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EFFECTS OF LOW AMBIENT CALCIUM LEVELS ON WHOLE-BODY Ca^{2+} FLUX RATES AND INTERNAL CALCIUM POOLS IN THE FRESHWATER CICHLID TELEOST, *OREOCHROMIS MOSSAMBICUS*

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SUMMARY

Calcium fluxes and internal calcium pools were measured in fed, rapidly growing, male tilapia, *Oreochromis mossambicus*, acclimated to 0·8 mmol l^{-1} (FW) and 0·2 mmol l^{-1} (LFW) Ca^{2+}. Plasma calcium levels were slightly and significantly higher in the LFW tilapia, but muscle calcium concentrations were independent of ambient Ca^{2+}. At the time of the experiments, the LFW fish were growing and accumulated calcium, although the calcium content of their hard tissues was reduced. The LFW fish had higher Ca^{2+} influx and efflux rates than the FW fish. The increase in the influx of Ca^{2+} in LFW fish was, however, substantially greater than the increase in the efflux of Ca^{2+}, giving these fish a more than four-fold increase in net Ca^{2+} influx from the water: for a 20-g tilapia net uptake rates of Ca^{2+} from the water were 390 and 1620 nmol Ca^{2+} h^{-1} for FW- and LFW-adapted fish, respectively. These values were calculated to represent at least 69 % of the total calcium accumulated by these growing fish. This indicates that even in low-calcium water, tilapia absorb a significant amount of their calcium requirement directly from the water.

The pool of readily exchangeable calcium in the bone of FW fish was estimated to be about 7 % of the total hard tissue calcium. In the fish acclimated to LFW, this percentage increased to about 15 % as total hard tissue mineralization decreased. This may indicate that tilapia can increase the mobility of their hard tissue calcium during periods of low calcium stress.

INTRODUCTION

Freshwater fish can and do accumulate calcium directly from the water by absorption across the gills (Simkiss, 1974; Milhaud, Rankin, Bolis & Benson, 1977;

Key words: teleost, bidirectional Ca^{2+} fluxes, low ambient calcium, internal calcium stores.

Mayer-Gostan *et al.* 1983), and in at least some species of fish this mode of calcium accumulation is sufficient to maintain normal growth, even when the fish are fed a calcium-deficient diet (Ogino & Takeda, 1976, 1978; Watanabe *et al.* 1980; Ichii & Mugiya, 1983). In fact, even when calcium is supplied with the food, direct absorption of calcium from the water *via* the gills prevails (Berg, 1970). This last uptake mechanism may be expected to function at full capacity in growing fish which, of course, must be supplied with all other nutritional requirements. Undernourished fish cannot be expected to grow and lay down mineralized tissue. Indeed, under conditions of starvation, fish show no callus formation in response to bone fracture, as was shown for *Carassius auratus* and *Tilapia macrocephala* (Moss, 1962); carp (*Carassius carassius*) are known to demineralize their scales during starvation (Ichikawa, 1953).

It is well known that net calcium uptake in freshwater fish is considerably higher than in seawater fish. This is frequently considered to point to the existence in fish of a compensatory calcium uptake mechanism *vis-à-vis* the availability of calcium in the water. This prompts the question whether the calcium levels in fresh waters – which show considerable variation – determine the magnitude of calcium accumulation in fish which inhabit such environments.

The study reported here was designed to investigate the role of ambient calcium on the net uptake of calcium from the water in growing specimens of the freshwater cichlid species *Oreochromis mossambicus*. Fish were acclimated to two levels of calcium in the water. Employing the isotopes ^{47}Ca and ^{45}Ca, Ca^{2+} influx and Ca^{2+} efflux were determined directly using a procedure which was developed earlier (Flik *et al.* 1985*a*). In addition, total, as well as readily exchangeable, calcium pools of the hard tissues were measured. The rates of uptake of calcium from the water were evaluated in relation to growth rates. A possible role for internal stores in the calcium homeostasis of the fish is discussed.

MATERIALS AND METHODS

Male tilapia, *Oreochromis mossambicus*, weighing 10–30 g were used throughout this study. They were obtained from laboratory stock and were held under conditions as described previously (Flik *et al.* 1985*a*).

^{45}Ca and ^{47}Ca were purchased as calcium chloride in aqueous solution (Amersham International plc, England). Specific activities were: for ^{45}Ca, 9·25–37·5 GBq mol^{-1} Ca; for ^{47}Ca, at least 0·74 GBq mol^{-1} Ca. Only reagent grade chemicals (Sigma) were used.

Acclimation

Fish used in the Ca^{2+} flux studies were acclimated to artificial fresh water prepared from demineralized water and containing (in mol l^{-1}): NaCl, 3·8; KCl, 0·06; $MgSO_4$, 0·2, and $CaCl_2$, either 0·8 or 0·2; the pH was adjusted with $NaHCO_3$ to 7·4 ± 0·2. The final osmolarity was 8–10 mosmol l^{-1}. Water containing 0·8 mmol l^{-1} $CaCl_2$ is referred to as fresh water (FW) and approximates to the Nijmegen city tap

water in which the laboratory stock of tilapia is kept and bred. Low-calcium fresh water, containing 0·2 mmol l^{-1} $CaCl_2$, is referred to as LFW. Fish were acclimated to LFW by transferring them first from FW to FW containing 0·4 mmol l^{-1} $CaCl_2$, and 1 day later to LFW. Throughout the adaptation period and during the experiments, both pH and Ca^{2+} concentrations of the water were monitored and adjusted as required. Ammonia levels were kept below 2 μmol l^{-1} NH_4^+ by charcoal filtration and by changing the water. Fish were maintained in their acclimation medium for at least 10 weeks before the start of flux determinations and were fed their normal ration of food. At the time of flux determinations the fish were growing at a rate of about 3–5 % body weight per month and they remained healthy.

