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Original Article

The skeletal deformity in response of dietary phosphorus and calcium level in the Caspian roach (*Rutilus rutilus caspicus*) larvae

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Abstract: Skeletal deformities are a common problem in fish hatcheries and commercial farms that affect growth, development and survival as well as the market value of the final product. Among the nutritional components, phosphorus (P) and calcium (Ca) are of special interest as they are directly involved in the development and maintenance of the skeletal system. Hence, the present study was carried out to investigate the effects of dietary P and Ca on the skeletal deformity, growth and carcass composition the Caspian roach (*Rutilus rutilus caspicus*) larvae. In this study, six semi-purified diets were formulated. The diets A, B, C, D and E were supplemented with 0.0, 0.4, 0.8, 1.2 and 1.6% available P supplied as a 1:1mixture of NaH_2Po_4/KH_2Po_4. These five diets were supplemented with 1% Ca, supplied as $CaCo_3$. Diets F was Ca-free and supplemented with 0.8% available P served as control level of P. Each diet was randomly assigned to triplicate groups of fish, and each group was stocked with 30 larvae and fed three times a day for 60 days. At the end experiment, there was no significant effect of dietary P (0 to 1.6%) or Ca (0 or 1%) supplementation on growth performance such as weight gain and FCR, carcass moisture, P and Ca. However, a significant difference found between treatments in carcass ash. Analysis of length, height and area of vertebrae in two regions of the vertebral column showed no significant difference between the dietary treatments. The skeletal abnormalities were highest incidence in the Caspian roach fed with a low P. Kyphosis placement of vertebrae was the most frequent abnormality.

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Introduction

Main morphological change of the fish larvae is occurred during the early developmental stages which can be affected by xenobiotic, environmental, genetic, nutritional and physiological factors. During this period, abnormalities such as the skeletal deformities are frequently observed in reared fish larvae as a result of deficiencies in above mentioned factors (Cahu et al., 2003; Lall et al., 2007). The incidence of skeletal deformities in cultured fishes is led to financial losses (Sadler et al., 2001). The most skeletal deformities in fishes include vertebral and spinal malformations such as kyphosis, lordosis, fusion of vertebrae and irregular column (Lall et al., 2007). Moreover, these deformities may be occurred as a combination of several types of deformities (Lall et al., 2007).

Nutrition has been suggested having a key role in skeletogenesis (Beattie and Avenell, 1992; Wallach, 2002). The effects of dietary factors related to nutrient deficiencies have received limited attention in fishes in despite many works on humans and terrestrial animals (Beattie and Avenell, 1992; Wallach, 2002). It has been suggested that nutritional deficiencies, including some vitamins e.g. C, B, A or D and minerals e.g. zinc, magnesium, phosphorous (P), calcium (Ca) and selenium, promote skeletal deformities in fish larvae under culture conditions

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Table 1. Formulation and composition of experimental diets (g/100 g dry weight).

Diet	A	B	C	D	E	F
Casein-dextrin basis[a]	82.5	82.5	82.5	82.5	82.5	82.5
Vitamin mixture	5	5	5	5	5	5
Mineral mixture without P and Ca[b]	2.5	2.5	2.5	2.5	2.5	2.5
Monobasic sodium and potassium phosphates (50/50)	0.00	1.68	3.36	5.04	6.72	3.36
α-Cellulose	7.50	5.82	4.14	2.46	0.78	4.14
Calcium carbonate	2.50	2.50	2.50	2.50	2.50	0.00
Available P	0.0	0.4	0.8	1.2	1.6	0.8
Available Ca	1.0	1.0	1.0	1.0	1.0	0.0

[a]Casein-dextrin basis (% diet): 52% casein); 0.65%, L-methionine; 0.85% , L-arginine; 8% soybean lecithin; 8% fish oil; 12% dextrin; 1% sodium alginate.
[b]Mineral mixture (g/kg mineral mix): KCl, 180; KI, 0.08; NaCl, 80; $CuSO_4{\cdot}5H_2O$, 6; $ZnSO_4{\cdot}7H_2O$, 8; $CoSO_4$, 0.04; $FeSO_4{\cdot}7H_2O$, 40; $MnSO_4{\cdot}H_2O$, 6; MgOH, 248; Na_2SeO_3, 0.06; NaF, 2. All ingredients were diluted with α-cellulose.

