EFFECTS OF FLUORIDE EXPOSURE ON THE GROWTH, METAMORPHOSIS, AND SKELETAL DEVELOPMENT OF *RANA CHENSINESIS AND RANA NIGROMACULATA* LARVAE

Jiayi Chen,[a] Lihong Chai[b,c] Hongfeng Zhao,[a] Minyao Wu,[a] Hongyuan Wang[a,*]

Xi'an, People's Republic of China

ABSTRACT: Chronic exposure to high fluoride ion levels (F) may lead to local tissue disturbances, known as fluorosis. The present study was conducted to determine the effects of sodium fluoride (NaF) on the growth, metamorphosis, and skeletal development in tadpoles of *Rana chensinesis* and *Rana nigromaculata*. The mortality, percentage of tadpoles completing metamorphosis, total length, body weight, and hindlimb length were determined. In addition, skeletal systems were investigated by using double staining methodology at Gosner stages 36, 42, and 46. The results showed that chronic exposure to 50 mg NaF/L significantly increased the mortality, inhibited metamorphosis, and delayed development in *R. chensinesis* and *R. nigromaculata* tadpoles. The NaF treatment also produced flexural tail malformations in *R. chensinesis* tadpoles but not in *R. nigromaculata* tadpoles, stimulated bone mineralization in *R. chensinesis* tadpoles, and retarded the deposition of calcium in *R. nigromaculata* tadpoles. In conclusion, our study suggests that fluoride may affect skeletal ossification differently in different frog species due to differences in sensitivity or the duration of the exposure.

Keywords Fluoride; Metamorphosis; *Rana chensinesis*; *Rana nigromaculata*; Skeletal development.

INTRODUCTION

Fluorine is one of common elements in the earth's crust but is not found naturally in the environment in its free state, due to its great chemical reactivity, and exists in inorganic fluorides, as the fluoride ion (F), or in organic fluoride compounds.[1,2] The primary natural sources of inorganic fluorides are volcanic eruptions, hot springs, soil during droughts, the weathering of rock, and the burning of fossil fuels, etc.[3] Fluoride is not an essential microelement for life and in excess can produce toxic effects in plants, animals, and humans.[4] Fluoride pollution, with increased fluoride concentrations in both water and the atmosphere, may be produced by human activities, such as metallurgy, the production of bricks, ceramics, and glasses, and the processing of nuclear fuel and manure.[5] Human activities may increase the fluoride concentration of surface water 100-fold up to 45 mg F/ L.[6]

Fluoride is known to interact with mineralized tissues such as bone and teeth and, when concomitant calcium deficit is present, may aggravate bone loss, via reductions in the mineralized trabecular and cortical bone mass, and cause fragility fractures.[7] Currently, many investigations have demonstrated that fluoride has effects on bone mineralization in both rats and humans.[8,9] Fluoride behaves as a

[a]College of Life Science, Shaanxi Normal University, Xi'an 710119, PR China; [b]School of Environmental Science and Engineering, Chang'an University, Xi'an 710054, PR China; [c]Key Laboratory of Subsurface Hydrology and Ecological Effect in Arid Region of Ministry of Education, Xi'an 710062, PR China; *For correspondence: Hongyuan Wang, PhD, College of Life Science, Shaanxi Normal University, Xi'an 710119, PR China; Tel: +86-029-85310266; Fax: +86-029-85310546; E-mail: hongyuanwang@snnu.edu.cn

cumulative toxic agent that can alter the accretion and resorption of tissues as well as affecting the homeostasis of bone mineral metabolism.[10,11] Fluoride exerts a biphasic action at the level of the osteoblasts, on bone mineralization, bone structure, and bone function. At low dosages, fluoride stimulates the formation of bone. However, at high concentrations, fluoride reduces the apposition rates of calcium ion and results in a prolongation of the mineralization time, thus delaying the development of the skeleton.[12,13] Moreover, at high dosages, fluoride may lead to the formation of abnormally mineralized bone of impaired quality.[14]

Bone is a composite tissue with a greater mineral component which consists of calcium phosphate and other salts deposited in a matrix, osteoid, primarily consisting of Type 1 collagen but also containing proteoglycans such as chondroitin sulfate and keratin sulphate. Calcification is crucial for the normal mineralization of bone.[15] Alizarin red can stain the calcium in bone red give an indication of the calcium concentration in the bone[16] while aleian blue can stain the chondroitin sulfate and keratin sulfate, in non-mineralized osteoid or cartilage, blue. In our experience, we have found the double-staining method with alizarin red and aleian blue to be useful for investigating the skeletal development of larvae exposed to fluoride at different development stages by clearly showing bone and shape and the degree of ossification.

Metamorphosis development is an important stage in the life history of amphibians and involves the reconstruction of the tissues and organs of the tadpole, including the remolding of the skeletal system and the growth of the limbs.[17,18] The reconstruction of the tadpole skeletal system is thought to play an essential role in amphibian metamorphosis, which enables adaptation to occur from the aquatic habitat to the land habitat. Although excessive fluoride can impair skeletal development in *Bufo gargarizans* tadpoles,[19] no studies had been done on the effects of fluoride on skeletal development in *R. chensinesis* and *R. nigromaculata.*

The aim of the present study was to investigate the effects of fluoride on skeletal development in *R. chensinesis* and *R. nigromaculata* tadpoles at metamorphosis. Using a control group with water with 0 mg NaF/L, we examined the effects of chronic fluoride exposure, with water at 50 mg NaF/L, on mortality, body size, and body mass in *R. chensinesis* and *R. nigromaculata* tadpoles. In addition, the skeletal systems at pre-metamorphosis, at metamorphic climax, and at the completion of metamorphosis were investigated by using double staining methodology.

MATERIALS AND METHODS

Experimental animals: The tadpoles studied originated from sexually mature male and female frogs, of the species *Rana chensinesis* and *Rana nigromaculata*, which were collected in February, from the Qinling Mountains, Shaanxi Province, PR China, and induced to spawn by each couple being placed in a 40 L glass aquarium.

