Effects of ovine prolactin on calcium uptake and distribution in Oreochromis mossambicus

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Flik, G., J. C. Fenwick, Z. Kolar, N. Mayer-Gostan, and S. E. Wendelaar Bonga. Effects of ovine prolactin on calcium uptake and distribution in Oreochromis mossambicus. Am. J. Physiol. 250 (Regulatory Integrative Comp. Physiol. 19): R161–R166, 1986.—Ovine prolactin stimulated the net uptake rate of Ca2+ from the water by 96%, produced frank hypercalcemia, and increased total bone calcium content in fed rapidly growing freshwater male tilapia, Oreochromis mossambicus. It did not, however, alter the size of the readily exchangeable bone calcium pool. The increase in calcium accumulation resulted from an increase in whole-body Ca2+ influx and a decrease in Ca2+ efflux. It is concluded that prolactin exerts an important control over Ca2+ exchange between the fish and its environment and that through its hypercalcemic action prolactin indirectly facilitates bone mineralization.

It is well established that fish have a remarkable capacity to extract Ca2+ directly from the water (25). Indeed, it has been estimated that in tilapia and goldfish at least 80% of the Ca2+ accumulated during growth is obtained from the surrounding aqueous environment (1, 7). The bulk of this uptake probably takes place via the chloride cells located in the branchial epithelium (24). It seems reasonable then to predict that branchial Ca2+ uptake mechanisms are an important element of calcium homeostasis in fish, that they must be under some control, and that at least part of this control is mediated by prolactin. This prediction is supported by the observation that ovine prolactin enhances high-affinity transport Ca2+-ATPase activity in American eel branchial epithelial plasma membranes (10) and that prolactin synthesis is stimulated in tilapia exposed to low ambient Ca2+ levels (28). Exposure to low ambient Ca2+ levels stimulates Ca2+ uptake from the water in killifish (18) and in tilapia (8). Concerning this last effect of low ambient Ca2+ levels, there are, however, deviating observations: Höbe et al. (14) reported that Ca2+-influx in rainbow trout and bullheads (Ictalurus nebulosus) was largely independent of ambient Ca2+ levels.

The present study was undertaken to investigate the effects of prolactin on Ca2+ exchange with the water in growing actively feeding tilapia. The hypothesis was that prolactin treatment would enhance net Ca2+ uptake in these fish and that the treated fish would be able to deposit Ca2+ in their bone more efficaciously. We analyzed the effects of ovine prolactin in both influx and efflux rates of Ca2+ in freshwater male tilapia. The internal distribution of the Ca2+ taken up was traced to determine the effect of prolactin on Ca2+ compartmentalization in the fish and to obtain an estimate of the readily exchangeable Ca2+ pools.

MATERIALS AND METHODS

Male tilapia, O. mossambicus, were obtained from laboratory stock and kept at 28°C in Nijmegen tapwater under conditions as described earlier (7). The Ca2+ concentration of the water was 0.8 mM in all cases. The fish were fed and continued to grow throughout the experiments.

Only reagent grade chemicals (Sigma, St. Louis, MO) were used. Ovine prolactin (spec act 31.5 IU/mg protein) was a generous gift of the National Institutes of Health (Hormonal Division, Endocrinological Department, Bethesda, MD). The radiotracers 45Ca and 47Ca were pur-
chased (Amersham International, UK) as CaCl$_2$ in aqueous solution. Specific activities were 9.25–37.5 and >0.74 GBq/mol Ca for $^{45}$Ca and $^{47}$Ca, respectively.

**Ca determinations.** Plasma total Ca was determined by atomic absorption spectrophotometry or by the use of a commercial Ca kit (Sigma) as described previously (7). Tissue total Ca was estimated after digestion of the tissue in concentrated HNO$_3$. Radiotracer activities were determined by $\gamma$-ray spectrophotometry ($^{45}$Ca) or by liquid scintillation counting ($^{47}$Ca).