(Lall et al., 2007). P and Ca are closely related to the development and maintenance of the skeletal system. Fishes can absorb Ca and P from water and their Ca requirement is met by their ability to absorb directly this element from water (Lall, 2002). Ca deficiency, unlike P deficiency is related to very low concentration of this element in water and not common, However, Ca deficiency induces a delay in the ontogeny of skeletal development without affecting final bone mineralization and leads to modifications in the shape and size of vertebrae (Fontagné et al., 2009). P deficiency in most fish species is led to poor growth, poor bone mineralization, skeletal deformities, low ash and high lipid content in the whole body, while excess P affects survival (Tacon, 1992; Lall, 2002).

Information about the larval nutritional requirements such as Ca and P is limited due to their fast-changing requirements during ontogeny and also; their requirements are slightly different from those of juveniles. Hence, understanding of biological effects and function of these components is essential and can allow to improve the quality of hatchery-reared larvae (Cahu et al., 2003). Therefore, a special attention should be paid to the level and availability of P in formulation of diets for normal vertebral development during the early development of fish larvae.

The Caspian roach (*Rutilus rutilus caspicus* Jakowlew, 1870), a commercially important, has declined greatly in the Iranian Caspian Sea due to overfishing and deterioration of its spawning grounds (Eagderi and Ghelichpour, 2012). Therefore, its artificial propagation in hatcheries to restock its natural population is necessary. The Iranian Fisheries Organization produces the Caspian roach larvae in the earthen pond till 1-3 g and then releases them to the rivers ending to the Caspian Sea (Kiabi et al., 1999). This study was conducted to investigate the effect of different levels of P and Ca on the skeletal deformity, growth and carcass composition of the reared Caspian roach larva with a view to determining the optimum P requirement for the growth of this fish.

Material and Methods

Design of experiment: the Caspian roach larvae were obtained (with a mean weight of 0.108 ± 0.01g) from the Sijval Fish Hatchery (Gorgan, Iran). Before the experiment, the larvae were acclimated to laboratory conditions in a single 1000 L tank for 10 days. During this period, water temperature (°C), pH, DO (mg L^{-1}) were 24 ± 2, 7.8 ± 0.2, 7 ± 1, respectively. After the acclimation period, the larvae were randomly divided into eighteen 100 L experimental tanks, each containing 30 larvae.