Determination of Ca^{2+} flux rates

Ca^{2+} flux rates were determined as described earlier (Flik *et al.* 1985*a*). A whole-body counter and ^{47}Ca (92·5–370 KBq l^{-1} water) as a tracer were used in influx studies; efflux rates of Ca^{2+} were determined in two ways yielding either (i) branchial or (ii) total efflux. (i) Branchial efflux was determined 4 days after intraperitoneal injection of $^{45}Ca^{2+}$ (37–74 KBq g^{-1} fish), on the basis of tracer appearance in small volumes of water (0·5–1 l) and the plasma ^{45}Ca specific activity at the end of the experiment; 1-day starved fish, with emptied urinary bladders were used. (ii) Total efflux was determined 4 days after $^{47}Ca^{2+}$ injection (92·5–185 KBq $fish^{-1}$) on the basis of apparently linear whole-body tracer loss in tracer-free water, and the plasma ^{47}Ca specific activity at that time. In the first type of determination, efflux rates of Ca^{2+} essentially represent branchial efflux rates (Flik *et al.* 1985*a*). In the second type of determination, efflux rates of Ca^{2+} include urinary and intestinal secretion. For both groups of fish tracer uptake, tracer retention after injection and tracer loss from the body to the water were plotted to ascertain the kinetics of these processes and to permit the application of flux rate calculations that we developed for FW tilapia (Flik *et al.* 1985*a*).

Analytical procedures

After severing the tail, blood was collected from the caudal peduncle (arterial as well as venous blood) into sodium-heparinized haematocrit capillaries. After centrifugation, plasma total Ca was determined by atomic absorption spectrophotometry, using 20 mmol l^{-1} $LaCl_3$ as diluent, or with a commercial calcium kit (Sigma) in the case of ^{45}Ca-containing samples.

Three types of bone sample and one muscle sample were taken from every fish. Triplicate samples of 10 scales each were taken from both sides at the mid-lateral region, posterior to the opercular slit. A sample of opercular bone was taken after removal of the skin and the connective tissue by rubbing with tissue paper. A sample of vertebral bone was taken after removal of adhering soft tissue by pressure-cooking for 1 min as suggested by Fleming (1973). Muscle samples carefully freed of bone, ribs and scales were taken from the dorsal region. All tissue samples were dried for at least 8 h at 90–100°C, dry weights were determined to the nearest 0·01 mg, and

the dried samples (5–50 mg) were dissolved in 0·5 ml concentrated HNO_3 at 60°C for 1 h. Next, the sample volume was brought up to 5 ml with doubly-distilled water. Total calcium of tissue digests was determined on 5–50 μl samples with the thymol-blue method of Gindler & King (1972). Calcium references were prepared from a calcium atomic absorption standard solution (1·0 mg ml^{-1} dilute HCl: Sigma).

For ^{45}Ca analysis, 1 ml of diluted tissue digest or 5–10 μl plasma in a volume of 1 ml water, were mixed with 4 ml Aqualuma (Lumac) and counted in a Rackbeta LSA, equipped with a d.p.m.-programme. All samples were assayed in triplicate. Tissue tracer content is presented as relative specific activity (SA_r), which is the ratio of tissue Ca-tracer specific activity (SA_t) to blood plasma Ca-tracer specific activity (SA_p).

Statistics and notations

Significance of differences between mean values was assessed applying Student's *t*-test for unpaired observations ($\alpha = 5\,\%$). Significance was accepted at the 2 % level. Linear regression analysis was based on the least-squares method. The symbols, definitions of symbols and units used were taken from Shipley & Clark (1972).

RESULTS

Flux rate determinations

In both FW and LFW the whole-body $^{47}Ca^{2+}$ content increases linearly for a 3-h period (Fig. 1A). In both FW and LFW, a rapid decrease in whole-body tracer content during the first 24 h after injections of the tracer was followed by a steady, slow and apparently linear decrease (Fig. 1B). LFW-adapted fish, however, retained significantly less ^{47}Ca than FW-adapted fish. From 24–100 h, the slopes of the tracer retention curves of the FW and LFW tilapia, fitted by linear regression analysis, were significantly different ($P < 0{\cdot}01$). These slopes were 136 ± 46 c.p.m. h^{-1} for FW tilapia (weight = $14{\cdot}3 \pm 0{\cdot}8$ g) and 313 ± 39 c.p.m. h^{-1} for LFW tilapia (weight = $18{\cdot}2 \pm 2{\cdot}1$ g). Apparently, the LFW tilapia lose tracer faster than tilapia kept in FW. In Fig. 1C, it is shown that tracer appearance in the water from fish previously injected with tracer was linear over a 6-h period in FW as well as LFW tilapia. The kinetics of the tracer movements in our set-up justify our calculation of Ca^{2+} flux rates (see Flik *et al.* 1985*a*).

In the body weight trajectory studied [weight (W) = 10–30 g], Ca^{2+} influx rates as well as Ca^{2+} efflux rates in LFW tilapia were significantly higher than the corresponding values for FW tilapia (Fig. 2). Fitting the relationships between flux rates and body weights of LFW tilapia by linear regression analysis (dashed lines) and using the forementioned relationships between flux rates and body weight for FW tilapia (that yielded essentially straight lines in this particular body weight trajectory), the following formulae were obtained for net fluxes: in FW tilapia, $F_{net} = F_{in} - F_{out} = 210 + 18(W - 10)$ nmol h^{-1} Ca^{2+} and in LFW tilapia, $F_{net} = 1040 + 48(W - 10)$ nmol h^{-1} Ca^{2+}. For a 20-g FW tilapia, F_{net} is 390 nmol h^{-1}

Ca^{2+} and, for a 20-g LFW tilapia, 1620 nmol h^{-1} Ca^{2+}, which indicates a 4·15-fold enhancement of Ca^{2+} uptake from the water in the latter fish.