**Ca$^{2+}$ flux determinations.** Unidirectional Ca$^{2+}$ fluxes between intact fish and water were determined and calculated as described in detail earlier (7). In brief, Ca$^{2+}$ influx rates were calculated from $^{47}$Ca$^{2+}$ accumulated in the body over a 3-h period (by the use of a whole-body counter) and the water $^{47}$Ca$^{2+}$ specific activity. Efflux rates of Ca$^{2+}$ from the fish were determined in two ways, yielding either total or branchial Ca$^{2+}$ efflux. Total efflux rates were calculated on the basis of apparently constant whole-body $^{47}$Ca$^{2+}$ loss rates over a 20-h period and plasma $^{45}$Ca specific activities in the middle of this period; in these fish $^{47}$Ca$^{2+}$ losses include urinary and intestinal losses. Branchial efflux rates of Ca$^{2+}$ were determined on the basis of tracer appearance in the water and plasma $^{45}$Ca specific activity 4 days after intraperitoneal injection of $^{45}$Ca$^{2+}$. In the latter type of efflux determination the fish were not fed for 1 day before the experiment, and the urinary bladder was emptied just before the experiment to exclude urinary and intestinal Ca$^{2+}$ excretion during the flux measurements (7); a constant tracer appearance rate in the water was taken as evidence that no significant tracer excretion via urine or feces occurred (which would have led to fluctuating tracer appearance rates in the water). To assess whether the injection procedure for the hormone administration affected the flux rates, the observed flux rates in the fish that received control injections were compared with the flux rates calculated for these fish on the basis of the relationships for flux rates and body weight (W) reported for untreated fish (7); these were influx, $F_{i} = 50W^{0.805}$ nmol Ca$^{2+}$/h and efflux, $F_{o} = 30W^{0.563}$ nmol Ca$^{2+}$/h.

**Hormone administration.** Prolactin was dissolved in 50 mM HCl and was injected intraperitoneally with a 26-gauge needle fixed to a Hamilton precision syringe. The dosage was 0.1 IU/g fish per 48 h; the injected volume was 50 $\mu$L max. Control fish received equal volumes of solvent. Injections were given at fixed time intervals. Ca$^{2+}$ flux rates were determined after a minimum of three hormone injections and always in the mornings of the day after the last injection. In the $^{45}$Ca$^{2+}$ efflux experiments the second injection consisted of a single combined injection of prolactin and tracer. The protocols for the different experiments are presented in Fig. 1.

**Calculations and statistics.** To compare flux rates of groups of fish with significantly different body weights (W$_i$), individual flux rates were converted to flux rates related to the mean body weight of the pertinent groups (W$_m$), taking into account the power relations for the respective fluxes and the body weights: $F(W_i) = aW_i^p \rightarrow F(W_m) = aW_m^p = F(W_j) \cdot (W_m/W_i)^p$.

To assess statistical significance of differences between mean values, the Mann-Whitney U test (one-tailed) was applied. A P value <0.05 was taken as significant. Linear regression analysis was performed according to the least-squares method.

**RESULTS**

**Tracer uptake and tracer retention.** As shown in Fig. 2A, $^{45}$Ca$^{2+}$ uptake from the water is significantly stimulated in prolactin-treated fish. $^{45}$Ca$^{2+}$ loss, however, after tracer loading from the water was not affected by prolactin treatment (Fig. 2B).

**Ca$^{2+}$ fluxes (Table 1).** Prolactin treatment increased Ca$^{2+}$ influx significantly by 39%. Measured influx rates in the control group (411 ± 128 nmol Ca$^{2+}$/h) did not differ significantly from Ca$^{2+}$ influx rates calculated according to $F_i = 50W^{0.805}$ nmol Ca$^{2+}$/h (357 ± 32 nmol Ca$^{2+}$/h; U = 29, P > 0.05), which indicates that the handling and the injection procedure did not affect influx measurements. Branchial Ca$^{2+}$ efflux rates were 38% lower in the prolactin-treated fish than in the control fish. Branchial efflux rates in the control fish (149 ± 49 nmol Ca$^{2+}$/h) did not differ significantly from efflux rates calculated according to $F_{o} = 30W^{0.563}$ (128 ± 11 nmol Ca$^{2+}$/h; U = 31, P > 0.05), and this was taken as evidence that the procedure itself did not noticeably influence the flux measurement. Total body efflux rates of Ca$^{2+}$ were reduced by 35% in prolactin-treated fish.