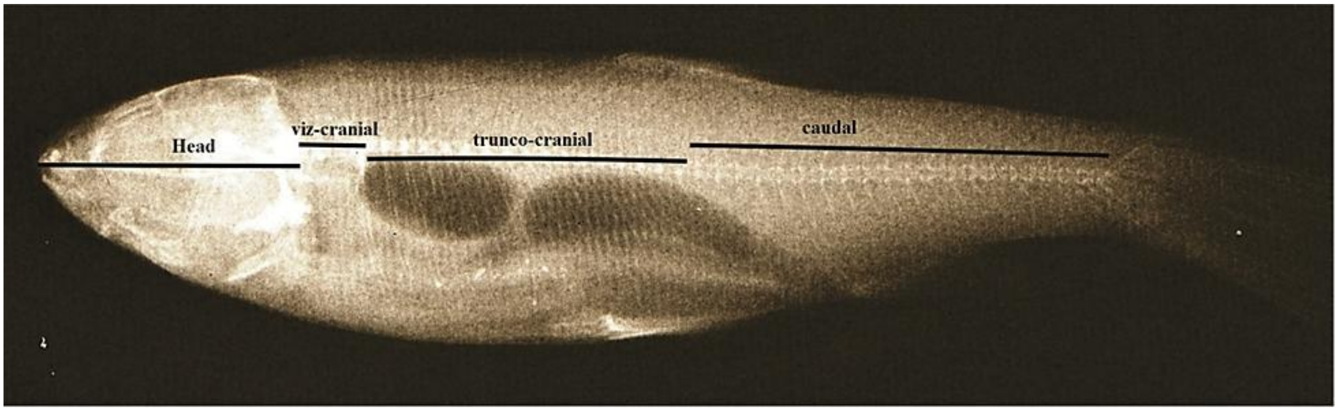

Figure 1. X-Ray image of Caspian roach larva showing three distinct regions considered during measurement of the vertebral column; viz-cranial, trunco-cranial and caudal.

Feeding and experimental diets: Six semi-purified diets were prepared with graded levels of P and Ca, based on Fontagne et al. (2009) (Table 1). The diets A, B, C, D and E were supplemented with 0.0, 0.4, 0.8, 1.2 and 1.6% available P supplied as a 1:1 mixture of NaH_2Po_4/KH_2Po_4. These five diets were supplemented with 1% Ca, supplied as $CaCO_3$. Diets F was Ca-free and supplemented with 0.8% available P served as control level of P. Each diet was provided to three replicate tanks. Every two weeks, samples of fish were taken from each tank to weigh fish and calculate the required food for the following week. Larvae were fed three times a day with experimental diets, 5% of their bw/d over a 60-days experiment period.

Sample collection: At the end of the experiment, the larvae were anaesthetized using the clove oil solution (1 ml diluted in 40 liters of water). Mean weight of larvae was measured using the sum of individual weight divided by the number of fish in each tank. Then, weight gain (gr) and FCR of treatment were calculated. Fifteen larvae from each treatment (five larvae per tank) were sampled to analysis the composition of carcass (five larvae) and examination of skeletal deformity (10 larvae).

Chemical analysis: Moisture determined by drying larvae in an oven at 105°C for 24 hrs. To determine the ash and mineral contents, dried samples were placed in a muffle furnace at 550°C for 24 hrs (AOAC, 2000). Minerals, including the P and Ca were measured using an atomic emission spectrophotometer.

Examination of vertebrae: Ten larvae were fixed in 5% formalin (from each treatment) and x-rayed using a mammography system (Payamed, mamo x-Ray, unit 100 kHZ, IRAN), with a manual exposure mode to give 10 mAs and 20 kV. The films of radiographies were digitized using Epson Perfection V600 Photo Scanner and archived as JPEG images for subsequent analysis. Three vertebrae in three anatomical regions were identified in the vertebral column of the Caspian roach based on Kacem et al. (2003) including, viz-cranial, trunco-cranial and caudal (Fig.1). The length (L), height (H) of vertebrae and vertebral area (area = $2\pi \times (H/2) \times L$) in trunco-cranial and caudal were measured using ImageJ (http://rsb.info.nih.gov/ij/). But viz-cranial was identified as the weberian apparatus were disregard.

Statistical Analysis: One-way ANOVA and Duncan's multiple range tests were performed using SPSS 20 to examine significance different between mean values of the dietary groups (Duncan, 1955).

Results

The weight gain (WG), FCR and carcass composition of the Caspian roach larvae fed with different levels of P and Ca are shown in Table 2. Dietary supplemented with different levels of available phosphorus (0, 0.4, 0.8, 1.2 and 1.6%) and calcium (0 and 1%) did not affect growth of the Caspian roach larvae. Although larvae in the group E with 1.2% available P had the highest WG and lowest FCR, but no significant differences in WG

