, but the contradictory data has done little to clarify the function of this hormone (Pang, 1971b; Lopez et al., 1976; Yamauchi et al., 1978; Glowacki et al., 1985; Fouchereau-Peron et al., 1987). Recently, the traditional hypocalcemic role of calcitonin has been challenged with the publication of data showing that salmon calcitonin can also be hypercalcemic (Wales, 1984; Glowacki et al., 1985; Fouchereau-Peron et al., 1987).

The findings of the present study are very diversified. In some instances calcitonin was hypocalcemic (Table 10), in some hypercalcemic (Tables 4, 6, 8, and 9) and in others it had no effect (Tables 4, 6, 8, 9, 10 and 12). It is noteworthy, however, that three of the four hypercalcemic responses occurred in seawater acclimated fish with the fourth appearing in a fresh-

water to seawater transfer experiment (Table 10). In all other freshwater to seawater transfer experiments, a bolus injection of calcitonin produced no effect on plasma total calcium concentrations.

The results obtained in the seawater acclimated eels are similar to those published by Wales (1984) and Fouchereau-Peron et al., (1987) but contradict those of Lopez et al., (1976), Wales and Barrett (1983), and Bjornsson and Deftos (1985). The discrepancy could be a result of the different doses injected. Wales found that a dose of 15 i.u. of salmon calcitonin (sCT) into a venous catheter produced hypercalcemia in seawater adapted eels after three hours (Wales, 1984) but doses of 50 or 100 i.u. (I.P.) of salmon calcitonin produced hypocalcemia (Wales and Barrett, 1983). Doses less than 10 i.u./fish produced no effect (Wales and Barrett, 1983). The opposite dose effect of salmon calcitonin was found in the rainbow trout (Fouchereau-Peron et al., 1987). In seawater and freshwater adapted trout low doses of sCT, injected into the ventral vessels of anaesthetized fish, resulted in hypercalcemia while higher doses produced hypocalcemia. The authors who report no effect (Pang, 1971b; Pang and Sawyer, 1975; Bjornsson and Deftos, 1985) or hypocalcemia (Lopez et al., 1976; Glowacki et al., 1985) in freshwater or seawater adapted fish also used salmon calcitonin but if the doses are converted to similar units it is found that these results generally arise from high doses of sCT.

In the present study, a dose of 2μg/fish of eel calcitonin

resulted in hypercalcemia in seawater adapted eels. Elsewhere, a similar dose of salmon calcitonin in European eels of the same acclimation state produced no change in blood calcium levels (Wales, 1984). This may reflect a difference in ability to respond to heterologous calcitonins.

Experiments involving freshwater eels injected with various CTs have produced data concluding that calcitonin is hypocalcemic (Chan et al., 1967; Pang, 1971b; Wales and Barrett, 1983). However, when freshwater eels are dosed with eel calcitonin, there is no change in plasma total calcium concentrations (Yamauchi et al., 1978; Hirano et al., 1981) and these findings are corroborated by the present study. The tracing of plasma calcium levels during the transfer between freshwater and seawater has also previously resulted in data showing that calcitonin has no effect on plasma calcium (Bjornsson and Deftos, 1985; Fouchereau-Peron et al., 1986). Transfer plasma data in Tables 4, 6, 7, 9, 10, 12 matches these previous data. Tables 8 and 10, however, contradict these earlier reports suggesting that changes in the plasma total calcium concentration are unreliable as an indicator of the effects of calcitonin on calcium homeostasis in eels.

The injection of calcitonin produced hypermagnesemia in approximately 75% of the experiments performed. The effect was observed most often in the seawater studies but was seen also in the freshwater and transfer experiments. Unfortunately, we know virtually nothing about magnesium regulation in vertebrates.

Hypernatremia, as a result of calcitonin injection or the combination of CT and surgery, was seldom seen in the seawater experiments, but was common, although sporadic, in the transfer studies. Potassium concentrations varied in only two experiments. These changes indicate that calcitonin may be involved more or less specifically in divalent ionoregulation.