Chemicals and reagents: The stock solutions were prepared by dissolving sodium fluoride (NaF), from Sigma-Aldrich Corporation (Sigma, St Louis, MO, USA), in dechlorinated tap water to obtain a nominal concentration of 50 mg NaF/L.

Exposure test: The control group was kept in dechlorinated tap water with 0 mg NaF/L while the experimental treatment group were kept in water with 50 mg NaF/L. After hatching, the *R. chensinesis* and *R. nigromaculata* embryos were collected and placed in individual 20 L glass aquaria with either 0 (control) or 50 (treatment) mg NaF/L (nominal concentrations). Using a complete randomized block design, at Gosner stage 26,[20] the tadpoles were divided into 16 tanks, 4 control tanks and 4 tanks with 50 mg NaF/L for each of the two species, with n=90 tadpoles in each tank. The temperature was maintained at 22ºC with photoperiods of 12 hr of light and 8 hr of darkness. The water conditions were pH 7.9, dissolved oxygen (DO) 7.2 mg/L, and specific conductivity 1477 µS/cm. During this time the dead embryos were removed twice daily. The data collected on the dead embryos was used to compute the embryonic survival rate. Any unconsumed food was siphoned from the tanks daily. The tadpoles were fed two to three times per day. The rearing water was completely renewed three times per week, starting at two weeks post-hatch.

Development, mortality, metamorphosis, and deformities: Daily records were maintained for hatching success, and the embryo and larval mortality. Deformities, mass, and the date of completed metamorphosis (Gosner stage 46) were monitored for all the individuals. We examined the effects of chronic fluoride exposure on the metamorphosis of *R. chensinesis* and *R. nigromaculata* tadpoles, at the fluoride concentrations of 0 and 50 mg NaF/L, from Gosner stage 26 to Gosner stage 46. The periods of tadpoles development were defined according to Gosner's 1960 classification[20] as pre-metamorphosis (Gosner stages 36–37), prometamorphosis (Gosner stages 39–40), metamorphic climax (Gosner stage 42), and completion of metamorphosis (complete tail resorption; Gosner stage 46). The sampling plan for this study was designed to encompass these periods. Thirty tadpoles from both control and treatment tanks were collected randomly for each of the two species, weighed, and measured for total length and hindlimb length at Gosner stages 36, 42, and 46, respectively.

Determination of the skeletal development: The double-staining method with alizarin red and aleian blue was used to investigate the skeletal development of the larvae exposed to different concentrations of NaF at different development stages. Three tadpoles, at Gosner stages 36, 42, and 46, were collected randomly from each aquarium. Both the control (0.00 mg NaF/L) and fluoride-treated (50 mg NaF/L) larvae were anaesthetized with 95% alcohol, decolorized with 30% H_2O_2, and defatted with acetone. After being eviscerated and cleared, each specimen was stained using the alizarin red-aleian blue double staining method.[21] Bones (containing calcium) were indicated in red and cartilage (containing chondroitin sulfate and keratin sulfate) in blue. The osteological terminology used follows Duellman and Trueb.[22] All the descriptions and illustrations were based on

photographs taken with a Cannon 7D digital camera attached to a Zeiss Discovery V12 stereoscope.

Statistical analysis: Statistical analyses were performed using the SPSS 16.0. Differences between treatments were tested by with one-way ANOVA of the total length, body weight, and hindlimb length as the dependent list, with the dose of NaF as the variable factor, at Gosner stages 36, 42, and 46. In addition, differences in the measured variables (mortality and percent metamorphosed) across each treatment were also tested using one-way ANOVA. The data were reported as mean±SE. For all analyses, a $p<0.05$ was considered to be a significant difference.

RESULTS

Mortality and metamorphosis rates in larvae of R. chensinesis and R. nigromaculata: Exposure to 50 mg NaF/L had a significant effect on the mortality of *R. chensinesis* and *R. nigromaculata* tadpoles ($p<0.05$). The mortalities of *R. chensinesis* tadpoles exposed to the 0 (control) and 50 mg NaF/L were 11.1% and 34.4%, respectively. Likewise, the mortalities of *R. nigromaculata* tadpoles in water with 0 and 50 mg NaF/[1] were 17.8% and 42.2%, respectively. For both species, the mortalities of the group exposed to 50 mg NaF/L were significantly greater ($p<0.05$) than the mortalities of the groups exposed only to dechlorinated water (Figure 1).

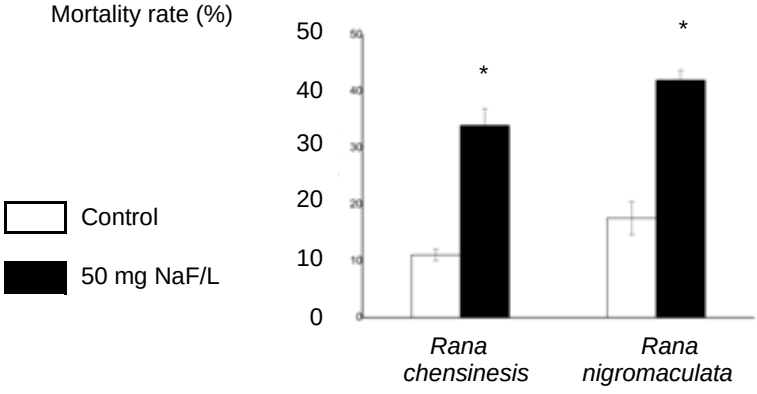

Figure 1. Mortality rate of *R. chensinesis* and *R. nigromaculata* tadpoles exposed to control water (dechlorinated water) and water with 50 mg NaF/L. Values are mean±SE. At the initiation of the exposure there were 16 tanks (4 control tanks and 4 tanks with 50 mg NaF/L for each of the two species) with n=90 tadpoles in each tank. Compared to the control group, *$p<0.05$.