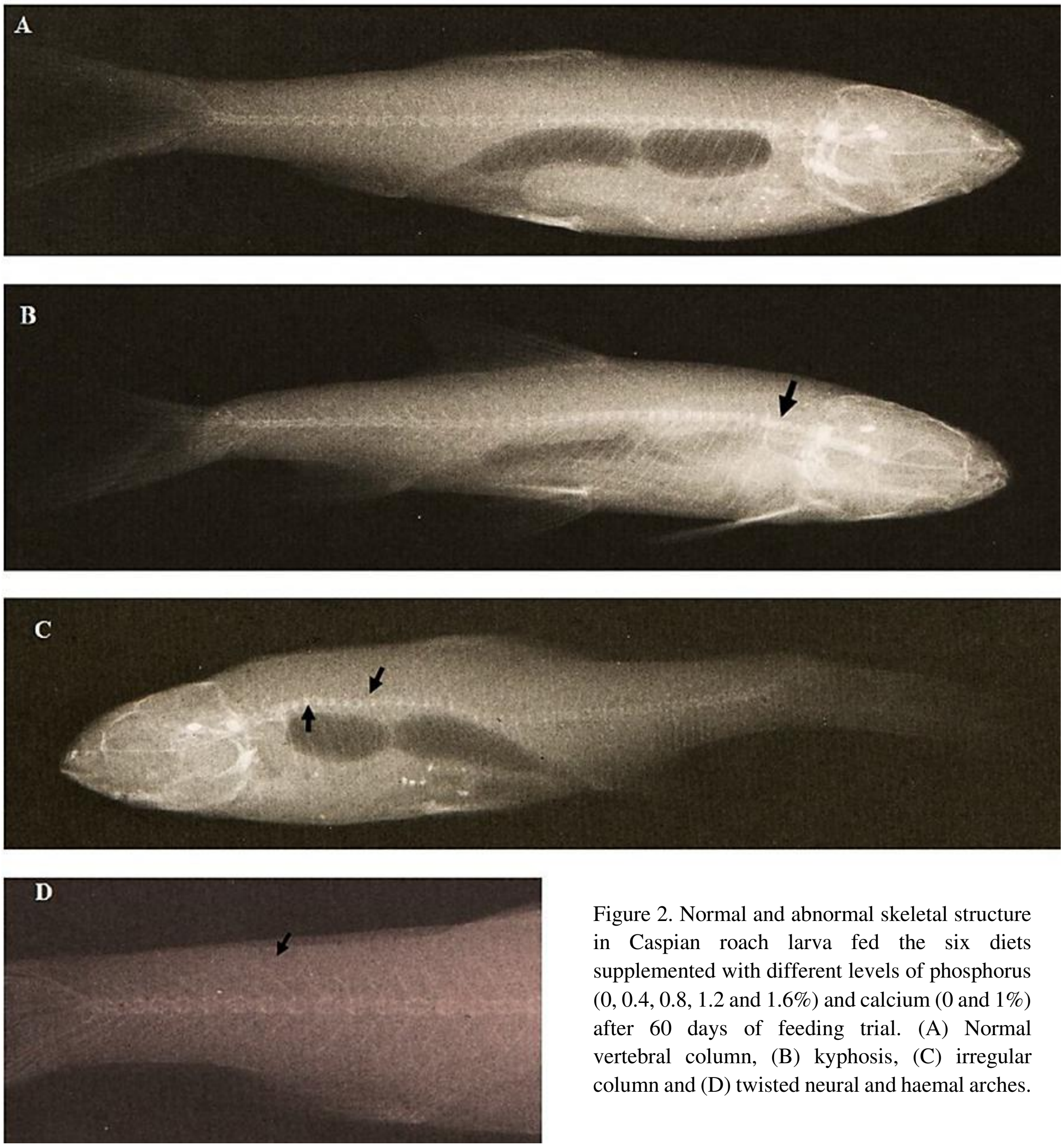

Figure 2. Normal and abnormal skeletal structure in Caspian roach larva fed the six diets supplemented with different levels of phosphorus (0, 0.4, 0.8, 1.2 and 1.6%) and calcium (0 and 1%) after 60 days of feeding trial. (A) Normal vertebral column, (B) kyphosis, (C) irregular column and (D) twisted neural and haemal arches.