Extrabranchial Ca^{2+} efflux rates

As shown in Fig. 1B, tracer loss from the body, although an exponential process, can be satisfactorily fitted by linear regression analysis of the data obtained between

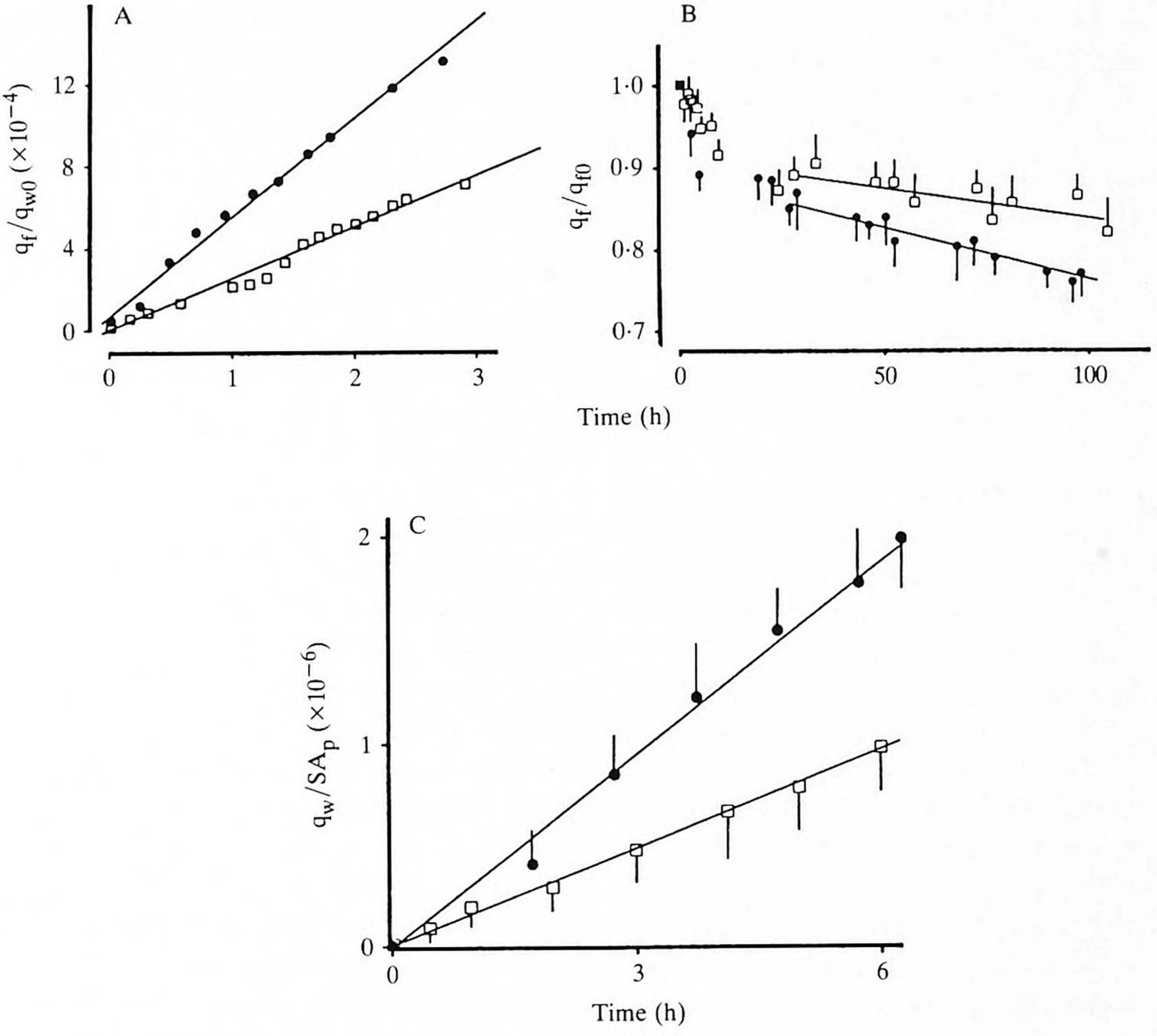

Fig. 1. (A) Whole body $^{47}Ca^{2+}$ uptake from the water, expressed as fish tracer content at time t (q_f) relative to the total radioactivity in the water at zero time (q_{w0}). Data concern one FW tilapia and one FLW tilapia only. (B) Whole-body tracer retention curves for tilapia injected intraperitoneally with $^{47}Ca^{2+}$. Retention is expressed as tracer retained in the body at time t (q_f) divided by the tracer content of the fish at zero (injection) time (q_{f0}). Mean values from seven FW and five LFW tilapia are given. Bars indicate standard deviation. (C) Tracer appearance in the water upon immersion of $^{45}Ca^{2+}$-injected tilapia in tracer-free water. Water tracer content (q_w) is expressed as the fraction of the plasma ^{45}Ca specific activity of the fish at the end of the experiment (SA_p). Mean values are given for 10 FW tilapia and for six LFW tilapia. Bars indicate standard deviation. LFW, ●; FW, □.

24 and 100 h. On the basis of tracer losses during this period and plasma ^{47}Ca specific activity at 62 h (halfway through this period), total Ca^{2+} efflux rates were calculated. In a previous paper it was shown that plasma tracer content over this period decreased linearly and concurrently with total body tracer content (Flik *et al.* 1985*a*). For FW tilapia, the measured total efflux rate of Ca^{2+} was 208 ± 71 nmol h^{-1} ($N = 5$; $W = 14{\cdot}3 \pm 0{\cdot}8$ g). The calculated branchial efflux rate of Ca^{2+} was (according to $F_{out} = 30W^{0{\cdot}563}$ nmol h^{-1}) 179 ± 10 nmol h^{-1}. For LFW tilapia, the measured total efflux was 557 ± 45 nmol h^{-1} ($W = 18{\cdot}2 \pm 2{\cdot}1$ g). This last value is significantly higher ($P < 0{\cdot}02$) than the measured branchial efflux rate, which was 383 ± 67 nmol h^{-1} ($W = 18{\cdot}5 \pm 3{\cdot}2$ g). Extrabranchial efflux rates of Ca^{2+}, calculated as the difference in mean total and mean branchial efflux rates, were 29 nmol h^{-1} for the FW tilapia and 178 nmol h^{-1} for the LFW tilapia; these extrabranchial effluxes represent 14 % and 31 % of the total Ca^{2+} efflux in the FW and LFW tilapia, respectively. For these calculations it was assumed that the specific activity of the ^{47}Ca lost from the body equalled the plasma ^{47}Ca specific activity.