No significant differences existed between the mean body weights of the groups of fish used for influx and total efflux determinations. This allows the net Ca$^{2+}$ uptake rate from the water ($F_{net}$) to be calculated directly from the observed flux values as $F_{net} = F_i - F_{o}$, being 411 - 178 = 233 nmol Ca$^{2+}$/h for the controls and 571 - 115 = 456 nmol Ca$^{2+}$/h for the prolactin-treated fish; in the prolactin-treated group $F_{net}$ is increased by 96%.

Mean body weights of the groups of fish used for branchial efflux experiments were significantly higher ($P < 0.01$ for controls and prolactin-treated fish) than those of the groups of fish used in the influx experiments. To allow the calculation of net branchial Ca$^{2+}$ influx rates ($F_{net}^{\pm}$) as $F_{net}^{\pm} = F_i - F_{o}$, measured individual influx rates were first normalized to the mean body weight (13.2 g) of the group of fish used for branchial efflux experiments, according to $F_{net}^{\pm} = F_i(W_i) \cdot x(13.2/W_i)^{0.805}$. Normalized influx rates, $F_i(13.2)$, were 480 ± 120 nmol Ca$^{2+}$/h for controls and 683 ± 195 nmol Ca$^{2+}$/h for prolactin-treated fish. Net branchial Ca$^{2+}$ uptake rates then come to $F_{o} = 480 - 149 = 331$ nmol Ca$^{2+}$/h for controls and to $F_{o} = 683 - 92 = 591$ nmol Ca$^{2+}$/h for prolactin-treated fish, $F_{net}$ being increased by 79% in the latter group.

In all cases the prolactin-treated tilapia showed significantly increased plasma Ca levels (Tables 1 and 3).

**Tissue Ca analyses (Tables 2 and 3).** Prolactin treatment significantly increased specific activities (SA) of $^{45}$Ca of vertebral bone and scales, but plasma $^{47}$Ca SA of prolactin-treated fish, although somewhat higher, was not significantly different from those of control fish (values were 354 ± 49 and 385 ± 52 counts • min$^{-1}$ • $\mu$mol$^{-1}$ Ca for controls and prolactin-treated fish, respectively). The relative specific activities (SA, 100 × SA$_{tissue}$/
prolactin lobes (27). In our opinion, prolactin must there­
fore be considered a hypercalcemic hormone in teleosts.

Since the degree of bone mineralization had also in­
creased, the prolactin-stimulated Ca" uptake from the
water must have caused this hypercalcemia.

**Prolactin and Ca²⁺ fluxes.** The Ca²⁺ influx rates pre­
sented in this study represent whole-body influx rates. How­ever, we advanced arguments that in freshwater
tilapia whole-body influx in fact can be equated with
branchial influx (7). The prolactin-induced hypercal­
cemia in freshwater tilapia is thus accompanied by a
stimulation of branchial Ca²⁺ influx. This observation
corroborates our report on stimulation of branchial
transport Ca²⁺-ATPase activity during prolactin-induced
hypercalcemia in American eels (11).

A similar enzyme activity was demonstrated in the
gills of tilapia, and moreover, this enzyme activity may
drive transbranchial transport of Ca²⁺ from the water to
the blood (9). It seems reasonable, therefore, to state
that prolactin treatment stimulates active transport
mechanisms in the gills and thereby promotes Ca²⁺ in­
flux. Whether the stimulatory effect of prolactin on
branchial Ca²⁺ uptake mechanisms is direct or indirect,
e.g., through the action of steroids, requires further in­
vestigation. Fleming and co-workers (6) have given evi­
dence that in *F. kансae* prolactin stimulates the produc­
tion of cortisol, the major mineralocorticoid in fish. It
has been suggested that branchial chloride cell densities
in tilapia are positively correlated with circulating cor­
tisol levels (12). Our unpublished measurements of chlor­
ide cell numbers in the branchial area show that, after

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SA plasma) for vertebrae and for scales in prolactin-treated
⁴⁷Ca²⁺-exposed (72 h) fish were increased by 17 and 19%,
respectively (Table 2). Thus prolactin stimulates the
deposition in the bone of Ca²⁺ taken up from the water.