Table 2. Weight gain, FCR and carcass composition of the Caspian roach larvae fed diets with different levels of P and Ca (% wet weight).

	A	B	C	D	E	F
Ash	1.37 ± 0.07 [a]	2.08 ± 0.34 [b]	2.28 ± 0.30 [b]	2.40 ± 0.39 [b]	2.67 ± 0.20 [b]	2.23 ± 0.18 [b]
Moisture	74.4 ± 0.28 [a]	74.4 ± 0.39 [a]	74.3 ± 0.91 [a]	74.4 ± 0.76 [a]	74.3 ± 0.13 [a]	74.6 ± 0.9 [a]
P	0. 23 ± 0.05 [a]	0.26 ± 0.08 [a]	0.31 ± 0.16 [a]	0.34 ± 0.03 [a]	0.32 ± 0.01 [a]	0.30 ± 0.12 [a]
Ca	0.19 ± 0.02 [a]	0.18 ± 0.04 [a]	0.25 ± 0.02 [a]	0.31 ± 0.04 [a]	0.22 ± 0.15 [a]	0.26 ± 0.1 [a]
WG (g)	0.784 ± 0.07[a]	0.807 ±0.05[a]	0.805 ± 0.18[a]	0.867 ± 0.05[a]	0.806 ± 0.09 [a]	0.77 ± 0.11 [a]
FCR	1.92 ± 0.17 [a]	1.86 ± 0.12 [a]	1.93 ± 0.49 [a]	1.74 ± 0.18 [a]	1.87 ± 0.22 [a]	1.97 ± 0.27 [a]

Means of triplicate values with similar superscript are not significantly different ($P>0.05$)

and FCR were found between dietary treatments ($P>0.05$). Also, no significant difference in carcass moisture, P and Ca between the dietary groups was found ($P>0.05$). However, there was significant difference between the treatments in ash content of the carcass ($P<0.05$). Analysis of the length, height and area of vertebrae in two identified regions in the vertebral column (Table 3) revealed no significant

Table 3. Length, height and area(pixel) of 3 vertebrae in 2 regions identified in the vertebral column of the Caspian roach sampled on day 60 and fed diets supplemented with different levels of available phosphorus ((0, 0.4, 0.8, 1.2 and 1.6%) and calcium (0 and 1%).

Column region	Diet	Vertebral length	Vertebral height	Vertebral area
Trunco-cranial	A	2.26 ± 3.4 a	26.0 ± 3.0 a	2132.4 ± 327.5 a
	B	25.3 ± 2.06 a	26.3 ± 2.2 a	2095.0 ± 220.3 a
	C	25.3 ± 2.7 a	25.3 ± 1.8 a	2024.2 ± 314.5 a
	D	27.07 ± 2.2 a	24.2 ± 1.9 a	2054.3 ± 157.3 a
	E	28.4 ± 1.2 a	27.3 ± 1.8 a	2438.3 ± 163.6 a
	F	25.3 ± 2.8 a	25.4 ± 2.0 a	2030.1 ± 352.1 a
Caudal	A	28.0 ± 1.4a	24.1 ± 1.9 a	2118.0 ± 86.4 a
	B	27.3 ± 1.6 a	26.1 ± 1.6 a	2132.4 ± 327.5 a
	C	26.4 ± 2.5 a	26.1 ± 1.9 a	2174.2 ± 335.9 a
	D	26.5 ± 1.9 a	25.7 ± 1.5 a	2137.4 ± 189.2 a
	E	26.6 ± 1.3 a	26.3 ± 1.9 a	2201.5 ± 287.8 a
	F	27.4 ± 1.2 a	26.5 ± 1.0 a	2281.9 ± 190.6 a