Tissue calcium analyses

LFW tilapia showed a slightly, but significantly, higher plasma total Ca level than FW tilapia (Table 1). In LFW tilapia, the bone calcium content was significantly lower than in FW tilapia, in all three types of bones. This difference was more pronounced in the skeletal bone (vertebrae, 13 %) and scalar bone (11·4 %) than in the dermal bone (operculum, 6·7 %). The calcium content of muscle on a dry weight basis did not differ significantly between the two groups of fish.

Tissue tracer content was determined at the completion of the efflux experiments, i.e. 80 ± 3 h after injection of $^{45}Ca^{2+}$. Relative specific activities (SA_r) for vertebrae, opercula and scales were significantly higher in LFW tilapia than in FW tilapia (Table 1). This difference in SA_r values was most pronounced in the scales (142 %) and amounted to 107 % and 108 % in vertebrae and opercula, respectively. SA_r values for muscle did not differ between the groups and were not significantly different from plasma SA values.

Table 2 presents the results obtained for four fish adapted to either FW or LFW and whose total skeletal, dermal and scalar bone was collected to determine the relative sizes of these subpools of bone. The sizes of these subpools, presented as a percentage of the total bone calcium pool (Q_{bone}), were both significantly different between the two samples of fish. No difference was observed with respect to Q_{bone}, expressed per body wet weight. Total bone mass, however, expressed as bone dry weight per body wet weight, was significantly higher in LFW than in FW tilapia ($P < 0{\cdot}02$). The total bone calcium content differed significantly between FW and LFW tilapia, averaging $5{\cdot}75 \pm 0{\cdot}02$ mmol g^{-1} for FW and $5{\cdot}22 \pm 0{\cdot}05$ mmol g^{-1} for LFW fish ($P < 0{\cdot}001$). For the FW tilapia, the difference between total body calcium (calculated as $Q_f = 357{\cdot}5W^{0{\cdot}965}$; Flik *et al.* 1985*a*), which comes to $3{\cdot}98 \pm 0{\cdot}38$ mmol Ca ($W = 12{\cdot}1 \pm 1{\cdot}2$ g), and Q_{bone} ($= 3{\cdot}76 \pm 0{\cdot}49$ mmol Ca) yields the soft tissue calcium pool ($Q_{soft} = Q_f - Q_{bone} = 0{\cdot}22$ mmol Ca), the latter being 5·42 % of Q_f. This calculated value for Q_{soft} did not differ significantly from