The injection of calcitonin in freshwater and seawater acclimated ligated eels as well as in transfer fish did not alter the amount of calcium accumulated during the four hour experimental period (Table 3). Thus it appears that the previously seen decrease in total body calcium accumulation after treatment with calcitonin (Kingsbury and Fenwick, to be published elsewhere) is lost after oesophageal ligation which renders seawater and transfer fish unable to drink (see gut accumulation values Table 3). In isolation, these data support the hypothesis that CT functions to reduce calcium influx by altering drinking patterns, but this conclusion is inconsistent with other observations.

Ligated fish which received an injection of calcitonin exhibited significantly lower gut accumulation of calcium, suggesting that calcitonin impairs the eel's ability to unload the isotope from the blood into the stomach. The reason behind this strategy in unknown as during the severe calcium challenge associated with direct transfer, it would be advantageous for the eel to secrete excess calcium into the gut for rapid removal. However, seawater acclimated eels do not increase their urine output or electrolyte excretion rate after injection with

calcitonin (Wales, 1984), and while this parameter has not been measured in fish after transfer to a more hypertonic medium, it is possible that ion excretion is high immediately following transfer and then, during the course of acclimation, the system is regulated back to basal levels.

When directly compared, the effect of calcitonin on calcium accumulation in SWA and SWT eels was not statistically different from that of ligation or sham oesophageal ligation (Table 5). However, in the seawater eels used in this experiment, CT significantly reduced the extent of hypercalcemia, hyperkalemia and hyperosmolarity relative to the ligated and control fish. This suggests that this hormone has an effect on ion balance in SWA eels but as plasma calcium changes are inconsistent further work on whole body fluxes is needed to clarify this effect.

The unchanged total body calcium accumulation in SWA sham or oesophageal ligated eels despite increases in plasma calcium (Table 7) suggests that the ability to drink is not as important to ion regulation in eels as previously described in the literature (Gaitskell and Chester Jones, 1971; Hirano, 1974; Kobayashi et al., 1983). Even during the more stressful transfer experiment oesophageal ligation of the eels did not result in any major disruption of ion homeostasis. This may be a result of the stress itself causing a rise in cortisol levels, a situation which would mask individual hormonal responses. However, the levels of cortisol during the transfer were not measured in the present study.

Ligation plus calcitonin in transferred fish resulted in a significant decrease in total animal calcium accumulation (see Table 9) and gut calcium accumulation (see Table 3), but no change in plasma calcium, compared to both ligated and ligated/carrier injected controls (see Table 9). This indicates that calcitonin exerts its effect on total animal calcium accumulation in freshwater fish transferred to seawater at a site other than the gut. However, as the effect generated by calcitonin was not statistically different from that seen in the carrier injected group it is unclear whether the CT or the solvent elicited the decrease. The calcitonin did not produce the same effect in seawater acclimated fish but it did produce hypercalcemia. This again suggests that CT is affecting an ion exchange site other than the gut.

Two other sites, the bone and the muscle, were examined to see if mobilization of calcium from these tissues was responsible for the hypercalcemia seen in some of the experiments. The action of CT on mammalian bone is achieved through the activation of adenyl cyclase and thus 3',5'-cyclic AMP production (Rotella et al., 1985; Gordeladze and Gautvik, 1986; Nicholson et al., 1986). However, unlike in rats, in fish calcitonin increases the degree of mineralization of the cellular matrix in the vertebral bone (Lopez et al., 1976). Additionally, cellular eel bone has been shown to be capable of supporting a higher plasma calcium concentration than can rat bone (Fenwick, 1974), but this effect is seen over a time span of 21 days. Further, the type of

teleost bone, be it cellular or acellular, does not confer sensitivity to calcitonin; cellular boned catfish and salmon do not always respond to CT (Pang, 1971; Suryawanski and Mahajan, 1976). In the present study, no change in either the bone or muscle ^{45}Ca specific activity was found after the injection of calcitonin. Although hypercalcemia has been obtained in eels within a few hours of transfer to seawater (Wales, 1984; Fouchereau-Peron, 1987) the present study, which concluded after four hours, did not produce hypercalcemia as a consequence of transfer. Thus, it is unclear whether changes in mobilization of bone Ca^{+2} could be a factor in producing an increase in plasma calcium.

