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Vitamin D_3-induced calcemic and phosphatemic responses in the freshwater mud eel *Amphipnous cuchia* maintained in different calcium environments

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Abstract

Vitamin D_3 (100 ng 100 g body weight^{-1} day^{-1}) was administered intraperitoneally (*ip*) to the freshwater mud eel *Amphipnous cuchia* kept in artificial freshwater, calcium-free freshwater, low-calcium freshwater (0.2 mmol/l $CaCl_2$) or calcium-rich freshwater (13.4 mmol/l $CaCl_2$) for 15 days. Analyses of serum calcium and phosphate levels were performed on days 1, 3, 5, 10 and 15 after the beginning of the experiment (six eels from each group at each interval). Administration of vitamin D_3 elevated the serum calcium [maximum elevation occurred at day 10 in artificial freshwater (vehicle: 10.55 ± 0.298, vitamin D: 13.90 ± 0.324), low-calcium freshwater (vehicle: 11.17 ± 0.220, vitamin D: 12.98 ± 0.297) and calcium-rich freshwater (vehicle: 11.24 ± 0.373, vitamin D: 14.24 ± 0.208) whereas it occurred at day 5 (vehicle: 8.42 ± 0.253, vitamin D: 11.07 ± 0.328) in calcium-free freshwater] and phosphate levels [maximum elevation at day 15 in artificial freshwater (vehicle: 4.39 ± 0.105, vitamin D: 5.37 ± 0.121), calcium-free freshwater (vehicle: 4.25 ± 0.193, vitamin D: 5.12 ± 0.181), low-calcium freshwater (vehicle: 3.93 ± 0.199, vitamin D: 5.28 ± 0.164) and calcium-rich freshwater (vehicle: 3.77 ± 0.125, vitamin D: 5.46 ± 0.151)] of the fish maintained in the above mentioned environmental media, but the responses were more pronounced in the fish kept in calcium-rich media.

Key words
- Vitamin D_3
- Calcium
- Phosphate
- Mud eel
- Teleost

Introduction

Endocrinologists have recently shown interest in evaluating the physiological actions of vitamin D_3 metabolites in fish. Several investigators have studied the presence of vitamin D metabolites in various teleosts (1-6) and the changes in the blood calcium and phosphate contents of fish after administration of vitamin D and/or its metabolites (7-15).

Teleost bone may or may not contain osteocytes and has been considered by some investigators to be metabolically inert and unable to contribute to calcium homeostasis. On this basis, we designed the present experiment to determine whether vitamin D_3 affects the serum calcium concentration of the freshwater mud eel *Amphipnous cuchia* when the external sources of calcium (environmental and dietary) are eliminated. For comparison, the effect of vitamin D_3 was also tested in eels adapted to low-calcium and calcium-rich environments.

Material and Methods

A total of 240 adult specimens of *Amphipnous cuchia* of both sexes weighing 180-230 g were collected locally during the resting phase and acclimated to the laboratory under conditions of natural photoperiod (11:58-12:38 h) and temperature (25.8 ± 1.8°C) for two weeks in plastic pools. The fish were fed live tadpoles during acclimatization. For the experiments, the eels were kept in identical glass aquaria each containing 20 l of the medium.