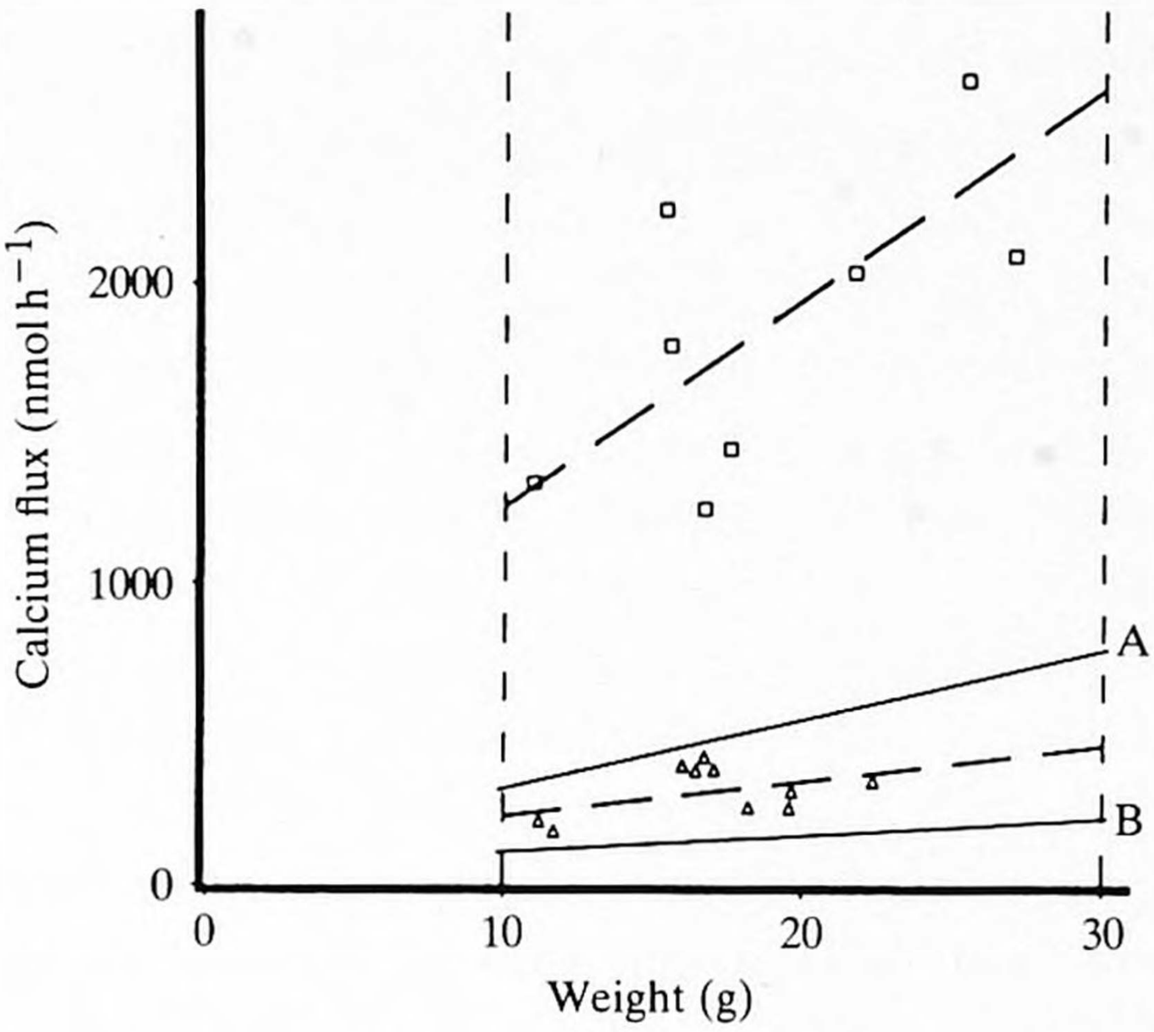

Fig. 2. Comparison of whole-body Ca^{2+} flux rates in FW and LFW tilapia. Influx and efflux rates of Ca^{2+} for FW tilapia are represented by $F_{in} = 50W^{0.805}$ nmol h^{-1} (line A) and by $F_{out} = 30W^{0.563}$ nmol h^{-1} (line B), respectively. For LFW tilapia individual Ca^{2+} influx rates (□, $N = 8$) and Ca^{2+} efflux rates (△, $N = 10$) are given. In the body weight range from 10 to 30 g the relationships between flux rates and body weight were fitted by linear regression analysis (dotted lines).

measured values for the soft tissue compartment of 0·24 ± 0·04 mmol Ca, which is 5·9 % of Q_f. The accuracy of these calculations, then, allows the calculation of Q_f for the LFW tilapia on the basis of tissue calcium contents and the relative sizes of the respective subpools as presented in Table 2.

Calcium accumulation in tilapia acclimated to FW or LFW

Table 3 presents data on growth and calcium accumulation in tilapia acclimated to FW or LFW. Three groups are considered: one FW group and two different groups of fish that were acclimated to LFW conditions. In all three cases significant weight increase and accumulation of calcium in the body occurred over the acclimation period as a whole, as indicated by the increase in mean body weight and mean total fish calcium pools. Mean body accumulation rates of calcium calculated as $\Delta Q_f/\Delta t$ are 383 nmol h^{-1} Ca for FW fish and 616 and 510 nmol h^{-1} Ca for the two groups of LFW fish, respectively.

DISCUSSION

Six major conclusions can be drawn from the data presented in this study.

1. Tilapia grown in FW (0·8 mmol l^{-1} Ca) showed a net uptake of calcium from the water as indicated by growth and accumulation of calcium in the body. This was also observed when fish were well-acclimated to a low-calcium medium (0·2 mmol l^{-1} Ca).

2. Net branchial Ca^{2+} influx rates in the latter fish increased more than four-fold.

Table 1. *Ca content and relative ^{45}Ca content (SA_r) of several tissues of FW and LFW tilapia, determined 80 ± 3 h after tracer injection*

	Calcium content		Relative ^{45}Ca content SA_r ($100 \times SA_t/SA_p$)	
	FW	LFW	FW	LFW
Tissue				
Plasma	2·77 ± 0·22	2·99 ± 0·13†	100	100
Muscle	$(12 \pm 6) \times 10^{-3}$	$(13 \pm 5) \times 10^{-3}$	94·7 ± 25·5	94·8 ± 33·3
Bone				
Vertebrae	5·40 ± 0·41	4·70 ± 0·48**	6·7 ± 2·0	13·9 ± 5·6**
Operculum	6·83 ± 0·31	6·37 ± 0·36*	5·8 ± 1·2	12·1 ± 4·4**
Scales	5·36 ± 0·25	4·75 ± 0·43**	8·6 ± 1·7	20·8 ± 5·6***

***$P < 0{\cdot}001$, **$P < 0{\cdot}01$, *$P < 0{\cdot}02$; † expressed in mmol l^{-1}.

Calcium content (mmol g^{-1}) of muscle and of bony tissues are compared on dry weight basis.

Values for the relative tracer content of the tissues are expressed as tissue ^{45}Ca specific activity (SA_t) relative to plasma ^{45}Ca specific activity (SA_p).

Mean values ± S.E. are given for seven FW and 12 LFW tilapia.

3. Both the branchial and extrabranchial Ca^{2+} efflux rates underwent significant increases during the acclimation process.

4. The low-calcium adapted tilapia showed slight, but significant, hypercalcaemia when compared with tilapia in normal fresh water.

5. The acclimation to low-calcium conditions was accompanied by a decrease in bone calcium content.

6. Finally, the pool of readily exchangeable calcium in bone tissue of tilapia well-adapted to low-calcium was significantly larger than that in tilapia from normal fresh water.

Table 2. *Comparison of calcium-containing compartments in tilapia acclimated for 12 weeks to FW or LFW conditions*

		FW tilapia (W = 12·1 ± 1·2 g)	LFW tilapia (W = 15·3 ± 1·8 g)
Compartment size:	Q_{skel}	1·56 ± 0·14 (41·3*)	1·87 ± 0·03 (40·3*)
	Q_{derm}	1·31 ± 0·25 (34·8*)	1·62 ± 0·12 (35·1*)
	Q_{scal}	0·90 ± 0·13 (23·9*)	1·14 ± 0·11 (24·6*)
	Q_{soft}	0·24 ± 0·04 (5·9§)	0·27 ± 0·05 (5·6§)
	Q_{bone}	3·76 ± 0·49 (94·1§)	4·63 ± 0·14 (94·4§)
	Q_f	4·00	4·90
Calcium content	Q_{bone}/W_{bone}	5·75 ± 0·02	5·22 ± 0·05‡
Relative mass	$W_{bone}/(10^{-2}W_f)$	5·38 ± 0·15	5·85 ± 0·25†

* % of Q_{bone}; § % of Q_{fish}; ‡ $P < 0{\cdot}001$; † $P < 0{\cdot}02$.