Although Hirano and Hasegawa (1984) reported that eel calcitonin had no effect on the drinking behaviour of Japanese freshwater eels, their method of delivery was via intra-arterial cannula, a method which had previously been shown to be ineffective in both fresh- and seawater acclimated eels (Hirano et al., 1981). While infusion of calcitonin into the ventral venous vessels has proven to be more likely to generate a response (Wales, 1984; Fouchereau-Peron et al., 1987), the present study employed intraperitoneal injection as a delivery method as this technique has previously been shown to be effective (Fenwick and Lam, 1988; Kingsbury and Fenwick, unpublished).

The fluctuating plasma ion concentrations and calcium influx values may be due to the non-continuous pattern of calcium regulation seen in teleosts (Wagner et al., 1985; Wagner and

McKeown, 1985) and rats (Perault-Staub et al., 1974). Research using young growing rainbow trout fry has resulted in the report of cycles of ^{45}Ca uptake (Wagner et al., 1985) and growth (Wagner and McKeown, 1985) encompassing an average of 11 days and 3-4 weeks respectively. Flux studies with adult tilapia, however, detected no significant change in calcium influx rates throughout the year (Flik et al., 1985). In this laboratory, we have discovered extensive variation in calcium influx in eels with time (Clarke, Kingsbury and Fenwick, in preparation). Rats experience large daily swings of plasma Ca^{+2}, a pattern that is precipitated, in part, by calcitonin (Perault-Staub et al., 1974). The hypocalcemia produced by CT release is part of the anticipatory regulation of calcium in rats which effects plasma Ca^{+2} prior to the absorption of calcium from the intestinal tract. Although a similar daily pattern has not been reported in fish, the existence of such a method of homeostasis might explain some of the variation in calcitonin mediated responses to changes in Ca^{+2}. In deference to a possible cyclical fluctuation in eel calcium regulation, control groups were included for each experiment. This excluded the possibility that any observed effect of calcitonin was due to variation in the eel's ability to respond to the exogenous hormone.

The regulation of intestinal activity in mammals and fish involves many hormones, including calcitonin. CT suppresses gastric emptying in humans (Jonderko et al., 1987) possibly allowing for more calcium absorption, while in dogs, salmon

calcitonin decreased the amount of water and sodium absorbed in the gut (Primi and Bueno, 1986). In rats, calcitonin affected calcium flux in brain cells and restored the fasted pattern of intestinal motility in fed animals (Fargeas et al., 1985) and can inhibit feeding (Levine et al., 1981). In fish, little has been done to determine the effects of CT on intestinal function. But Nakamura and Hirano (1986) have reported that calcitonin had no effect on calcium or water absorption in stripped non-everted eel intestines.

It is interesting to note that removal of any body tissue after anaesthetization resulted in the disappearance of the significant decreases in calcium accumulation or plasma calcium seen in previous experiments which did not involve tissue removal before whole animal homogenization (see Table 10). Differences in the amount of tissue removed or leakage of bodily fluids due to the technique of removal could result in enough experimental error to mask any changes in calcium accumulation in the body or blood. Individual responses to the effects of surgery also caused inconsistent results (see Table 7) as did the time of year the experiment was performed. Data collected from several researchers in our laboratory indicates that while plasma calcium concentrations remain somewhat stable over a year, large fluctuations in whole body calcium influx occur from one season to the next and even between successive months (Clarke, Kingsbury and Fenwick, in preparation). Careful planning to conduct similar experiments in the same season along with higher N values may

help to reduce the error.