Compared to the control groups who were exposed to dechlorinated water only, exposure to 50 mg NaF/L significantly delayed ($p<0.05$) the rates of metamorphosis from tadpole to frog of *R. chensinesis* and *R. nigromaculata*. The metamorphosis rates of *R. chensinesis* exposed to 0 and 50 mg NaF/L were 54.4% and 28.9%, respectively. Similarly, the metamorphosis rates of *R. nigromaculata*

tadpoles, exposed to 0 and 50 mg NaF/L, were 57.8% and 32.2%, respectively. (Figure 2).

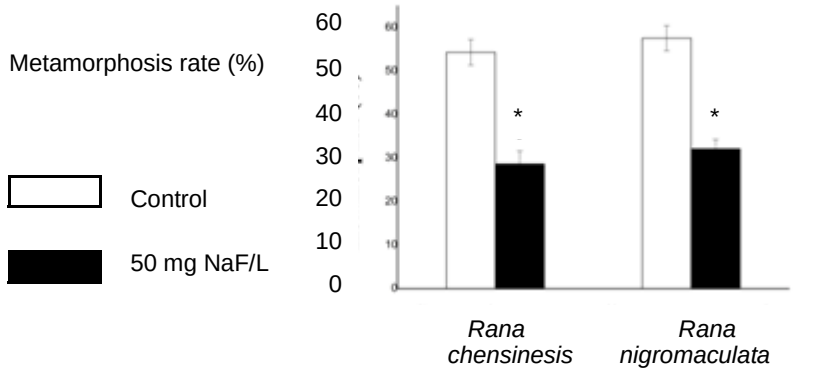

Figure 2. Metamorphosis rate of *R. chensinesis* and *R. nigromaculata* tadpoles exposed to control water (dechlorinated water) and water with 50 mg NaF/L. Values are mean±SE. At the initiation of the exposure there were 16 tanks (4 control tanks and 4 tanks with 50 mg NaF/L for each of the two species) with n=90 tadpoles in each tank. Compared to the control group, *p<0.05.

Effect of sodium fluoride on growth and development in larvae of R. chensinesis and R. nigromaculata: The total length of the *R. chensinesis* tadpoles was significantly reduced in the treated group compared with the control group at Gosner stages 36 and 42 ($p<0.01$ and $p<0.05$, respectively, one-way ANOVA). However, the total length of the individuals that reached completed metamorphosis at Gosner stage 46 during exposure to 50 mg NaF/L was not significantly reduced compared to the untreated control group ($p= 0.524$, Figure 3).

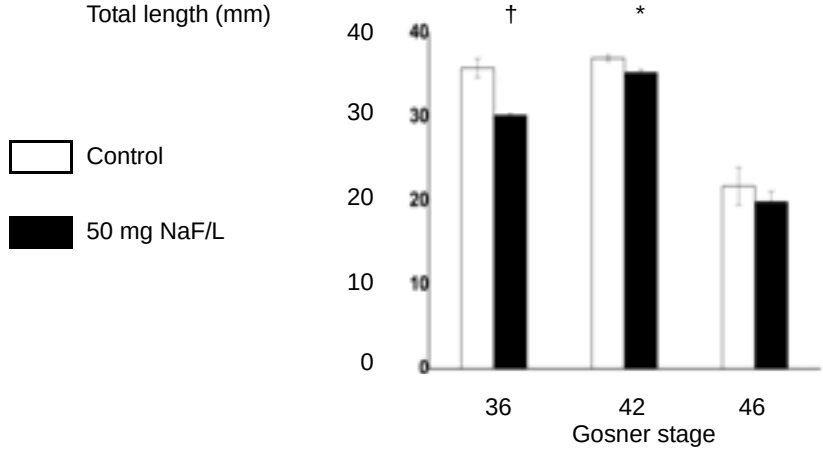

Figure 3. Total length of *R. chensinesis* at Gosner stages 36, 42, and 46. Values are mean±SE. Compared to the control group (one-way ANOVA): *p<0.05, †p<0.01.

At Gosner stages 36 and 46, significant reductions in weight were observed in the treated groups of the *R. chensinesis* tadpoles, relative to untreated control group, but no significant reductions were observed at Gosner stage 42 (Figure 4).

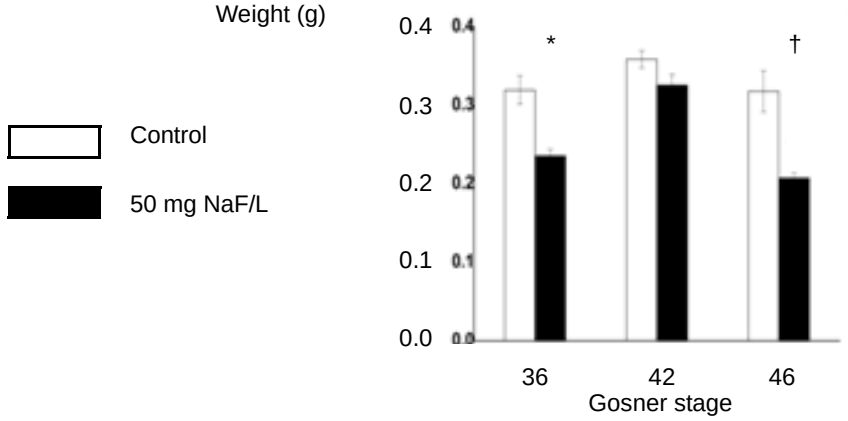

Figure 4. Weight of *R. chensinesis* at Gosner stages 36, 42, and 46. Values are mean±SE. Compared to the control group (one-way ANOVA): *p<0.05, †p<0.01.

There were no signs of reduced hindlimb length in the treated groups of the *R. chensinesis* tadpoles compared to the control group at Gosner stages 42 and 46, but significant diminutions were observed at Gosner stage 36 ($p<0.01$, Figure 5).

