Vitamin D₂ Is Not Biologically Active for Rora (Labeo rohita) as Vitamin D₃

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Summary The present investigation was directed towards finding the
relative biopotency of vitamin D₃ and D₂ in fish. The freshwater column
feeder fish Labeo rohita (Rora) was used for the study. The feeding of
Rora with graded levels of vitamin D₂ (550, 1,100 and 1,650 i.u./kg diet)
and vitamin D₃ (1,100 and 1,650 i.u./kg diet) resulted in no behavioural
or morphological changes in comparison with the group fed a vitamin
D-deficient diet. Also, the growth rate, feed efficiency, mortality rate,
carcass protein, total lipids, calcium and phosphorus were found to remain
unaltered in the vitamin D-deficient fish and fish fed any form of the
vitamin. Further, there is no difference in any of the above parameters
between the different doses of vitamin D₃ or vitamin D₂. Thus, the results
of this study indicate that both of the forms of vitamin D (D₂ or D₃) are
not biologically active for Rora (Labeo rohita) as a representative of
freshwater fish.

Key Words carcass, fish, Rora, vitamin D₂, vitamin D₃

It is well known that vitamin D exists in two major forms, vitamin D₃, the
animal form and vitamin D₂, of plant origin. While vitamin D₃ is synthesized by
the action of sunlight on 7-dehydrocholesterol (7-DHC) (provitamin D₃) in the
skin of land animals, vitamin D₂ is produced upon ultraviolet (UV) irradiation of
the plant sterol, ergosterol (provitamin D₂) (1–5). Vitamin D₃ derived either
endogenously from photobiogenesis in the skin or exogenously from the diet
undergoes two sequential hydroxylations to become biologically active. The first
occurs in the liver at position C-25 to form 25-hydroxy vitamin D₃ (25-OH-D₃),
the circulating form of vitamin D, and the next occurs in the kidney at position
C-1 to form 1,25-dihydroxy vitamin D₃ [1,25-(OH)₂D₃], the most active metabolite

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of vitamin D and which plays an important role in calcium homeostasis (6, 7). Thus, in terrestrial animals, vitamin D is an essential nutrient required for normal growth and mineralization, and also, the effects of its deficiency and hyper-vitaminosis D are well studied in these animals (7–9).

Vitamin D is known to occur abundantly in certain fish (5, 10–12). Recently, we have reported that vitamin D in fish may mainly be derived from their dietary sources, i.e. plankton, which were found to be very rich sources of both vitamin D$_2$ and D$_3$ (13), but not by photochemical synthesis (14), or a non-photochemical pathway may not be operating in these animals (15). Thus, not only vitamin D$_3$, but also vitamin D$_2$ appears to enter fish through their food sources. However, it is not clear whether both of these forms are biologically active in fish.

Earlier studies have shown biological discrimination towards vitamin D$_2$ and D$_3$ in some species of terrestrial animals. Vitamin D$_2$ was not found to be biologically active in chick and New World monkeys (16–18). However, the biopotency of vitamin D$_3$ and D$_2$ in fish is not clearly known. These aspects were studied in the freshwater fish Rora (Labeo rohita) and reported in the present paper.

MATERIALS AND METHODS

**Chemicals.** The sources of vitamin D$_3$ and D$_2$ have already been given earlier (13, 19). All other chemicals were of analytical grade procured locally. The purity standards of vitamin D$_3$ and D$_2$ were assessed by their UV spectra as described earlier (13, 19).

**Experimental design.** Four-week-old Rora fry weighing 63.08 ± 7.95 mg (mean ± SD) used in the experiment were obtained from the government hatchery at Ananthapur, India. The fish were then acclimatized to the laboratory conditions for a week in large plastic pools of 1,000 L capacity before commencement of the experiment. The plastic pools were placed in such a way that the fish received natural light for about 12 h a day. However the pools were not kept under direct sunlight and the water was clear. The pools were aerated 18–20 h/d. During the period of acclimatization, all of the fish were fed a basal diet deficient in vitamin D.

The acclimatized fish were then randomly divided into six groups of 250 each and fed the following diets containing graded levels of vitamin D$_3$ or vitamin D$_2$, as shown in Table 1. Triplicate groups of fish were used for each dietary treatment.

The basal diet employed for the study (Table 2) was that formulated by Mahajan and Yadave (20) which did not have any added vitamin D.

The ambient water had the following characteristics: temperature: 24.7 ± 3.2°C, pH: 7.8 ± 0.3, dissolved O$_2$: 8.6 ± 0.5 ppm, calcium: 34.7 ± 5.9 ppm, phosphorus: 0.76 ± 0.05 ppm; mean ± SD.