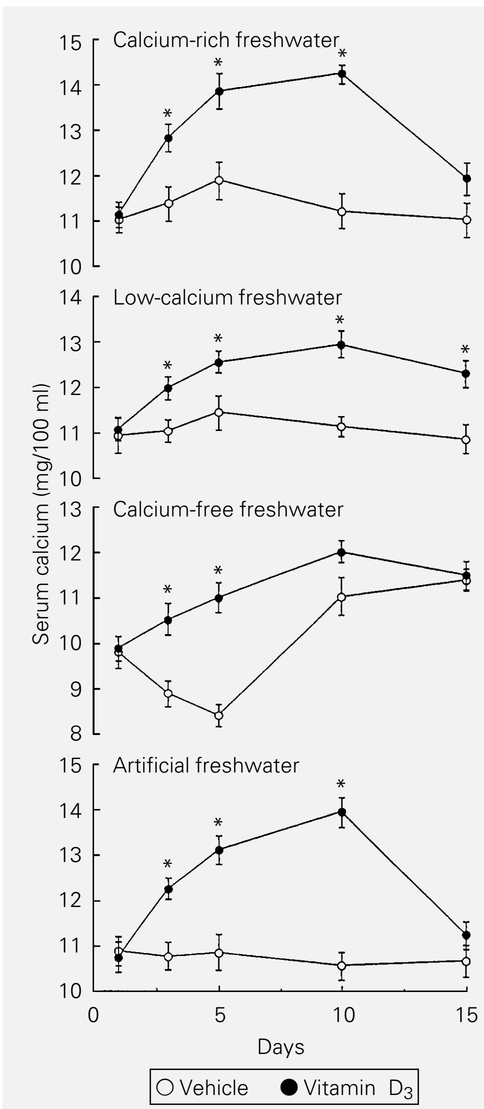

Figure 1 - Changes in serum calcium levels of *A. cuchia* kept in artificial freshwater, calcium-free freshwater, low-calcium freshwater or calcium-rich freshwater and treated with vehicle or vitamin D_3. Each value represents the mean ± SEM for six specimens. Asterisk indicates significant differences (P<0.05) compared to the vehicle-injected group (Student *t*-test).

After acclimatization, the eels were divided into eight groups of 30 animals each and submitted to the following treatments:

Group A: injected *ip* with vehicle (0.1 ml of 95% ethanol 100 g body weight^{-1} day^{-1}) and kept in artificial freshwater.

Group B: injected *ip* with 100 ng of vitamin D_3 100 g body weight^{-1} day^{-1} and kept in artificial freshwater.

Group C: injected *ip* with vehicle (0.1 ml of 95% ethanol 100 g body weight^{-1} day^{-1}) and kept in calcium-free freshwater.

Group D: injected *ip* with 100 ng of vitamin D_3 100 g body weight^{-1} day^{-1} and kept in calcium-free freshwater.

Group E: injected *ip* with vehicle (0.1 ml of 95% ethanol 100 g body weight^{-1} day^{-1}) and kept in low-calcium freshwater.

Group F: injected *ip* with 100 ng of vitamin D_3 100 g body weight^{-1} day^{-1} and kept in low-calcium freshwater.

Group G: injected *ip* with vehicle (0.1 ml of 95% ethanol 100 g body weight^{-1} day^{-1}) and kept in calcium-rich freshwater.

Group H: injected *ip* with 100 ng of vitamin D_3 100 g body weight^{-1} day^{-1} and kept in calcium-rich freshwater.

Vitamin D_3, administered to groups B, D, F and H, was dissolved in 95% ethanol. The eels were not fed 24 h before and during the experiment.

Different artificial media were prepared as follows: a) artificial freshwater: distilled water containing 2.10 mM NaCl, 0.45 mM Na_2SO_4, 0.06 mM KCl, 0.8 mM $CaCl_2$, 0.20 mM $MgCl_2$. pH of the solution was adjusted to 7.6 with $NaHCO_3$; b) calcium-free freshwater: same as above without $CaCl_2$; c) low-calcium freshwater: same as artificial freshwater except that only 0.2 mM $CaCl_2$ was added; d) calcium-rich freshwater: 13.4 mM $CaCl_2$ was added to the artificial freshwater.

Six eels from each group were anesthetized with MS222 and blood samples were taken by sectioning the caudal peduncle 4 h after the injection on days 1, 3, 5, 10 and 15 after treatment. The sera were separated and analyzed for calcium and phosphate levels according to the methods of Trinder (16) and Fiske and Subbarow (17), respectively.

Data are reported as mean ± SEM for six specimens and the Student *t*-test was used to determine statistical significance. Each experimental group was compared to its specific time control group.

Results

Artificial freshwater (groups A and B)

In vehicle-injected eels (group A) the serum calcium levels exhibited almost no change throughout the experiment (Figure 1). No change was observed in serum calcium level on day 1 following vitamin D_3 treatment (group B). From day 3 to day 10 the serum calcium level increased progressively, and returned to normal levels on day 15. In vehicle-injected eels (group A), the serum phosphate level remained unchanged throughout the experiment (Figure 2). There was no significant change in serum phosphate level up to day 3 in vitamin D_3-treated eels (group B) as compared to the vehicle-injected specimens. A progressive increase occurred thereafter from day 5 to the end of the experiment.