Starvation and artificial photoperiod have also been known to produce changes in ion flux. For example, in isolated perfused trout head from starved SWT fish, net sodium influx decreases due to delayed chloride cell modifications (Nance et al., 1987). However, eels naturally undergo a phase of decreased feeding during the winter months (Tesch, 1977) and display a degree of lethargy when the water temperature drops below 10°C. Thus, the eels used in this study are in a quasi-normal physiological state of starvation and their metabolism is presumably adjusted to compensate. Eels are also sensitive to lighting conditions (Tesch, 1977). The seawater fish used in this study were acclimated in aquaria exposed to 24 hours of light to minimiz(escape as eels are extremely active in the dark between the hours of one and three a.m. (Medcof, 1966; Hain, 1975). However, because control groups were chosen from the same stock of fish any photoperiod effect was, in part, negated.

Eel calcitonin also did not affect total animal ^{45}calcium efflux in freshwater eels transferred to seawater (Table 12) and this result contradicted those of Dacke (1975) and Leung (1979). However, Leung injected bovine CT into $CaCl_2$ loaded eels and Dacke used highly potent salmon calcitonin, factors which could account for the discrepancy in results.

Differences in values in identical groups between experiments may also be due to the fishes response to the varying $[Ca^{+2}]$ in the artificial seawater. 70% seawater was initially

chosen as this concentration is intermediate to that experienced by eels during their migration and to lessen the shock of transfer. Exact [Ca^{+2}] differed between the experiments due to oversight and slight changes in the physiological responses, either gill morphology or drinking rates, could be responsible for the variable accumulation rates and plasma values.

Responses to other calcium regulatory hormones immediately after transfer may, too, have impacted on the results. For example, there appears to be an interaction between CT and Vitamin D. Recently, it has been reported that injections of Vitamin D_3 in rats resulted in significant increases in plasma CT levels in spite of the fact that plasma Ca^{+2} concentrations were stable (Segond et al., 1985). Further, porcine calcitonin in rats decreased the Vitamin D induced increase in calcium re-absorption from the intestine and prevented hypercalcemia (Olson et al., 1972) indicating that the two hormones are involved in direct negative feedback regulation of calcium homeostasis in mammals. In fish, Vitamin D_3 has produced hypercalcemia in fed but not unfed eels (Fenwick and Belanger, 1984). But, Vitamin D_3 did not increase the level of hypercalcemia above that seen in unfed-stanniectomized controls (Fenwick and Belanger, 1984). The authors concluded that food is requisite for a hypercalcemic response to Vitamin D_3 in eels and that Vitamin D_3 produces hypercalcemia in teleosts, as in mammals, by increasing the intestinal absorption of Ca^{+2}. However, the eels used in the present research were maintained unfed throughout the course of

the study so that any hypercalcemic response seen can probably be attributed to the action of calcitonin and not Vitamin D_3.

The hypercalcemic substance, prolactin, could also affect or counter-act the hypocalcemia produced by calcitonin. Indeed, in rats, the two hormones operate in a negative feedback loop with injection of one stimulating production of the other (Aleshin et al., 1985). However, in fish, injections of prolactin do not normally produce changes in calcium levels until several days post-treatment (Bern et al., 1981). Nevertheless, the hypercalcemia seen in some experiments in the present study suggests that the four hour time course of the fluxes may be sufficient time for this feedback system to begin restoring control levels of Ca^{+2}.

The structure of the freshwater eel ultimobranchial bodies obtained in this study is similar to that documented elsewhere (Yamane, 1977; Robertson, 1986). The overall structure of the endocrine gland following an acute change from freshwater to seawater remained the same as the freshwater controls but individual cells increased in length and produced large, membrane bound, ovoid bodies, a phenomenon not yet characterized.

The morphological changes in the ultimobranchial bodies following transfer to seawater matches previous data showing that after rapid transfer, intact eels are able to maintain calcium accumulation levels, similar to those exhibited by the intact controls, but only if injected with calcitonin (see Table 10, Expt. II). When not supplied with exogenous CT after transfer,

the eels seem to be unable to produce sufficient hormone to prevent an increase in both calcium accumulation or plasma total calcium. However, the changes seen in the ultimobranchial tissue indicate that cellular modifications are occurring and these may be associated with increased production. This indicates that the four hour time frame allowed for a response to an environmental disturbance may be too short to allow for endogenous hormone production.