Compartment size of the skeletal bone (Q_{skel}), dermal bone (Q_{derm}), scalar bone (Q_{scal}), total bone compartment ($Q_{bone} = Q_{skel} + Q_{derm} + Q_{scal}$), and soft tissue compartments (Q_{soft}) and of the complete fish ($Q_f = Q_{bone} + Q_{soft}$) is expressed in mmol Ca. Bone calcium content is expressed on the basis of dry weight (W_{bone}) in mmol g^{-1}. Total bone mass is presented as the total bone dry weight relative to the body weight of the fish [$W_{bone}/(10^{-2}W_f)$].

In all cases mean values ± S.E. are given for four fish.

Ca^{2+} flux rates

Tilapia acclimated to low-calcium conditions accumulate calcium in their body at a comparable rate to the controls. This indicates that this species is capable of establishing a positive calcium balance even after a four-fold reduction of ambient calcium levels to $0{\cdot}2\,mmol\,l^{-1}$. It has been shown, however, that in tilapia bone demineralization and loss of total body calcium take place in the first 2 weeks after transferring them to a low-calcium environment (Wendelaar Bonga & Flik, 1982). This suggests that the positive calcium balance, which was established after 10 weeks, was re-established after a period of negative calcium balance. Apparently, the acclimation of calcium metabolism to LFW conditions is a relatively slow process. Indeed, in three fish exposed for only 1 week to LFW, Ca^{2+} influx rates were still roughly the same as those of FW tilapia (unpublished result). How do the Ca^{2+} flux rates observed in tilapia compare with values reported for other species? By linear extrapolation to fluxes per h per kg fish, it turns out that the Ca^{2+} flux rates in tilapia are high. The following influx rates ($\mu mol\,h^{-1}\,kg^{-1}$ fish) were calculated. For tilapia adapted to FW and LFW, 28 and 116, respectively; for both rainbow trout, *Salmo gairdneri*, and bullheads, *Ictalurus nebulosus*, adapted to ambient calcium levels in the range of $0{\cdot}085$–$0{\cdot}325\,mmol\,l^{-1}$, 5–7·5 (Hōbe, Laurent & McMahon, 1984); for the goldfish, *Carassius auratus*, 15 (Berg, 1968, 1970); for the killifish, *Fundulus heteroclitus*, 32·5 (Pang, Griffith, Maetz & Pic, 1980) and 10–50 (Mayer-Gostan *et al.* 1983); and for *Fundulus kansae*, 27 (Fleming, 1973). Efflux rates presented in this way come to 8·1 and 15·7 for FW and LFW tilapia, respectively; to 5–7·5 for rainbow trout; and to 1–5 for bullheads (Hōbe *et al.* 1984). Influx rates of Ca^{2+} in FW tilapia are of the same order only as the values for the two *Fundulus* species. When comparing Ca^{2+} flux rates in tilapia to Ca^{2+} flux rates reported for other species, a comment must be made concerning the presentation of Ca^{2+} flux rates in the literature. To our knowledge it has not been recognized before that Ca^{2+} flux rates in fish are not necessarily directly related to body weight. Hence, flux rates linearly extrapolated to fluxes per e.g. 0·1- or 1-kg fish may be misleading, especially when small fish are used. For example, when the proper relationships are observed (Flik *et al.* 1985*a*), net Ca^{2+} influx in a 20-g tilapia in FW is calculated as $F_{net} = F_{in} - F_{out} = 558 - 162 = 396\,nmol\,h^{-1}$, or $= 13\,000 - 1466 = 11\,534\,nmol\,h^{-1}$

Table 3. *Growth and calcium accumulation in FW and LFW tilapia*

Fish	*N*	W_0 (g)	Δt (h)	ΔW (g)	ΔQ_f (mmol Ca)
FW	7	$19{\cdot}95 \pm 2{\cdot}03$	1464	1·82	0·561
LFW	7	$19{\cdot}40 \pm 6{\cdot}42$	1944	3·66	1·197
LFW	5	$24{\cdot}10 \pm 3{\cdot}75$	2424	3·60	1·237

W_0 represents the body weight at the start of the experiments; mean values ± S.E. are given.
Δt refers to the duration of the acclimation period.
ΔW represents the mean increase in body weight per fish over the pertinent period.
ΔQ_f refers to the calculated increase in total body calcium per fish over the pertinent period.

for a (hypothetical) tilapia of 1 kg. Values obtained by linear extrapolation (multiplying by 50) would yield $F_{net} = 27\,900 - 8100 = 19\,800$ nmol h^{-1} kg^{-1} tilapia and over-estimate true flux rates. However, even though the data published in the literature may be under- or over-estimates of true flux rates, it is clear that the values we observed for LFW tilapia exceed every Ca^{2+} flux rate determined so far for teleost fish.

The very high value for net influx rates in LFW tilapia that we obtained by bidirectional flux measurements compares well with an uptake rate of Ca^{2+} from the water derived from growth rates reported by Ichii & Mugiya (1983) for rapidly growing goldfish, fed a calcium-deficient diet. The most important conclusion from our results is that in tilapia the branchial calcium uptake system has a great capacity for adaptation and can supply almost all the calcium requirement of the fish and does permit growth in relatively soft water. It is also evident from our results that to assess uptake of Ca^{2+} from the water by flux rate determination, both influx and efflux should be determined, as the magnitude and the routes of Ca^{2+} movement depend on ambient Ca^{2+} concentrations: branchial and extrabranchial efflux rates as a percentage of total body efflux rates were estimated to represent 86 % and 14 % in FW tilapia and 69 % and 31 % in LFW tilapia.