Calcium-free freshwater (groups C and D)

The serum calcium levels of vehicle-injected specimens (group C) decreased progressively from day 1 to day 5 (Figure 1), and increased thereafter from day 10 to the end of the experiment. On day 1 following vitamin D_3 treatment (group D) the serum calcium level remained unchanged as compared to the vehicle-injected group. From day 3 to day 10 the eels exhibited progressive hypercalcemia with a slight decrease on day 15. In vehicle-injected specimens (group C) there

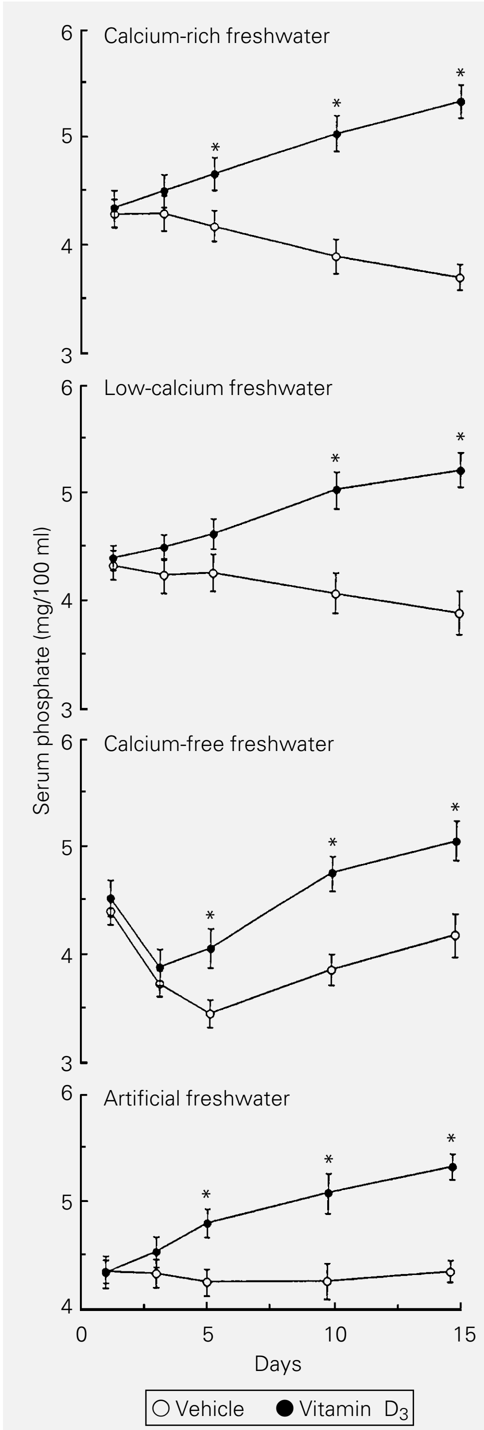

Figure 2 - Changes in serum phosphate levels of *A. cuchia* kept in artificial freshwater, calcium-free freshwater, low-calcium freshwater or calcium-rich freshwater and treated with vehicle or vitamin D_3. Each value represents the mean ± SEM for six specimens. Asterisk indicates significant differences ($P<0.05$) compared to the vehicle-injected group (Student *t*-test).

was progressive hypophosphatemia from day 3 to day 5, followed by an increase from day 10 to day 15 (Figure 2). Up to day 3 following vitamin D_3 treatment (group D), the serum phosphate level remained almost similar to that of vehicle-injected eels. From day 5, the level increased progressively until day 15.

Low-calcium freshwater (groups E and F)

The serum calcium level of vehicle-injected specimens (group E) was slightly increased on day 5, and progressively decreased between day 10 and day 15 (Figure 1). There was no change in the serum calcium level of vitamin D_3-treated specimens (group F) on day 1. The level increased progressively from day 3 to day 10. On day 15, the level exhibited a slight decrease although it was still above normal. The serum phosphate level of vehicle-injected specimens (group E) was slightly decreased on day 10 and day 15 (Figure 2). Up to day 5 following vitamin D_3 treatment (group F) the serum phosphate level remained unchanged. On day 10, the levels exhibited a significant increase which persisted until day 15 (Figure 2).