The membrane-bound ovoid bodies in the SWT ultimobranchial tissue, and the darkly staining regular objects in them, have not been identified. It is possible that the ovoid bodies contain colloid material in preparation for cystolysis similar to that seen in frogs (Boschwitz, 1969). The formation of cysts may represent the near exhaustion of the ultimobranchial gland following such an acute change in external calcium.

The lack of a multitude of stained hormone granules in the transferred fish glands presents a question. Has most of the calcitonin been secreted in response to the calcium stress or did the stain not mark the majority of granules? It is also possible that the sections examined were superficial and thus few granules were seen. Immunohistochemical work to localize the calcitonin granules has determined that the majority of granulation is in the basal portion of the cytoplasm (Sasayama et al., 1984). Although no granulation was detected in the experimental group it is possible that the lead citrate and uranyl acetate stains do not stain calcitonin secretory granules in eels. However, the

staining technique employed in the present study has previously resulted in the detection of CT secretory granules in zebrafish, Brachydanio rerio (Yamane, 1978) and Masu salmon (Yamane and Yamada, 1976).

In conclusion, eel calcitonin does not reduce calcium accumulation in freshwater eels transferred to seawater or in seawater acclimated eels by causing a decrease in the amount of environmental water ingested. The data procured during this study lends credence to the hypothesis that calcitonin, if it works at all, works at the site of the gills and indicates that future work should be directed at this area. In addition, data has been presented which suggests that calcitonin plays a minor role in calcium regulation under the experimental conditions utilized. Very surprisingly, the data also suggests that the gut plays a much less important role in osmoregulation in eels than is generally assumed. In fact, the survival of ligated eels for four days in seawater seriously challenges the established dogma concerning the primacy of the gut in seawater acclimated fish and deserves much more attention.

Although the ultimobranchial bodies of eels respond to the calcium challenge associated with transfer to seawater as indicated by the histological data showing the manufacture of ovoid membrane bodies in the ultimobranchial cells, it appears that the administration of exogenous eel calcitonin does not affect calcium homeostasis.

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APPENDIX A

Summary of Honors data involving the effects of calcitonin in freshwater and seawater acclimated eels and in eels rapidly transferred from freshwater to 70% seawater[a].

Acclimation State	CT dose[b]	Total Body Calcium Accumulation		Plasma total Ca^{+2}	
		Control	Injected	Control	Injected
FW[c]	0.02	0.48 ± 0.16	0.21 ± 0.09	2.1 ± 0.1	3.1 ± 0.1[e]
	0.2	0.59 ± 0.24	0.35 ± 0.14	2.0 ± 0.3	2.1 ± 0.1
	1.0	0.73 ± 0.28	0.67 ± 0.31	5.0 ± 0.2	5.4 ± 0.2
	1.6	1.19 ± 0.17	1.08 ± 0.21	4.8 ± 0.1	4.7 ± 0.3
	10.0	0.38 ± 0.04	0.53 ± 0.11	3.7 ± 0.1	3.5 ± 0.4
FW-SW[c]	2.0	5.14 ± 0.78	1.02 ± 0.20[d]	3.7 ± 0.1	4.1 ± 0.2
	2.0	2.49 ± 0.60	0.58 ± 0.13[d]	5.1 ± 0.3	4.7 ± 0.1
	2.0	1.84 ± 0.48	0.36 ± 0.10[d]	4.7 ± 0.1	4.7 ± 0.1
SWA[c]	2.0	1.00 ± 0.17	0.36 ± 0.10[d]	4.6 ± 0.1	4.2 ± 0.2

a accumulation values are mean μmol kg^{-1} hr^{-1} ± S.D.
plasma Ca^{+2} are mean mMol/L ± S.D.
b doses are μg/fish
c FW = freshwater
FW-SW = freshwater acclimated fish transferred to 70% seawater
SWA = seawater (70%) acclimated fish
d significantly ($P < 0.05$) lower than the control value of the same acclimation state
e significantly ($P < 0.05$) higher than the control value of the same acclimation state

A P P E N D I X B

Pilot research was performed using the neotonous salmander, the axolotl, Ambystoma mexicanum, to determine its response to bolus injections of calcitonin.