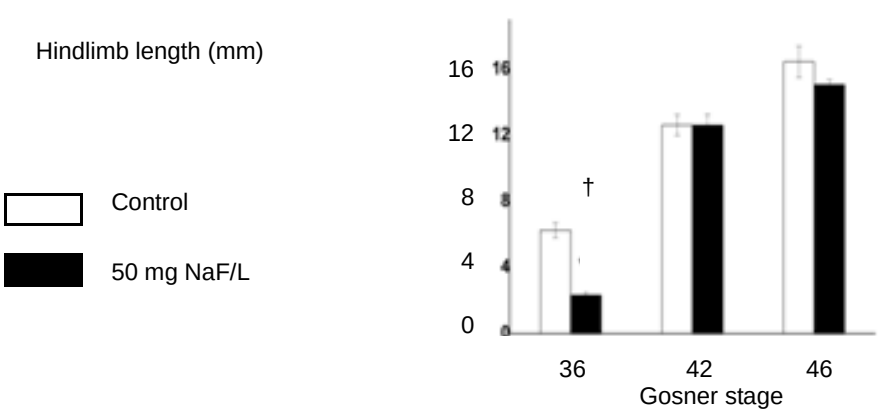

Figure 5. Hindlimb length of *R. chensinesis* at Gosner stages 36, 42, and 46. Values are mean±SE. Compared to the control group (one-way ANOVA): *p<0.05, †p<0.01.

In the *R. nigromaculata* tadpoles, growth was inhibited with 50 mg NaF/L at Gosner stages 42 and 46 with significant reductions in total length ($p<0.05$ for both stage 42 and 46, one-way ANOVA), compared to the control values, but the total length for stage 36 was not significantly altered by the treatment (Figure 6).

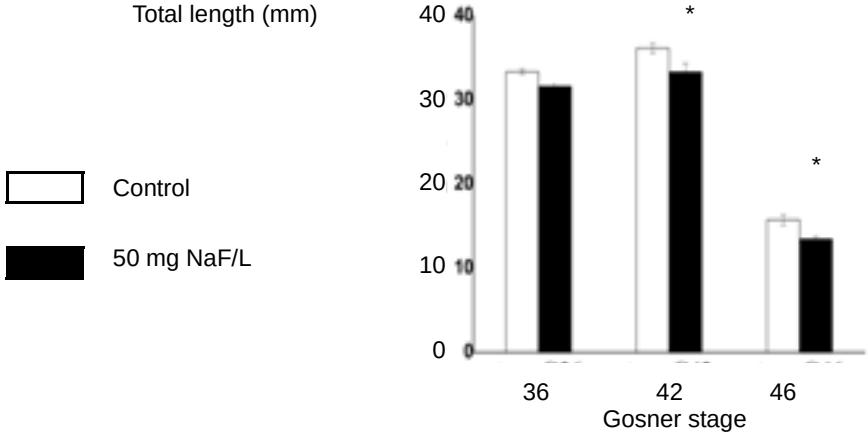

Figure 6. Total length of *R. nigromaculata* at Gosner stages 36, 42, and 46. Values are mean±SE. Compared to the control group (one-way ANOVA): *p<0.05, †p<0.01.

In the *R. nigromaculata* tadpoles, at Gosner stage 46, the average weight of the individuals that reached the completion of metamorphosis during exposure to 50 mg NaF/L was significantly reduced relative to that of the individuals in the control group but no significant reductions were observed at Gosner stages 36 and 42 (Figure 7).

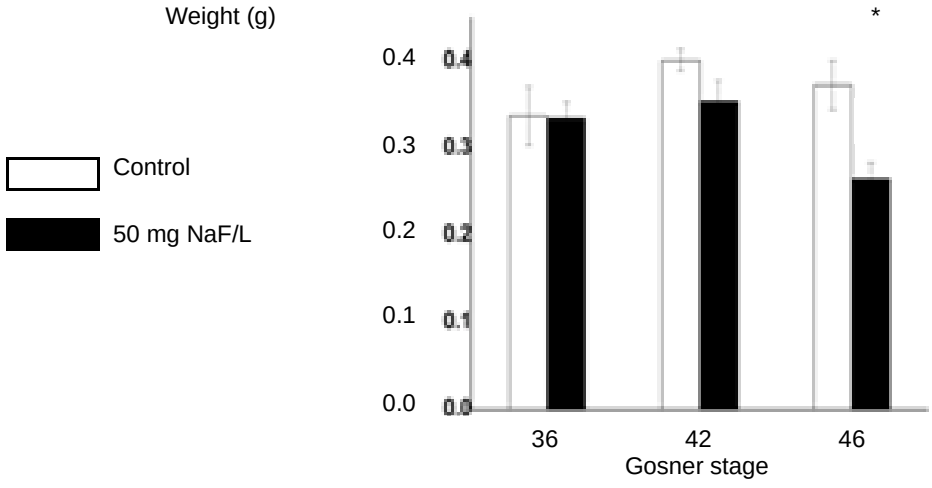

Figure 7. Weight of *R. nigro maculata* at Gosner stages 36, 42, and 46. Values are mean±SE. Compared to the control group (one-way ANOVA): *p<0.05, †p<0.01.

In the *R. nigromaculata* tadpoles, the hindlimb length was significantly lower in the group treated with 50 mg NaF/L compared to the untreated control group, at Gosner stages 36, 42, and 46 respectively ($p < 0.05$ for all stages, Figure 8).

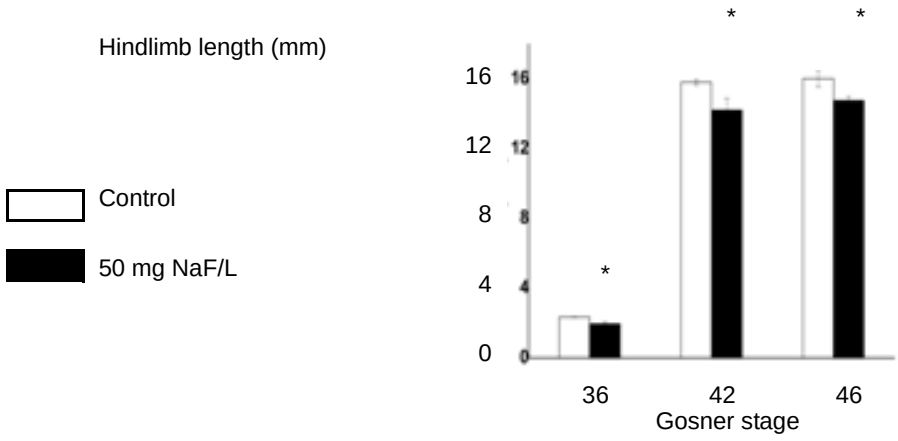

Figure 8. Hindlimb length of *R. nigro maculata* at Gosner stages 36, 42, and 46. Values are mean±SE. Compared to the control group (one-way ANOVA): *p<0.05, †p<0.01.