As we have discussed elsewhere, integumental Ca^{2+} exchange may involve both transcellular and paracellular routes (Flik *et al.* 1986). For transcellular Ca^{2+} uptake in fish gills, we recently proposed a model on the basis of our studies on Ca^{2+} transport ATPase activity in plasma membranes of branchial epithelium of eel and tilapia (Flik, Wendelaar Bonga & Fenwick, 1983, 1984*a*; Flik, van Rijs & Wendelaar Bonga, 1985*b*). According to this model, Ca^{2+} to be transported from the water to the blood enters the cell passively down its electrochemical gradient, is buffered in the cytosol by Ca^{2+}-binding proteins, and is subsequently pumped into the blood by an active Ca^{2+} transport mechanism. With this model in mind, we now suggest that at least three events are involved in the changes in integumental Ca^{2+} fluxes, when tilapia are acclimated to LFW.

First, it has been demonstrated in tilapia that an inverse relationship exists between ambient Ca^{2+} and prolactin cell activity (Wendelaar Bonga, Loewik & van der Meij, 1983; Wendelaar Bonga, Flik, Loewik & van Eys, 1985). Prolactin secretion is enhanced in LFW tilapia (Wendelaar Bonga *et al.* 1985). Prolactin stimulates Ca^{2+} tracer influx in American eel gill arches (Ma & Copp, 1981), stimulates transport Ca^{2+}-ATPase activity in American eel gill plasma membranes (Flik *et al.* 1984*b*), and stimulates uptake of Ca^{2+} from the water in intact tilapia (Flik *et al.* 1986). Therefore, the enhanced prolactin secretion that occurs under LFW conditions may stimulate Ca^{2+} transport mechanisms in the branchial epithelium and by so doing increase the Ca^{2+} transport capacity of the gills. Such an adaptation, in concert with increased entry of Ca^{2+} at the apical membranes, probably accounts for the observed increased transcellular Ca^{2+} influx in the gills.

Secondly, in LFW tilapia the chloride cell density is tripled, when compared to FW tilapia (unpublished results). As originally suggested by Payan, Mayer-Gostan & Pang (1981), we too consider the chloride cells of the gills as their Ca^{2+} transporting

units (Flik *et al.* 1984*a*). Thus, an increase in chloride cell density in the branchial epithelium would lead to an increase of transcellular Ca^{2+} influx capacity.

Thirdly, a decrease in ambient Ca^{2+} causes enhanced permeability to monovalent ions (Dharmamba & Maetz, 1972) and osmotic water permeability (Wendelaar Bonga & van der Meij, 1981; Wendelaar Bonga *et al.* 1983) of tilapia gills as well as of the gills of Japanese eel, *Anguilla japonica* (Ogawa, 1974; Ogasawara & Hirano, 1984*a*), *Fundulus kansae* (Potts & Fleming, 1970), rainbow trout, *Salmo gairdneri* (Ogawa, 1974) and brown trout, *Salmo trutta* (Oduleye, 1975). Although no data are available in the literature on gill Ca^{2+} permeability, the possibility that the Ca^{2+} permeability of the epithelium increases in LFW tilapia cannot be excluded. It is relevant to mention that in chick gut the permeability to Ca^{2+} of apical membranes is determined by and negatively correlated with mucosal Ca^{2+} concentrations (Ebel & Guenther, 1980; Bikle, Zolock & Morrissey, 1981). If in tilapia a decrease in ambient Ca^{2+} would, indeed, enhance the permeability to Ca^{2+} of the apical membranes of the branchial epithelium, this process would facilitate Ca^{2+} permeation at the apical membranes and thus promote transcellular Ca^{2+} influx rates in the gills. However, increased permeability may also lead to increased Ca^{2+} loss. Such loss has been described, e.g. for paracellular 'secretion' of Ca^{2+} in rat ileal epithelium (Nellans & Kimberg, 1978, 1979), which is determined by and negatively correlated with luminal Ca^{2+} concentrations. By analogy, then, low calcium concentrations in the ambient water of tilapia would allow intercellular Ca^{2+} to diffuse out of the animal. Thus, branchial efflux rates of Ca^{2+} following paracellular routes could be increased as a result of lowered ambient Ca^{2+}. The above-mentioned increase in chloride cell density in LFW fish may, however, also contribute to an increase in Ca^{2+} efflux, since it implies extension of the paracellular flux route. Recently, Ogasawara & Hirano (1984*b*) reported for *Anguilla japonica* that the gill permeability to water is positively correlated with the number of chloride cells in the gills and that the number of junctional complexes in the epithelium may determine its permeability to water. Similar conclusions were drawn by Sardet, Pisam & Maetz (1979) from their studies on a variety of freshwater and marine fish.

Our result of increased uptake rates of Ca^{2+} from the water in LFW tilapia contrasts with results of Berg (1968, 1970) on goldfish adapted to low-calcium water. He concluded that branchial exchange rates of Ca^{2+} in this species are independent of ambient calcium levels. Under low-calcium conditions, increased intestinal absorption of calcium compensated for increased extrabranchial calcium loss, to satisfy the total calcium demand. Apparently, the goldfish adjusts its intestinal calcium absorption instead of its branchial Ca^{2+} uptake in soft water. For two other species of freshwater fish, bullheads and rainbow trout, it was reported that whole-body calcium exchange rates were largely independent of ambient Ca^{2+} (Hōbe *et al.* 1984). However, altered influx rates of Ca^{2+} as an adaptive response to varying ambient Ca^{2+} levels have been reported earlier for *Fundulus heteroclitus* (Mayer-Gostan *et al.* 1983). Thus, such adaptive responses of the branchial calcium uptake system seem to be of wider occurrence.

The observation of increased extrabranchial Ca^{2+} efflux rates in LFW tilapia is in line with the observation of increased integumental osmotic water permeability at low ambient Ca^{2+} (Wendelaar Bonga & van der Meij, 1981): increased osmotic water uptake at low ambient Ca^{2+} may enhance urine production; increased urine production leads to extra Ca^{2+} loss from the body in American eels (Fenwick, 1981). Hence, increased urine production as a response to enhanced water uptake in LFW tilapia could explain, at least partly, the increase in extrabranchial Ca^{2+} efflux under these conditions.