Table 3 lists the effects of prolactin on the Ca content
and the ⁴⁷Ca SA, values of plasma and three types of
bone, 80 ± 3 h after tracer injection. The SA, values,
reflecting the readily exchangeable Ca pool of the tissue,
were not affected by prolactin treatment. However, skel­
etal, dermal, and scalar bone showed significant increases
in Ca content in prolactin-treated fish.

**DISCUSSION**

**Prolactin-induced hypercalcemia.** Ovine prolactin pro­
duced a state of frank hypercalcemia in freshwater-
adapted tilapia. This confirms earlier reports concerning
tilapia (26) and other teleosts, including sticklebacks (*G.
aculeatus*) (26), American eels (*A. rostrata*) (11), rainbow
tROUT (*S. gairdneri*) (15), and the killifish (*F. heteroclitus*)
(22). We believe that the effect observed was a physio­
logical rather than a pharmacological response to prolac­
tin, because the fish used in the present study were kept
in water with a Ca²⁺ concentration of 0.8 mM, at which
concentration the endogenous prolactin production is
submaximal (28). Also, in tilapia kept under identical
conditions, homologous prolactin can cause hypercal­
cemia, as judged by the effect of ectopically implanted
prolactin lobes (27). In our opinion, prolactin must there­
fore be considered a hypercalcemic hormone in teleosts.
injection of ovine prolactin or after implantation of tilapia prolactin cell implants, chloride cell densities are increased in these fish. Recently, Edery et al. (4) reported the presence of prolactin receptors in the branchial epithelium of the species used in this study and showed that these receptors specifically bind ovine prolactin. Thus a direct action of (ovine) prolactin on the gills should also be considered.

In fish residing in hypocalcic fresh waters, Ca$^{2+}$ efflux will result from passive diffusion of Ca$^{2+}$ through the integument and from urinary and intestinal outflow of Ca$^{2+}$. As discussed earlier (7), integumental Ca$^{2+}$ efflux essentially equals branchial efflux of Ca$^{2+}$. Data on total body Ca$^{2+}$ efflux and integumental Ca$^{2+}$ efflux, then, allow discrimination between branchial and extrabranchial efflux rates. Branchial and total body efflux rates determined in the present study for control fish agree well with those reported elsewhere (7, 8).

TABLE 1. Effects of ovine prolactin on Ca$^{2+}$ flux rates in freshwater tilapia

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Prolactin</th>
<th>% Change</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole-body Ca$^{2+}$ influx rates (n = 9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish wt, g</td>
<td>10.7±1.3</td>
<td>10.7±1.3</td>
<td>NS</td>
<td></td>
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<tr>
<td>Plasma Ca, mM</td>
<td>2.82±0.17</td>
<td>3.15±0.18</td>
<td>+12</td>
<td>&lt;0.001</td>
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<tr>
<td>F$_{in}$, nmol Ca$^{2+}$/h</td>
<td>411±128</td>
<td>571±148</td>
<td>+39</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>F$_{in}$(13.2), nmol Ca$^{2+}$/h</td>
<td>480±120</td>
<td>683±195</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Branchial Ca$^{2+}$ efflux rates (n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish wt, g</td>
<td>13.2±0.2</td>
<td>13.2±0.2</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Plasma Ca, mM</td>
<td>2.85±0.13</td>
<td>3.16±0.12</td>
<td>+11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>F$_{re}$, nmol Ca$^{2+}$/h</td>
<td>149±49</td>
<td>92±44</td>
<td>-38</td>
<td>&lt;0.025</td>
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<tr>
<td>Total Ca$^{2+}$ efflux rates (n = 9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish wt, g</td>
<td>10.6±1.3</td>
<td>11.3±1.3</td>
<td>NS</td>
<td></td>
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<tr>
<td>Plasma Ca, mM</td>
<td>2.89±0.14</td>
<td>3.11±0.11</td>
<td>+8</td>
<td>&lt;0.001</td>
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<tr>
<td>F$_{tot}$, nmol Ca$^{2+}$/h</td>
<td>178±54</td>
<td>115±16</td>
<td>-35</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are means ± SD. Normalization of individual influx rates [F$_{in}$(W)] to influx rates of 13.2-g tilapia (mean body wt of fish used for determination of F$_{re}$) was carried out according to F$_{in}$(13.2) = F$_{in}$(W)/(13.2 W)$^{0.86}$. F$_{re}$, whole-body influx rate; F$_{re}$, branchial efflux rate; F$_{tot}$, total efflux rate.