The morphology of R. chensinesis and R. nigromaculata tadpoles:

The morphological alterations of *R. chensinesis* and *R. nigromaculata* tadpoles in the control group and the treated group were recorded at the different developmental stages. Compared to the normal morphology of *R. chensinesis* from tadpole to frog in the control group (Figure 9A), exposure to the 50 mg NaF/L induced significant deformities, involving asymmetric or bent tails in *R. chensinesis* (Figure 9B). In contrast, no malformations appeared in *R. nigromaculata* with the NaF treatment (Figure 9C).

Figures 9A,B, and C. Photograph illustrating the morphology of *R. chensinesis* and *R. nigromaculata* tadpoles exposed to 0 and 50 mg NaF/L at the different developmental stages. A: dorsal view of representative developmental stages from the control group in *R. chensinesis*; B: dorsal view of representative developmental stages from the treated group in *R. chensinesis*; and C: dorsal view of representative developmental stages from the treated group in *R. nigromaculata.*

Effect of sodium fluoride on bone formation in the larvae of R. chensinesis and R. nigromaculata: The skeletal systems of the tadpoles were demonstrated using the double staining method at different developmental stages in the control and treatment groups. At Gosner stage 36, no ossification was observed in the chondrocranium of the control *R. chensinesis* group, suggesting that ossification was not well developed (Figure 10a). In contrast, ossification of the parasphenoid bones occurred in the 50 mg NaF/L *R. chensinesis* group (Figure

10b). The parasphenoid and exoccipital bones were well ossified indicated by red staining in the control *R. chensinesis* specimen at stage 42 (Figure 10c). In the 50 mg NaF/L *R. chensinesis* group, greater ossification was observed in the parasphenoid, frontoparietal and exoccipital bones compared to control group (Figure 10d). At stage 46, ossification of the parasphenoid, frontoparietal and exoccipital bones increased significantly in the 50 mg NaF/L *R. chensinesis* group compared with that in the control group (Figures 10e and 10f).

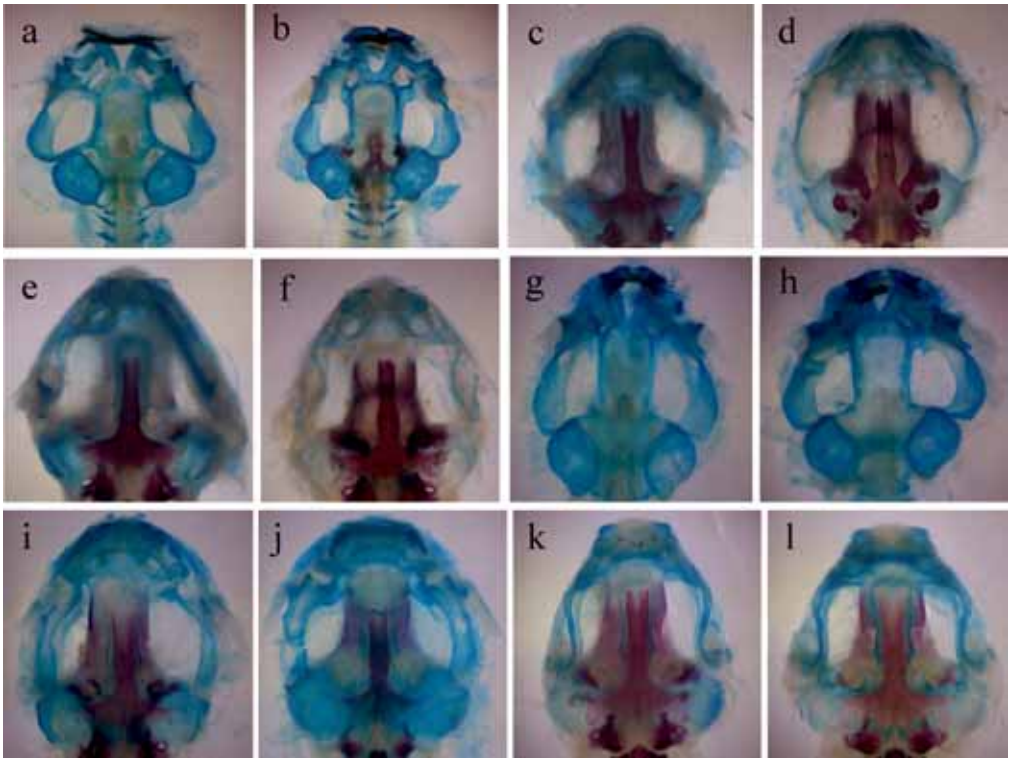

Figure 10. Double staining of the cranial skeleton of *R. chensinesis* and *R. nigromaculata* larvae at different development stages, ventral view. a: the cranial skeleton of *R. chensinesis* at stage G36 in the control group; b: the cranium skeleton of *R. chensinesis* at stage G36 exposed to 50 mg NaF/L; c: the cranial skeleton of *R. chensinesis* at stage G42 in the control group; d: the cranial skeleton of *R. chensinesis* at stage G42 exposed to 50 mg NaF/L; e: the cranial skeleton of *R. chensinesis* at stage G46 in the control group; f: the cranial skeleton of *R. chensinesis* at stage G46 exposed to 50 mg NaF/L; g: the cranial skeleton of *R. nigromaculata* at stage G36 in the control group; h: the cranial skeleton of *R. nigromaculata* at stage G36 exposed to 50 mg NaF/L; i: the cranial skeleton of *R. nigromaculata* at stage G42 in the control group; j: the cranial skeleton of *R. nigromaculata* at stage G42 exposed to 50 mg NaF/L; k: the cranial skeleton of *R. nigromaculata* at stage G46 in the control group; and l: the cranial skeleton of *R. nigromaculata* at stage G46 exposed to 50 mg NaF/L.