As in fish, eel calcitonin elicited a significant decrease in calcium influx but no corresponding change in any of the plasma ions measured.

The conclusion drawn from this data indicates that axolotls may regulate plasma calcium in a manner more similar to teleosts than amphibians.

This work also serves as an assay for the lot of calcitonin used in the experiments contained in the main body of this thesis. Biological activity was not impaired during the lyophilization procedure as the exogenous hormone produced changes in experimental parameters in two diverse species.

THE EFFECT OF EEL CALCITONIN ON CALCIUM INFLUX AND PLASMA ION LEVELS IN AXOLOTLS, *Ambystoma mexicanum*

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Kingsbury, D.L. and J.C. Fenwick. The Effect of Eel Calcitonin on Calcium Influx and Plasma Ion Levels in Axolotls, Ambystoma mexicanum

Plasma total calcium and calcium influx, measured during four hour calcium^{45} influx experiments, were both lower in control axolotls, Ambystoma mexicanum, during August when compared to July. A single intraperitoneal injection of 10μg of synthetic eel calcitonin significantly reduced the calcium influx rates during both months but the effect was greater in July ($P<0.001$) than in August ($P<0.05$). Plasma levels of calcium, sodium, potassium and magnesium were not affected. In paedomorphic wholly-aquatic amphibians, eel calcitonin may work in the same way as it does in fish by reducing the uptake of dissolved calcium from the ambient medium.

INTRODUCTION

Fish (Sasayama et al., 1984) and amphibians (Robertson, 1986) both possess ultimobranchial tissue and produce and release calcitonin (Oguro et al.,1981) which is routinely described as having hypocalcemic action in mammals (Copp et al.,1962). Although the findings have not been consistent (for review see Taylor, 1985), calcitonin has also been shown to be hypocalcemic in both fish (Mathur, 1979; Wales, 1984) and amphibians (McWhinnie and Scopelliti, 1978; Sasayama, 1978; Oguro et al., 1983).

However, the basic mechanism of calcium regulation in fish and amphibians seems to be different. Most adult anurans have parathyroid glands and appear to regulate their plasma calcium by moving calcium between their plasma and body calcium stores. Fish, on the other hand, have constant access to the calcium dissolved in their surroundings and they use environmental calcium both as a source and a sink for plasma calcium regulation (Fenwick and Wendelaar Bonga, 1982).

This difference is reflected in the effect of calcitonin. In fish, calcitonin reduces calcium uptake from the environment (Milhaud et al., 1977; Peignoux-Deville et al., 1978; Milet et al., 1979), but in amphibians, calcitonin inhibits the PTH stimulated mobilization of internal calcium stores (Robertson, 1977). Certainly, the observations of Sasyama (1978), who showed that plasma calcium levels in ultimobranchialectomized tadpoles became markedly hypercalcemic in high calcium environments,

suggest that calcitonin may reduce calcium influx. And further, the fact that the parathyroids only appear at metamorphosis in some urodeles and do not appear at all in some neotonous salamanders (Clark, 1983) such as the mudpuppy (Necturus) strongly suggests that during their wholly aquatic phase, amphibians may regulate their plasma calcium in the same way as fish regulate theirs.

Accepting these assumptions, we wondered if the observed different sites of action of calcitonin represent genetic differences or physiological opportunities. To test this, we measured the effect of calcitonin on calcium uptake in the axolotl, Ambystoma mexicanum, a neotonous salamander which habitually lives submerged in water and which possesses well developed external gills.