Internal calcium stores

Blood plasma and soft tissues

Our finding of an elevated plasma calcium content in tilapia 10 weeks after the start of acclimation to LFW confirms a report by Wendelaar Bonga *et al.* (1985). Five days after transference to LFW, tilapia show a significant hypocalcaemia (Wendelaar Bonga, Flik & Fenwick, 1984); it therefore appears that the restoration of plasma calcium in response to reduced ambient Ca^{2+} is preceded by an initial drop in plasma calcium levels. This restoration of plasma calcium levels is most probably mediated by an enhanced production of the hormone prolactin (Wendelaar Bonga *et al.* 1984). This conclusion is further substantiated by the fact that exogenous prolactin induces hypercalcaemia in several species of freshwater teleosts: the killifish, *Fundulus heteroclitus*, the stickleback, *Gasterosteus aculeatus*, tilapia and American eels (Pang, Schreibman, Balbontin & Pang, 1978; Wendelaar Bonga & Flik, 1982; Flik *et al.* 1984*b*).

Reducing ambient Ca^{2+} concentrations changed the amount of calcium in the soft tissue compartment from 5·9 % to 5·6 % of the total amount of calcium in the fish, a change which is not statistically significant. The size of the soft tissue calcium compartment under freshwater conditions of the goldfish is 6 % (Berg, 1968) and of *Fundulus kansae* 3·2 % (Fleming, Brehe & Hanson, 1973). Muscle calcium content in tilapia seems not to be affected by changes in plasma calcium levels.

Bones and scales

The values for bone (and muscle) calcium contents of tilapia adapted to FW are approximately the same as those reported earlier (Wendelaar Bonga & Flik, 1982; Wendelaar Bonga & Lammers, 1982). Tilapia kept in LFW, however, showed decreased calcium density of bone. Nevertheless, the fish increased their total body calcium pool (Q_f) under LFW conditions, connected with growth, but bone calcium density in these fish was lower than in FW tilapia. Thus, although tilapia increase the uptake of Ca^{2+} from the water and re-establish a positive calcium balance under LFW conditions, the degree of bone mineralization is maintained at a lower level. This observation of growth connected with a changed degree of bone mineralization seems a beautiful adaptation of the fish to a calcium poor environment. The question still to be answered is why the degree of bone mineralization is maintained at a lower level when the uptake of Ca^{2+} from the water is very much enhanced. The possi-

bility that phosphate metabolism may also be influenced by ambient Ca^{2+} levels in LFW tilapia could give at least a partial explanation for this phenomenon. Mobilization of calcium from acellular bone has been reported for *Fundulus kansae* (Brehe & Fleming, 1976), goldfish and killifish (Mugiya & Watabe, 1977), *Lepomis macrochirus* (Weiss & Watabe, 1978), *Tilapia macrocephala* (Weiss & Watabe, 1979) and for *Oreochromis mossambicus* (Urasa, Flik & Wendelaar Bonga, 1984). Both Weiss & Watabe, and Urasa and co-workers came to the conclusion that the need for phosphate and not calcium was the primary trigger for bone demineralization. Rodgers (1984) reports for brook trout, *Salvelinus fontinalis*, that low ambient Ca^{2+} levels impair absorption of dietary Ca^{2+}, and that mobilization of bone minerals from scales and fins occurs under such conditions. An attractive possible explanation for the demineralization of bone at low ambient Ca^{2+} would be that calcium and phosphate resorption in the gut are impaired, which urges the fish to mobilize bone mineral (or limit bone mineralization, or both) to provide for its phosphate requirements. The capacity of *Lepomis macrochirus* specifically to mobilize calcium phosphate but not calcium carbonate, as reported after oestrogen treatment (Weiss & Watabe, 1978), supports this hypothesis.

The degree of demineralization was higher in vertebral and scalar bone than in opercular bone. This can be related to two differences in histophysiology of these bones. First, vertebrae and scales are a cancellous, less dense type of bone than the opercular bone (Moss, 1963; Lanzing & Wright, 1976). In those cases where shifts in physico-chemical Ca^{2+} exchange processes at the bone surface constitute the basis of bone resorption, Ca^{2+} mobilization occurs more intensely in the less dense type of bone (Amprino, 1952*a*,*b*). Secondly, Rowland (1966) has shown that bone structures with the greatest exposure to circulating fluids are the primary sites for Ca^{2+} exchange processes between blood and bone. W. Vogel (personal communication) has shown that the scales of tilapia are very well provided with the so-called secondary vessel system. This system, that branches off from the primary blood vessels in the skin and covers the scales, could allow for an efficient exchange of minerals between the surface of the bone and the plasma.

The SA_r values determined for the various tissues give an indication of the amount of readily exchangeable calcium of the tissue. Four days after injection of the tracer, SA_r values for muscle approximated to 100, which means that this tissue exchanged its calcium rather rapidly and completely with the plasma. Brehe & Fleming (1976) came to the same conclusion for the calcium exchange rate of the soft tissue compartment of *Fundulus kansae*. SA_r values for bones in FW tilapia ranged from 5·83 % (operculum) to 8·61 % (scales). These values further parallel the values for bone calcium content, which corroborates the thesis that the bone density, at least partly, determines the size of the exchangeable pool. Assuming that 4 days after tracer injection bone SA_r values represent the percentage of readily exchangeable calcium of the bone, we can calculate that the readily exchangeable calcium contents for FW and LFW tilapia are 12 % and 19 %, respectively. In FW tilapia, the soft tissue compartment and total bone compartment provide for 47 % and 53 % of the readily exchangeable calcium, respectively, and in LFW tilapia these figures are 27 %

and 73 %, respectively. Apparently, in LFW fish the bone provides for an important, enlarged, readily exchangeable calcium pool. This increase in readily exchangeable calcium in the body of LFW tilapia may fulfil a calcium buffer function under conditions of increased whole-body turnover of Ca^{2+}.

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