The fish were fed ad libitum once a day and were allowed 6 h to feed for a period of 240 d. The fish were weighed at 30 or 45 d intervals in batches of 25–30 to study the growth. Also, the hepatosomatic index, i.e. the % liver weight to body weight, was measured at similar intervals using 5 fish from each dietary treatment.
Table 1. Vitamin D$_2$ and D$_3$ administration to various groups of fish.

| Group No. | Vitamin D status | Dose (i.u./kg diet) |
|-----------|------------------|---------------------|
| 1         | Vitamin D-deficient | 0                   |
| 2         | Vitamin D$_2$     | 550                 |
| 3         | Vitamin D$_2$     | 1,100               |
| 4         | Vitamin D$_3$     | 1,650               |
| 5         | Vitamin D$_3$     | 1,100               |
| 6         | Vitamin D$_3$     | 1,650               |

The various experimental groups are shown in the table. All six dietary treatments of fish were fed basal diet + graded levels of either vitamin D$_3$ or D$_2$. The composition of basal diet given in Table 2.

Table 2. Composition of the basal diet.

| Ingredient               | %   |
|--------------------------|-----|
| Casein                   | 22.9|
| Gelatin                  | 14.5|
| Dextrin                  | 33.7|
| Soyabean oil             | 5.4 |
| Vitamin premix*          | 0.6 |
| Mineral premix           | 2.4 |
| Cellulose powder         | 20.5|

The composition of the basal diet is shown in the table. All ingredients except casein and dextrin were obtained locally. Casein and dextrin were obtained from ICN Pharmaceutical Inc., Life Science Group, Cleveland Ohio, USA. Vitamin and mineral premix as given in Mahajan and Yadave (20).

* Vitamin premix did not have any added vitamin D.

A record was maintained of mortality and feed efficiency, calculated on the basis of feed consumed vs. average body weight throughout the experimental period. Five fish were used for the measurement of feed efficiency for each dietary treatment and each time interval.

At the end of the experimental period, fish were taken at random from each tank, sacrificed and the following parameters were estimated: carcass lipid, protein, calcium and phosphorus levels.

*Behavioural and morphological changes.* The behaviour of the fish was judged by swimming pattern, reaction to disturbance and eagerness to feed when diet was given. The morphological observations included skin colour, fin structure, possible infection of any kind, shape of body, etc.

*Carcass, protein and total lipids.* The fish ($n = 20$) from each dietary treatment were dried to a constant weight at 100°C, the dried carcass was powdered and...
about 1 g of powder in duplicate was used for the estimation of protein and total lipids.

Carcass protein was estimated following the Kjeldahl method. The total lipids were extracted with ether using a soxlet apparatus. The ether was evaporated and the residual lipids weighed.

*Carcass, calcium and phosphorus.* About 1 g of the carcass powder (in duplicate) was ashed in a muffle furnace at 600°C for 24 h. The carcass ash was dissolved in 6 N HCl and the volume was made up to 100 mL with water. Calcium and phosphorus were determined as described earlier (19) using appropriate diluted solutions.

*Statistical analysis.* Values are indicated as the M ± SE. The statistical differences between the various experimental groups, fed/not fed vitamin D$_3$ or vitamin D$_2$, were assessed by analysis of variance (ANOVA) (21). A normal curve test was utilised for differences in mortality rates (21).

The experiments were approved by the ethical committee on the use of animals for research.

**RESULTS**

**Behavioural, morphological changes and growth**

No behavioural or morphological changes were observed in any of the groups of fish throughout the experimental period. There were no signs of tetany or lethargy in any of the experimental fish, either given or not given vitamin D.

The growth curves of fish from the various experimental groups are shown in Fig. 1. As can be seen from the figure, no significant effect on growth rate was observed throughout the experimental period in fish fed the vitamin D-deficient diet (group 1) or supplemented with vitamin D at various doses (groups 2–6). No change in growth was observed at any time interval between fish given either vitamin D$_2$ or vitamin D$_3$ [(groups 2–4) and (groups 5 and 6)] (one-way ANOVA).

Also, no significant difference was observed in the feed efficiency or mortality rates among the various experimental groups (groups 1–6). The data on feed efficiency and mortality rates of fish fed diet devoid of vitamin D, or supplemented with either vitamin D$_2$ or D$_3$ (1,650 i.u./kg diet), i.e. groups 1, 4 and 6, are shown in Table 3.