From the ventral view, the chondrocrania of *R. nigromaculata*, in the control and 50 mg NaF/L groups, were completely cartilaginous at stage 36, (Figures 10g and 10h). In the crania of larvae at stage 42, the ossified parasphenoid, frontoparietal, exoccipital, and prootic bones were observed clearly in the specimens in the control *R. nigromaculata* group (Figure 10i). In contrast, only the ossified parasphenoid and exoccipital were found in the 50 mg NaF/L *R. nigromaculata*

group (Figure 10j). At Gosner stage 46, more ossification was visible in the parasphenoid, frontoparietal, and exoccipital bones in the control group compared to the 50 mg NaF/L *R. nigromaculata* group (Figures 10k and 10l).

In anurans (frogs and toads), the vertebral column is divided into three regions, namely, presacral, sacral, and postsacral regions. The presacral region consists of eight discrete vertebrae; the sacral region is composed of a single vertebra, and the postsacral region is formed by the urostyle. At Gosner stage 42 in the *R. chensinesis* tadpoles, there was no distinct difference in the ossification of the vertebral column between the treated and the control groups (Figures 11a and 11b). At Gosner stage 46 in the *R. chensinesis* tadpoles, in the control group most of the arches had begun to ossify with the transverse process remaining cartilaginous, as indicated by their staining blue, while in the 50 mg NaF/L group both the arches and the transverse process were completely ossified with no cartilage remaining (Figures 11c and 11d).

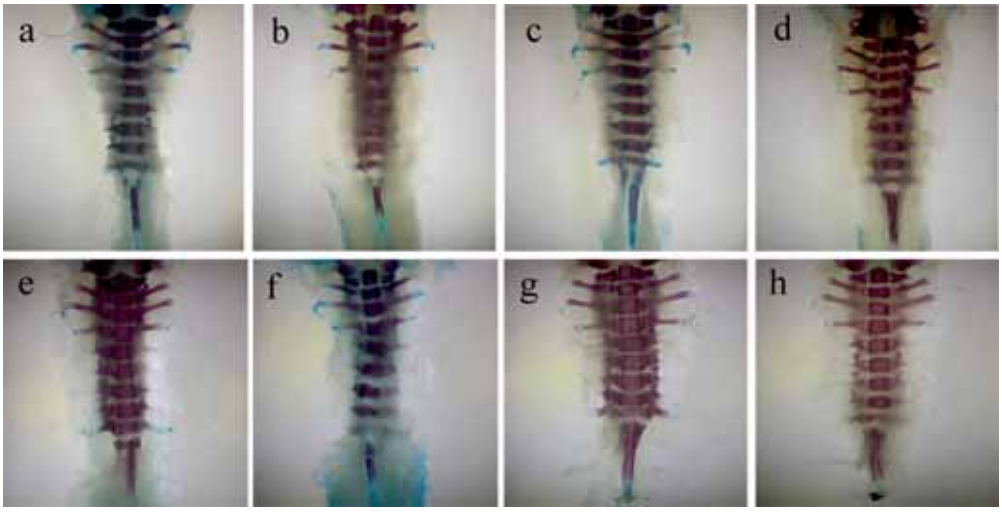

Figure 11. The axial skeleton in ventral view. a: the axial skeleton of *R. chensinesis* at stage G42 in the control group; b: the axial skeleton of *R. chensinesis* at stage G42 exposed to 50 mg NaF/L; c: the axial skeleton of *R. chensinesis* at stage G46 in the control group; d: the axial skeleton of *R. chensinesis* G46 exposed to 50 mg NaF/L; e: the axial skeleton of *R. nigromaculata* at stage G42 in the control group;. f: the axial skeleton of *R. nigromaculata* at stage G42 exposed to 50 mg NaF/L; g: the axial skeleton of *R. nigromaculata* at stage G46 in the control group; h: the axial skeleton of *R. nigromaculata* G46 exposed to 50 mg NaF/L.

At Gosner stage 42 in the *R. nigromaculata* tadpoles, most of the arches and transverse process were ossified in the control group (Figure 11e). However, compared to the control group, ossification of the vertebral column was obviously less in the 50 mg NaF/L group (Figure 11f). At Gosner stage 46 in the *R. nigromaculata* tadpoles, there was no obvious difference in the ossification of the vertebral column between the treated and control groups (Figures 11g and 11h).

The pectoral girdle is composed of the clavicle, the coracoid, the scapula, the cleithrum, the sternum, and the omosternum. The forelimb bones are the humerus, the antibrachium, the carpus, the ossa metacarpalia, and the phalanges. At Gosner

stage 42 in the control group, the ossification centers of the coracoid, the scapula, the humerus, and the antibrachium were distinctly observed in the *R. chensinesis* tadpoles (Figure 12a).