Calcium-rich freshwater (groups G and H)

The serum calcium level of vehicle-injected eels (group G) was slightly elevated on day 3 and day 5, declining thereafter until the end of the experiment (Figure 1). There was no change on day 1 in the serum calcium level of vitamin D_3-injected specimens (group H) as compared to the vehicle-injected group. The level was significantly increased on day 3 and continued to increase progressively until day 10. However, at the end of the experiment the level declined. The serum phosphate level of vehicle-injected eels (group G) remained unchanged until day 5 and then tended to decline on day 10 and day 15 (Figure 2). In vitamin D_3-treated specimens (group H), the first perceivable change in serum phosphate level was an increase on day 5 which continued progressively until day 15.

Discussion

In *A. cuchia* vitamin D_3 acted as an inducer of hypercalcemia and hyperphosphatemia when the fish were kept in artificial freshwater and low-calcium freshwater. However, these responses were greater when the eels were maintained in calcium-rich freshwater. Earlier investigators working on sharks, rays and cyclostomes (18) and on lungfish (19) have reported that administration of vitamin D_3 fails to affect blood calcium contents. Lopez et al. (20) injected $1,25(OH)_2D_3$ into *Anguilla anguilla* and found that the plasma calcium concentrations were not affected by the administration of the metabolite. MacIntyre et al. (21) noticed hyperphosphatemia among eels treated with $1,25(OH)_2D_3$ but no change in calcium levels. According to them, $1,25(OH)_2D_3$ mediates phosphate homeostasis in marine fish which live in an environment rich in calcium but poor in phosphorus. The observed hypercalcemic and hyperphosphatemic effects of vitamin D_3 in *A. cuchia* are in good agreement with earlier reports of similar responses after vitamin D and/or maintenance of the fish in a calcium-rich environment (7-12,15). The present study also agrees with the reports of other investigators who have noticed hypercalcemia (9-11,15) and hyperphosphatemia (9,10,15,21) after administration of $1,25(OH)_2D_3$. Lafeber et al. (22) injected trout and eel with 0.68 M $CaCl_2$ solution (100 µl 100 g fish^{-1} day^{-1}) and noticed increased plasma calcium levels. A pronounced hypercalcemia has also been recorded after injecting the American eel *Anguilla rostrata* with calcium chloride solution (23). These studies support the hypercalcemia observed here in *A. cuchia* maintained in calcium-rich freshwater.

In calcium-free freshwater, administration of vitamin D_3 to *A. cuchia* induced hypercalcemia and hyperphosphatemia. The hypercalcemia observed in *A. cuchia* cannot be attributed to calcium absorption at the intestinal mucosa level since the eels were not fed and the surrounding medium lacked calcium.

In the present study vitamin D_3 treatment resulted in hypercalcemia and hyperphosphatemia in all media tested, a fact possibly explained by increased resorption of bone and/or mobilization of calcium and phosphate from soft tissues.

There was a decline in the serum calcium and phosphate levels of vehicle-injected *A. cuchia* maintained in calcium-free freshwater. Wendelaar Bonga et al. (24) also noticed significant hypocalcemia in tilapia after 5 days of transfer to a low-calcium environment, which they attributed to the increased efflux of this ion through the gill. Moreover, Flik et al. (25) have suggested that low-calcium concentration in the ambient water of tilapia may allow intercellular Ca^{2+} to diffuse out of the animal. They have further explained that branchial efflux routes of Ca^{2+} following paracellular routes may be increased as a result of lower ambient Ca^{2+}. The hypocalcemia observed in *A. cuchia* maintained in calcium-free freshwater also confirms data reported by Wendelaar Bonga and van der Meij (26) who noticed increased integumental water permeability at low-ambient Ca^{2+}. At low-ambient Ca^{2+} the increased water uptake may increase urine production which leads to extra Ca^{2+} loss from the body (27).

In vehicle-injected *A. cuchia* kept in calcium-free freshwater, the serum calcium level was reduced up to day 5 and was slightly elevated on day 10 and day 15. This restoration of plasma calcium is most probably mediated by an enhanced production of prolactin, as previously suggested by Wendelaar Bonga et al. (24). According to Flik et al. (28), prolactin stimulates Ca^{2+} uptake from the water in intact tilapia. In the present study, there was no calcium available to the eels from the surrounding medium; therefore, the restoration of calcium can be attributed to bone demineralization and/or increased mobilization from soft tissues. Since in the present study we did not analyze bone calcium content, we cannot emphatically state that bone demineralization occurred in *A. cuchia*.

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