**Hepatosomatic index**

The changes in hepatosomatic index, which indicates the relative size of liver to the body of fish, are given in Table 4. As shown in the table, the hepatosomatic index of the fish from all of the experimental groups tended to decrease (not statistically significant) with their increase in body weight. The data was analysed by two-way ANOVA.
Fig. 1. Growth curves for *Labeo rohita* (Rora) receiving diets with various levels of vitamin D$_3$ or D$_2$. 1: vitamin D-deficient diet; 2, 3, 4: 550, 1,100 and 1,650 i.u. vitamin D$_2$/kg diet; 5, 6: 1,100 and 1,650 i.u. vitamin D$_3$/kg diet. No statistical difference was observed between the six experimental groups each day (ANOVA).

**Carcass protein, lipid, calcium and phosphorus**

Table 5 gives the data on carcass protein, lipid, calcium and phosphorus of the various experimental groups of fish. It can be observed from the table that there were no significant differences in the carcass protein or lipid values in the groups fed the vitamin-D deficient diet or supplemented with vitamin D$_2$ or D$_3$ (one-way ANOVA).

The carcass calcium was found to be about 54 mg/g in the group fed a diet devoid of vitamin D (group 1) and is not different from the other groups (groups 2–6). Also, it was found that the carcass phosphorus remained unaltered in the groups whether given/not given vitamin D (one-way ANOVA).
Table 3. Feed efficiency and mortality of fish.

| Group No. | Dose (i.u./kg diet) | Feed efficiency (%) | Mortality rate (%) |
|-----------|---------------------|---------------------|-------------------|
| 1         | Vitamin D-deficient diet | 0                | 1.10 ± 0.09       | 21.0             |
| 4         | Vitamin D₂           | 1,650              | 1.10 ± 0.02       | 21.0             |
| 6         | Vitamin D₃           | 1,650              | 1.11 ± 0.04       | 20.6             |

The values are shown as % feed efficiency, i.e. % feed consumed vs. average body weight of fish and % mortality, after feeding fish with/without vitamin D for 240 d (M ± SE). Five fish were used for feed efficiency. No significant differences were observed among the groups. The mortality rates among the various groups did not differ significantly. A normal curve test was utilised for differences in mortality rates.

Table 4. Hepatosomatic index in fishes at various time intervals.

| Group No. | Dose (i.u./kg diet) | Sampling time in days |
|-----------|---------------------|-----------------------|
|           |                     | 30  | 60  | 105 | 150 | 195 | 240 |
| 1         | Vitamin D-deficient diet | 0  | 2.34| 2.46| 2.41| 2.59| 1.74| 1.65|
|           |                      | ±0.39%| ±0.19%| ±0.32%| ±0.75%| ±0.77%| ±0.31%|
| 2         | Vitamin D₂           | 550 | 2.19| 2.54| 2.57| 2.17| 1.82| 1.64|
|           |                      | ±0.62| ±0.28| ±0.46| ±0.47| ±0.21| ±0.24|
| 3         | Vitamin D₂           | 1,100| 2.42| 2.27| 2.38| 2.21| 1.73| 2.05|
|           |                      | ±0.47| ±0.46| ±0.40| ±0.39| ±0.58| ±0.33|
| 4         | Vitamin D₂           | 1,650| 2.40| 2.68| 2.60| 1.96| 1.68| 1.98|
|           |                      | ±0.38| ±0.31| ±0.31| ±0.35| ±0.43| ±0.21|
| 5         | Vitamin D₃           | 1,100| 2.71| 2.40| 2.30| 2.10| 1.81| 1.45|
|           |                      | ±0.33| ±0.33| ±0.52| ±0.40| ±0.33| ±0.22|
| 6         | Vitamin D₃           | 1,650| 2.64| 2.55| 2.41| 2.14| 1.87| 1.61|
|           |                      | ±0.42| ±0.46| ±0.79| ±0.49| ±0.40| ±0.15|

The values are shown as hepatosomatic index, i.e. % liver weight to body weight (M ± SE). Five fish were used for this experiment. No significant difference was observed among the groups when data were analysed by two-way ANOVA.
Table 5. Carcass, protein, lipid, calcium and phosphorous levels in various groups of fish.

| Group No. | Dose (i.u./kg diet) | Carcass |
|-----------|---------------------|---------|
|           |                     | Protein (g/100 g) | Lipids (g/100 g) | Calcium (mg/g) | Phosphorus (mg/g) |
| 1         | Vitamin D-deficient diet (20) | 0 | 67.46 ± 1.96 | 7.08 ± 0.33 | 54.06 ± 1.76 | 30.28 ± 1.17 |
| 2         | Vitamin D$_2$ (20) | 550 | 67.57 ± 2.45 | 7.89 ± 0.81 | 54.81 ± 1.26 | 30.91 ± 1.26 |
| 3         | Vitamin D$_2$ (20) | 1,100 | 68.09 ± 0.99 | 8.29 ± 0.60 | 55.27 ± 1.63 | 31.02 ± 1.73 |
| 4         | Vitamin D$_2$ (20) | 1,650 | 67.42 ± 0.93 | 7.83 ± 0.51 | 54.96 ± 3.08 | 30.41 ± 1.77 |
| 5         | Vitamin D$_3$ (20) | 1,100 | 68.46 ± 0.87 | 8.08 ± 0.31 | 55.05 ± 1.68 | 30.88 ± 0.83 |
| 6         | Vitamin D$_3$ (20) | 1,650 | 68.73 ± 2.04 | 8.16 ± 0.90 | 55.28 ± 2.18 | 30.88 ± 1.52 |