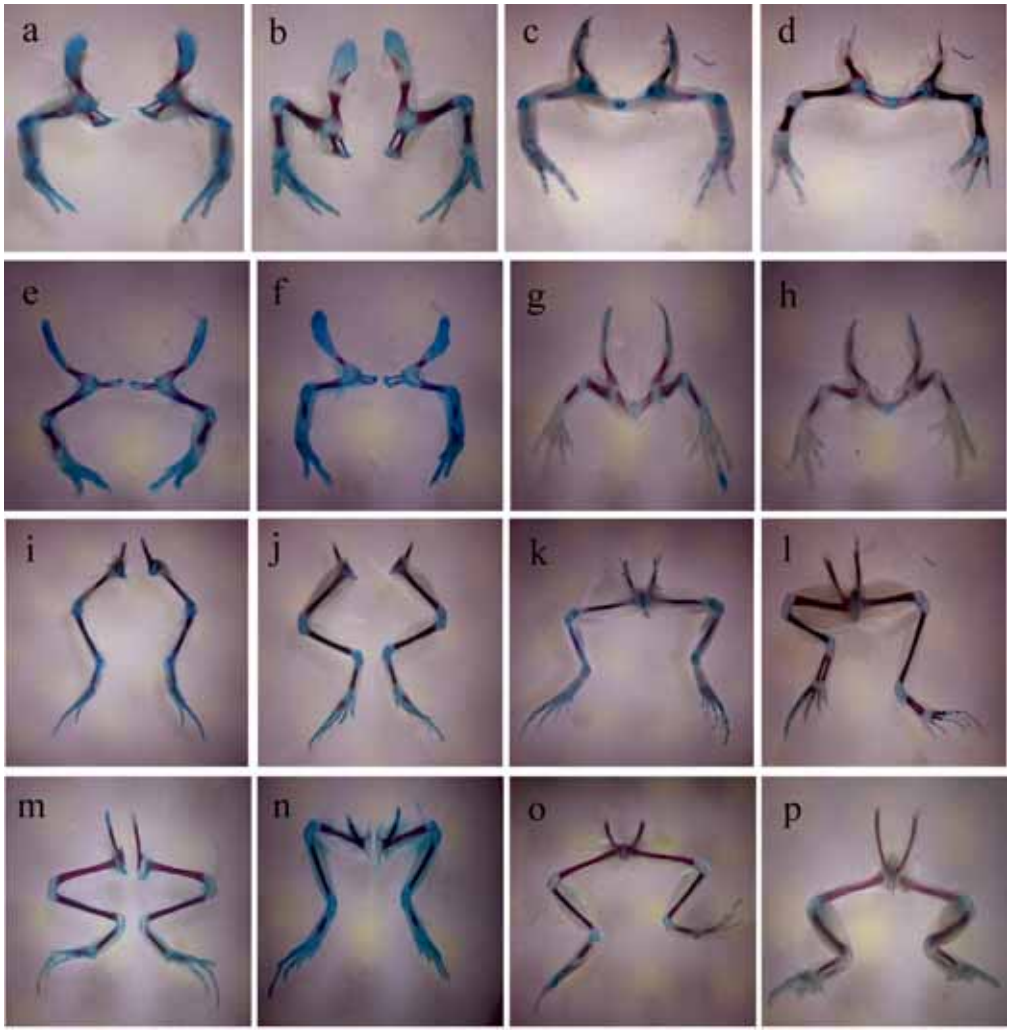

Figure 12. The forelimb and hindlimb skeleton in ventral view. a: the forelimb skeleton of *R. chensinesis* at stage G42 in the control group; b: the forelimb skeleton of *R. chensinesis* at stage G42 exposed to 50 mg NaF/L; c: the forelimb skeleton of *R. chensinesis* at stage G46 in the control group; d: the forelimb skeleton of *R. chensinesis* at stage G46 exposed to 50 mg NaF/L; e: the forelimb skeleton of *R. nigromaculata* at stage G42 in the control group; f: the forelimb skeleton of *R. nigromaculata* at stage G42 exposed to 50 mg NaF/L; g: the forelimb skeleton of *R. nigromaculata* at stage G46 in the control group; h: the forelimb skeleton of *R. nigromaculata* at stage G46 exposed to 50 mg NaF/L; i: the hindlimb skeleton of *R. chensinesis* at stage G42 in the control group; j: the hindlimb skeleton of *R. chensinesis* at stage G42 exposed to 50 mg NaF/L; k: the hindlimb skeleton of *R. chensinesis* at stage G46 in the control group; l: the hindlimb skeleton of *R. chensinesis* at stage G46 exposed to 50 mg NaF/L; m: the hindlimb skeleton of *R. nigromaculata* at stage G42 in the control group; n: the hindlimb skeleton of *R. nigromaculata* at stage G42 exposed to 50 mg NaF/L; o: the hindlimb skeleton of *R. nigromaculata* at stage G46 in the control group; and p: the hindlimb skeleton of *R. nigromaculata* at stage G46 exposed to 50 mg NaF/L.

At Gosner stage 42 in the *R. chensinesis* tadpoles, compared with the control group, more ossification of the pectoral girdle and the forelimb bones was observed in the 50 mg NaF/L group (Figure 12b). At Gosner stage 46 in the *R. chensinesis* tadpoles in the control group, only some bones such as the scapula were ossified and only slight ossification of the diaphysis was present in the humerus (Figure 12c). In contrast, at Gosner stage 46 in the *R. chensinesis* tadpoles in the 50 mg NaF/L group, the clavicle, the coracoid, the scapula, the humerus, and the antibrachium were fully ossified in the 50 mg NaF/L group (Figure 12d)

At Gosner stage 42 in the *R. nigromaculata* tadpoles in the control group, the ossification of the coracoid, the scapula, the humerus, and the antibrachium were distinctly observed (Figure 12e). In contrast, at Gosner stage 42 in the *R. nigromaculata* tadpoles in the 50 mg NaF/L group, there was less ossification of the pectoral girdle and the forelimb bones (Figure 12f). At Gosner stage 46 in the *R. nigromaculata* tadpoles, there was significantly less ossification of the pectoral girdle and the forelimb bones in the 50 mg NaF/L group compared with the control group (Figures 12g and 12h).

In a typical anuran, the pelvic girdle consists of paired, highly ossified ilia, ischia, and pubes. The hindlimb bones are the femur, the tibio-fibula, the tibiale, the fibulare, and the pes. At Gosner stage 42 in the *R. chensinesis* tadpoles in the control group, the ossification centers of the ilium, the femur, the tibio-fibula, and the tarsus were distinctly observed (Figure 12i). In comparison to the control group, the *R. chensinesis* tadpoles in the 50 mg NaF/L group had more ossification of the pelvic girdle and the hindlimb bones (Figure 12j). At Gosner stage 46 in the *R. chensinesis* tadpoles in the control group, the ossification of diaphyses appeared in the ilium, the femur and tibiofibula while, in contrast, the pelvic girdle and the hindlimb bones were fully ossified in 50 mg NaF/L group (Figures 12k and 12l).