MATERIALS AND METHODS

Animals: Artificially raised, sexually mature, male and female axolotls, Ambystoma mexicanum, (120 ± 5 grams) were taken from the University of Ottawa Axolotl Colony in July and August, 1987. The animals were raised for two weeks prior to the experiment in water containing 1.5% NaCl, 0.25% KCl, 0.5% $CaCl_2$, 1.0% $MgSO_4$, and 1.0% $NaHCO_3$. The axolotls were fed raw beef heart and their water was replaced every two days. The animals were fasted for 48 hours prior to the experiments.

Experimental Protocol: Twenty-four hours before the experiment the axolotls were placed in black plexiglass flux boxes (volume= 1L; 7cm H X 8cm W X 31cm L) containing 500ml of the above medium. Immediately prior to the start of the experiment, the axolotls were removed from the flux boxes and injected intraperitoneally with either 10 μg of eel calcitonin in 0.2ml of solvent containing 1% sodium acetate trihydrate and 0.1% glycine in distilled water (treated axolotls) or solvent alone (controls). The animals were immediately returned to the flux boxes. During the injection the animals were out of water for no more than one minute.

Sufficient radiocalcium was then added to each of the boxes to give 1.20×10^5 DPM ml^{-1} in July and 6.0×10^4 DPM ml^{-1} in August (specific activity (July: 4.04×10^5 DPM $umol^{-1}$; August: 2.19×10^5 DPM $umol^{-1}$). One 1ml water sample was taken 10 minutes after the start of the flux and two 1ml samples were taken at the end of the four hours in order to calculate the

specific activity of the ambient medium.

The axolotls were killed by over-anaesthetization in MS-222 (10g/L containing 10 mM $CaCl_2$ to displace surface bound Ca^{45}). After weighing, terminal blood samples were taken from the bulbous arteriosus. Plasma was separated by centrifugation (2 minutes, 5000 x g) and stored at -20°C until analyzed. The bodies were frozen at -20 °C until homogenized.

Homogenization: Frozen axolotls were microwaved for 4 minutes at the high setting and were then homogenized with 200ml of City of Ottawa tap water in a Waring Commercial blender. One aliquot (10-15 ml) of the homogenate was weighed and dried at 75°C for twelve hours. The dry samples were subsequently ashed in a Fisher Isotemp muffle furnace, Model 184A, for five to seven hours at 550 °C. The ashes were dissolved overnight in 5ml of 1N HCl. The resulting solution was neutralized with 833µl of 6N NaOH. Three 1ml samples of this solution, each mixed with 4ml of distilled water and 10ml of Aqueous Counting Scintillant (ACS II, Amersham Canada, Ltd., Oakville, Ontario), were counted for their ^{45}Ca content in an LKB Wallac 1215 Rackbeta Liquid Scintillation Counter with a built in DPM program.

Ions: Plasma calcium, sodium, potassium and magnesium were measured, after appropriate dilution, using a Varian SpectraAA-10 atomic absorption flame emission spectrophotometer.

Calculations: Unidirectional calcium influx (J_{in}) was calculated by the following formula:

[1]
$$J_{in} = \frac{WBC \times 1000}{SA \times T \times W}$$
$$= \mu mol\ Ca\ kg^{-1}\ hr^{-1}$$

Where WBC = whole body ^{45}Ca counts; SA = specific activity of the ambient medium at the end of four hours; T = duration of the flux in hours; W = weight of the animal in grams.

Statistics: Data were analyzed using a two-way ANOVA. Significances between individual pairs of means were tested using the Student-Newman-Keul's Test.

RESULTS

In July and August synthetic eel calcitonin significantly ($P<0.001$ and $P<0.05$ respectively) reduced calcium influx (Table 1). Calcium influx of the buffer injected control axolotls was significantly ($P<0.05$) higher in July compared to the buffer injected control axolotls in August. However, the calcium influx of the calcitonin injected axolotls was significantly ($P<0.05$) higher in August than in July (Table 1). The interaction (ANOVA) between the effect of CT and the month of treatment was also significant ($P<0.05$).

Plasma total calcium levels were significantly lower ($P<0.05$) in both groups of August axolotls but neither this parameter, nor those of plasma sodium, potassium or magnesium were affected by the calcitonin injection.