TABLE 2. Effects of ovine prolactin on tissue 45Ca specific activities

<table>
<thead>
<tr>
<th>Relative Specific Activity</th>
<th>Control</th>
<th>Prolactin</th>
<th>% Change</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>100</td>
<td>100</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Vertebræ</td>
<td>6.37±0.37</td>
<td>7.45±0.38</td>
<td>17</td>
<td>&lt;0.005</td>
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<tr>
<td>Scales</td>
<td>7.38±0.47</td>
<td>8.80±0.41</td>
<td>19</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

Values are means ± SD for 5 fish after 72-h exposure to 45Ca$^{2+}$-containing water. Relative 45Ca specific activities (S$_{Ar}$ = 100 x S$_{tissue}$/S$_{plasma}$) represent tissue specific activities relative to plasma specific activities.

TABLE 3. Effects of ovine prolactin on tissue Ca content and relative specific activity values

<table>
<thead>
<tr>
<th>Ca Content</th>
<th>Control</th>
<th>Prolactin</th>
<th>Relative Specific Activity</th>
<th>Control</th>
<th>Prolactin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>2.85±0.13</td>
<td>3.16±0.12</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Vertebræ</td>
<td>5.20±0.69</td>
<td>5.83±0.69</td>
<td>16.9±9.9</td>
<td>18.6±11.8</td>
<td></td>
</tr>
<tr>
<td>Operculum</td>
<td>5.93±0.37</td>
<td>6.75±0.83</td>
<td>16.2±7.1</td>
<td>16.9±9.1</td>
<td></td>
</tr>
<tr>
<td>Scales</td>
<td>4.83±0.38</td>
<td>5.41±0.66</td>
<td>26.9±7.6</td>
<td>24.2±11.7</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD for 10 fish injected intraperitoneally 80 ± 3 h before with 45Ca$^{2+}$. Relative 45Ca specific activities (S$_{Ar}$ = 100 x S$_{tissue}$/S$_{plasma}$) represent tissue specific activities relative to plasma specific activities. * P < 0.001. † P < 0.05. ‡ P < 0.025.
branchial, intestinal, and renal tissue of freshwater tilapia (4). From a quantitative point of view, however, our data indicate that, although the effects of prolactin on Ca\textsuperscript{2+} fluxes may extend to extrabranchial sites, the gills are, in this respect, the most important target for this hormone.

Our Ca\textsuperscript{2+} efflux data implicate that integumental permeability to Ca\textsuperscript{2+} is reduced by prolactin. Earlier (7) we concluded that in tilapia this permeability to Ca\textsuperscript{2+} is determined by the constitution and numbers of the paracellular pathways of the epithelium. In the same species the significance of the paracellular routes for the permeability to ions was first shown for Na\textsuperscript{+} efflux by Dharmamba and Maetz (2). Prolactin decreases branchial permeability to water and ions in tilapia (2), freshwater Japanese eel (A. japonica) (21), European eel (A. anguilla) (16), killifish (F. heteroclitus) (17) and green molly (Poecilia latipinna) (5). We now suggest that the control of Ca\textsuperscript{2+} fluxes may extend to extrabranchial sites, the gills are, in this respect, the most important target for this hormone.