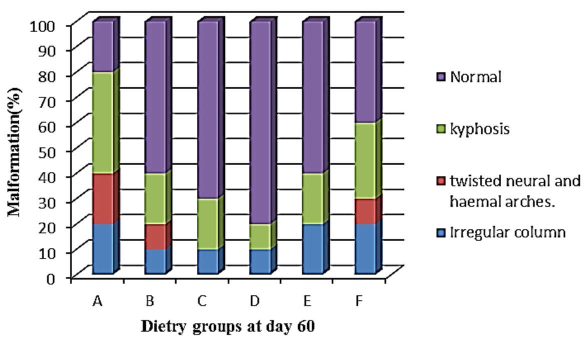

Figure 3. Percentage of the Caspian roach larvae sampled on day 60 fed diets supplemented with different levels of available P (0, 0.4, 0.8, 1.2 and 1.6%) and Ca (0 and 1%) which showed malformation include kyphosis, irregular column and twisted neural and haemal arches (n=10 for each dietary group).

difference between the dietary groups ($P>0.05$).

Skeletal abnormalities were grouped into three categories viz. twisted haemal and neural arches, kyphosis, irregular column. These abnormalities showed in Figure 2. The frequency of each abnormality was calculated as a proportion of the total number of abnormalities per dietary group (n=10) (Fig. 3). The highest incidence of internal abnormalities was observed in the treatment fed with a low P. The lowest abnormality was observed in group D (1.2% available P). Kyphosis placement of vertebrae was the most frequent abnormality.

Discussion

Skeletal abnormality due to its impact on larva quality is led to financial losses; hence it is considered as an important issue for finfish industry and animal welfare (Koumoundouros, 2001; Sfakianakis et al., 2006). An approach to solve this problem is to determine accurately of larval nutritional requirements such as Ca and P which are key parameters influencing the skeletogenesis during the early development of fish (Negm et al., 2013).

The present study investigated the effects of different dietary level of P and Ca on the skeletal deformity, growth and carcass composition in the Caspian roach larvae with a view to determine its optimal P requirement. The results revealed that the whole-body ash was significantly lower in the larvae fed a low P supplement (group A), whereas, the whole-body moisture not affected by any dietary groups indicating P is necessary for bone mineralization. The ash content of bone is considered to be the most sensitive criterion for evaluating dietary P utilization (Ye et al., 2006). Nwanna et al. (2008) determined that P deficiency produced lower ash in channel catfish (*Heterobranchus bidorsalis*). Similar results have also been reported in Channel catfish (Andrews et al., 1973), Sunshine bass (Brown et al., 1993) and Haddock (Roy and Lall, 2003), which are in agreement with our results. The results also showed that growth parameters, including WG and FCR were not affected by different dietary P and Ca levels; however, the results suggest that the best WG were obtained with diet D containing 1.2% available P. It is well recognized that the need for P, to gain maximum bone strength and bone ash content, are higher than that to gain maximum WG (Sauveur and Perez, 1987).

At the end of experiment, the mineral content of fish whole body, P and Ca contents showed no significant different among P dietary groups. Calcium supplement, also, had no significant effect on WG, FCR and carcass composition, including moisture, ash, phosphorus and calcium, which is in agreement with the generally accepted view that most fish can absorb Ca directly from the aquatic environment or from food ingredients to meet their requirements (Baeverfjord et al., 1998).

The results of the present study showed that the dietary P deficiency induced skeletal deformities in the Caspian roach larvae. In addition, sizes of vertebrae such as length, height and area of vertebrae were identified in two regions, i.e. the trunco-cranial and caudal regions showed no differences among treatments. However, some skeletal abnormalities such as twisted haemal and neural arches, kyphosis and irregular vertebral column were observed with dietary P deficiency that is in agreement with other studies (Baeverfjord et al., 1998; Vielma and Lall, 1998; Helland et al., 2005; Uyan et al., 2007). Visible external abnormalities are principally associated with the spine and differed in incidence between the fish fed with different diets (Fontagné et al., 2009).

In conclusion, the present study revealed that P deficiency affects bone calcification in the Caspian roach larvae. Thus, it is necessary to pay attention to the level and availability of P in the formulation of diets for normal vertebral development during the early development of Caspian roach.

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