DISCUSSION

The ability of calcitonin to decrease calcium influx in axolotls is similar to the effect reported in some teleosts (Milhaud et al, 1977; Peignoux-Deville et al, 1978; Milet et al., 1979) and suggests that these neotonous salamanders regulate their plasma calcium in a fashion which is more similar to that of fish than conventional amphibians. This conclusion is supported by the observation that calcitonin produced no consistent changes in plasma calcium, sodium, potassium or magnesium concentrations in axolotls contrary to its effects in typical amphibians (Boschwitz and Bern, 1971; Sasayama, 1978; Oguro et al, 1980; Oguro et al, 1983).

Plasma total calcium is generally a poor index of calcium turnover. Several studies on fish reported that calcitonin was hypocalcemic (Mathur, 1979; Wales, 1984; Glowacki, 1985) while others report a hypercalcemic response (Glowacki, 1985; Fouchereau-Peron et al., 1987). Reptilian, amphibian and mammalian studies are also inconsistent in that CT has been found to be either hypocalcemic or to have no effect (Hadley, 1984; Taylor, 1985; Srivastav et al, 1986).

These preliminary studies were conducted in the late summer to coincide with the time of the maximal hypocalcemic effect of amphibian ultimobranchial glands as described in frogs (Robertson, 1977). However, whereas the peak of Rana ultimobranchial activity is in August, axolotls exhibited a greater percent decrease in calcium influx in response to calcitonin in July,

suggesting that the peak of ultimobranchial activity varies between different amphibians. Calcium cycles have also been reported in fish, but the periods are shorter (Wagner et al., 1985), suggesting correlations to factors other than seasons. Additional research is needed to document any existence of a calcium cycle in axolotls and if present, its periodic nature. A short cycle would more fully support our conclusion that axolotl calcium regulation is more similar to the teleost model than the amphibian.

The conclusion that the function of calcitonin is a product of an organism's external environment and not a product of its phylogenetic placement provides a novel perspective for the study of comparative calcium regulation. Additional experiments using other neotonous salamanders, as well as air-breathing fish could broaden the generality of this conclusion.

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TABLE 1

EFFECT OF SYNTHETIC EEL CALCITONIN ON CALCIUM INFLUX IN AXOLOTLS, *Ambystoma mexicanum*[a]

Date	μg/animal	Buffer Injected	Calcitonin Injected
July	10	3.28 ± 0.93	0.16 ± 0.04[b]
August	10	1.27 ± 0.16[d]	0.83 ± 0.09[c,d]

[a]Data are means ± S.E.M. (N = 7) expressed as μmol kg^{-1} hr^{-1}.

[b]Significantly ($P<0.05$) lower than buffer injected.

[c]Significantly ($P<0.001$) lower than buffer injected.

[d]Significantly ($P<0.05$) different than July values.

TABLE 2

EFFECT OF EEL CALCITONIN ON PLASMA TOTAL CALCIUM, SODIUM, POTASSIUM AND MAGNESIUM IN AXOLOTLS, *Ambystoma mexicanum*[a]

	Calcium (mM)		Sodium (mM)		Potassium (mM)		Magnesium (mM)	
Date	Controls	CT[b]	Controls	CT[b]	Controls	CT[b]	Controls	CT[b]
July	1.76 ± 0.05	2.00 ± 0.05	107.9 ± 3.6	104.4 ± 0.9	4.13 ± 0.07	3.40 ± 0.54	0.77 ± 0.09	0.77 ± 0.07
August	1.50 ± 0.03[c]	1.54 ± 0.10[c]	101.6 ± 2.2	102.0 ± 1.3	3.01 ± 0.21	2.88 ± 0.15	0.67 ± 0.05	0.73 ± 0.02

[a]Data are means ± S.E.M.; For sodium, potassium and magnesium

N = 6 for July, 1987 groups and N = 7 for August, 1987 groups.

N = 7 for all calcium data.

[b]Injected with a single dose of 10 pg of synthetic eel calcitonin.

[c]Significantly ($P<0.05$) lower than the July group.