Ca\textsuperscript{2+} in bone occurs when tilapia are treated with prolactin. Our Ca\textsuperscript{2+} efflux data implicate that integumental permeability to Ca\textsuperscript{2+} is reduced by prolactin. Earlier (7) we concluded that in tilapia this permeability to Ca\textsuperscript{2+} is determined by the constitution and numbers of the paracellular pathways of the epithelium. In the same species the significance of the paracellular routes for the permeability to ions was first shown for Na\textsuperscript{+} efflux by Dharmamba and Maetz (2). Prolactin decreases branchial permeability to water and ions in tilapia (2), freshwater Japanese eel (A. japonica) (21), European eel (A. anguilla) (16), killifish (F. heteroclitus) (17) and green molly (Poecilia latipinna) (5). We now suggest that the control of Ca\textsuperscript{2+} fluxes may extend to extrabranchial sites, the gills are, in this respect, the most important target for this hormone.

Prolactin and internal Ca\textsuperscript{2+} reservoirs. The hypercalcemia and increased Ca content of the bony tissues, after prolactin-stimulated Ca\textsuperscript{2+} uptake in tilapia, show that in this species plasma Ca\textsuperscript{2+} freely exchanges with the bone and can be stored there. From the present results it may be concluded that prolactin-induced hypercalcemia enhances bone mineralization. Clearly, the acellular bone of tilapia is intimately associated with calcium metabolism and acts as an internal compartment for Ca\textsuperscript{2+} storage. No separate determination of plasma-free and protein-bound Ca\textsuperscript{2+} was carried out in this study; it has been shown, however, that in tilapia prolactin-induced hypercalcemia is accompanied by elevated levels of free Ca\textsuperscript{2+} (2). Apparently, it was the plasma-free Ca\textsuperscript{2+}, increased by the prolactin treatment, that was deposited into the bony tissues. These phenomena in tilapia resemble the Ca\textsuperscript{2+} exchange process in otoliths of rainbow trout, reported by Mugiya (20). Mugiya showed that the degree of calcium deposition in the otoliths was positively correlated with total plasma Ca levels. From his in vitro studies he concluded that the Ca\textsuperscript{2+} exchange between the otoliths and the endolymph parallels the fluctuations in free Ca\textsuperscript{2+} levels in the endolymph.

In our study the analysis of the prolactin effect on bone was restricted to analysis of the bone Ca contents. It has been demonstrated earlier that prolactin treatment of tilapia does not affect the lining cells (osteoblasts) of its bone, at least when judged on the basis of the ultrastructural features of this tissue (26). The observed effect of prolactin on fish bone, therefore, seems noncellular and is different from that exerted by, e.g., growth hormone on bony tissue in mammals. Growth hormone stimulates the activity of the lining osteoblasts and thereby promotes growth of bone. However, in tilapia both calcitonin and 24,25-dihydroxyvitamin D\textsubscript{3} exert their effect on bone through activation of the lining cells (29, 30), which indicates that, in fish also, lining cells are involved in bone metabolism. The SA, values for bones in the controls and in the prolactin-treated tilapia are very similar, which indicates that the increase in bone Ca content is not noticeably accompanied by a relative increase in the bone readily exchangeable Ca pool. This then further supports our conclusion that true storage of Ca\textsuperscript{2+} in bone occurs when tilapia are treated with prolactin.

In conclusion, the results presented in this paper confirm that prolactin is a hypercalcemic hormone in freshwater teleosts and show that in tilapia the hypercalcemic effect of prolactin results from mainly a dual action on the integument, viz. stimulation of branchial Ca\textsuperscript{2+} uptake and reduction of integumental permeability to Ca\textsuperscript{2+}. In terrestrial vertebrates calcium homeostasis depends on parathormone that is hypercalcemic and stimulates cell-mediated Ca resorption from the bone. In fish, calcium metabolism differs essentially from that in land-living vertebrates, and it seems that in freshwater fish calcium homeostasis depends on the control of Ca\textsuperscript{2+} exchange with the water by the hypercalcemic hormone prolactin.

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Prolactin and calcium uptake in freshwater tilapia