The carcass protein, lipid, calcium and phosphorous were calculated and compared to each value of fish fed diet without vitamin D or with vitamin D (D$_2$ or D$_3$) (M±SE). The figures in parentheses indicate the number of fish used in the experiment. No significant difference was observed among the groups.

DISCUSSION

Although both the forms of vitamin D (D$_2$ and D$_3$) are known to enter fish through their dietary sources (13, 22), the relative efficiency of vitamin D$_2$ vs. D$_3$ is not clear. Earlier studies are mostly confined to catfish (Ictalurus punctatus) and rainbow trout (Salmo gairdneri) (23–26).

In the present study, the relative biopotency of vitamin D$_2$ and D$_3$ was examined in Rora by feeding fish with graded levels of either vitamin D$_3$ (1,100 and 1,650 i.u./kg diet) or vitamin D$_2$ (550, 1,100 and 1,650 i.u./kg diet) and comparing the growth responses of fish with those fed diet devoid of vitamin D. Feeding fish the vitamin D-deficient diet for 1 week must have depleted the liver vitamin D, though they may not be vitamin D-deficient. However, we did not assess the liver vitamin D content. The absence of any significant differences in any of the parameters studied in either fish fed diet devoid of vitamin D or those fed any form of vitamin D (D$_2$ or D$_3$) at various doses for a long feeding period of 240 d, provides evidence of a lack of biological discrimination of vitamin D$_2$ and D$_3$ by fish.

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These findings in Rora are similar to earlier studies in catfish (25, 26) and rainbow trout (24), which have shown that both forms of vitamin D are equally efficacious at dietary levels up to 1,000–1,500 i.u./kg diet. These researchers have further reported that, beyond these levels (>1,000 i.u./kg diet), vitamin D$_3$ was more potent than vitamin D$_2$. But in their studies, no tetany or hypocalcemia was observed in fish not fed/fed vitamin D below or beyond 1,000–1,500 i.u./kg diet. However, a decrease was observed in weight gain and feed efficiency in fish fed a diet containing vitamin D$_2$ more than D$_3$ beyond 1,000–1,500 i.u./kg diet, based on which the authors claim that vitamin D$_3$ is more potent than vitamin D$_2$ at higher levels (>1,000 i.u./kg diet). The argument may not sound to draw such a conclusion since vitamin D-related known parameters (serum and bone calcium and phosphorus levels) did not seem to alter such a case. Further, fingerlings of fish were used for the studies. In the present study, the fry stage of fish were fed vitamin D$_2$ or D$_3$ of various levels to give a more clear view of the efficacy of vitamin D$_2$ vs. D$_3$ in fish. The absence of any changes in the various growth or vitamin D-related parameters (carcass calcium and phosphorus levels) in fish fed not only lower, but also higher levels of both vitamin D$_2$/D$_3$ (1,650 i.u./kg diet) for a long feeding period of 240 d, compared to short feeding periods as in earlier studies [98 d (25), 168 d (24) and 196 d (26)], clearly indicates that both forms of vitamin D are biologically inactive in Rora, as a representative of freshwater fish.

There was a tendency for a decrease (not statistically significant) in the hepatosomatic index of Rora in all of the experimental groups, which may be explained on the basis that liver weight does not increase in the same proportion as the body weight.

Our earlier studies using Rora have shown no differences in vitamin D-deficient fish (vitamin D deficiency was judged by the absence of any detectable vitamin D in the liver of fish grown in dark and fed a diet devoid of vitamin D for six months) and those fed vitamin D$_3$ (1,650 i.u./kg diet) in vitamin D-related (bone and carcass calcium and phosphorus contents) and growth parameters (carcass protein and lipid, hepatosomatic index, feed efficiency and mortality rates), indicating that vitamin D$_3$ is not biologically active in this fish (27). The present study is in line with earlier observations showing that not only vitamin D$_3$ but also the other form of vitamin D, vitamin D$_2$, is biologically inactive in Rora, as a representative of freshwater fish.

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