At Gosner stage 42 in the *R. nigromaculata* tadpoles in the control group, the ossification of the ilium, the ischia, the femur, the tibio-fibula, and the tarsus were distinctly observed in control group while less ossification of the pelvic girdle and the hindlimb bone was present in the 50 mg NaF/L[1] group (Figures 12m and 12n). At Gosner stage 46 in the *R. nigromaculata* tadpoles, no obvious differences were found between the treated and the control groups (Figures 12o and 12p).

DISCUSSION

In the present study, the increased mortality in *R. chensinesis* and *R. nigromaculata* tadpoles with exposure to 50 mg NaF/L indicates that NaF may be seriously hazardous for amphibian survival. In addition, fluoride significantly inhibited metamorphosis and produced developmental delays in the *R. chensinesis* and *R. nigromaculata* tadpoles, including decreased length and weight. In both species, exposure to 50 mg NaF/L resulted in reductions, compared to the control groups, in the total length, the hindlimb length, and the weight, at one or more of the Gosner stages 36, 42 and 46. Our observations of reduced body weight and total length with NaF exposure are consistent with the finding by Goh and Neff

that NaF exposure reduced the head-tail length and the eye diameter of *Xenopus* embryos.[12]

We assessed the effects of NaF on tadpole morphology, in order to explore whether chronic exposure would induce tadpole deformities, and found that NaF produced flexural tail deformities in *R. chensinesis* tadpoles. Developmental and morphological abnormalities (e.g., visceral, mouth, eye, and limb deformities) have been observed in anuran tadpoles exposed to several different classes of compounds.[23-26] The increased incidences of deformities could result in reduced fitness by affecting foraging or predator avoidance, and cause reduced survival and reproduction of adults.[27] In contrast to the deformities found in the *R. chensinesis* tadpoles, no malformations appeared in the *R. nigromaculata* tadpoles with NaF exposure. The *R. nigromaculata* embryos metamorphose into frogs approximately 7–8 weeks postfertilization, whereas the *R. chensinesis* embryos need as long as 100 days to complete metamorphosis. As the time for the *R. nigromaculata* tadpoles to metamorphose to frogs was significantly shorter than that required for the *R. chensinesis* tadpoles, the occurrence of deformities in tadpoles may be related to duration of the NaF-exposure time.

Sodium fluoride can also lead to the development of skeletal fluorosis with effects on bone mineralization, bone cells, and bone architecture.[28] The long-term excessive intake of fluoride can disrupt the balance of bone deposition and remodeling activities leading to skeletal fluorosis.[29] Our results showed that fluoride can stimulate bone mineralization in *R. chensinesis* tadpoles. Increased ossification was observed in the parasphenoid, frontoparietal, and exoccipital bones in the tadpoles exposed to 50 mg NaF/L, compared to control group. In addition, most of the arches and transverse process in vertebral column ossified earlier in the 50 mg NaF/L group compared to control group.

However, in the *R. nigromaculata* tadpoles, fluoride can retard deposition of calcium in bone. In the NaF-treated group, the ossification of the parasphenoid, frontoparietal, and exoccipital bones was significantly less than that in the control group. Furthermore, the ossification of the vertebral column was also obviously less in the NaF group compared to the control group. In a similar experiment on *B. gargarizans*, it was found that fluoride delayed the development of the skeleton by reducing the apposition rates of the calcium ion, leading to a prolongation of the mineralization lag time and the bone formation period.[19]

CONCLUSIONS

The present study is the first to examine the effects of fluoride on *R. chensinesis* and *R. nigromaculata* during the entire larval development period. Chronic exposure to NaF increased the mortality, inhibited metamorphosis, and delayed development in *R. chensinesis* and *R. nigromaculata* tadpoles. Exposure to NaF increased the incidence of deformities in *R. chensinesis* but no malformations appeared in the *R. nigromaculata* tadpoles. In addition, fluoride can stimulate bone mineralization in *R. chensinesis* tadpoles and retard the deposition of calcium in *R. nigromaculata* tadpoles. Thus, our study suggests that fluoride has different effects

in the two species on the ossification of bone. As the postfertilization time to metamorphosis differed in the two species and the increased ossification occurred in the species, *R. chensinesis,* with the longest time to metamorphosis, we hypothesize that the increased skeletal ossification with NaF in *R. chensinesis,* may be associated with an increased sensitivity to fluoride or an increased duration of the exposure. Thus our study suggests that fluoride may affect skeletal ossification differently in different frog species due to differences in sensitivity or the duration of the exposure

ACKNOWLEDGEMENTS

The current work was supported by the National Science Foundation of China (No. 41401570, No. 31201726) and the special fund of the Shaanxi Normal University (No. GK20153051).

REFERENCES

1 Hardisson A, Rodriguez MI, Burgos A, Flores LD, Gutierrez R, Varela H. Fluoride levels in publicly supplied and bottled drinking water in the island of Tenerife, Spain. Bull Environ Contam Toxicol 2001;67:163-70.
2 Camargo JA. Fluoride toxicity to aquatic organisms: a review. Chemosphere 2003;50:251-64.
3 Weinstein LH, Davison AW. Fluorides in the environment: effects on plants and animals. Cambridge, MA, USA: CABI Publishing; 2004.
4 Dhar V, Bhatnagar M. Physiology and toxicity of fluoride. Indian J Dent Res 2009;20:350-5.
5 Roy RL, Campbell PGC, Premont S. Geochemistry and toxicity of aluminium in the Saguenay River, Quebec, Canada, in relation to discharges from an aluminium smelter. Environ Toxicol Chem 2000;19:2457-66.
6 Ren FH, Jiao SQ. Distribution and formation of high-fluorine groundwater in China. Environ Geology 1988;12:3-10.
7 Simon MJ, Beil FT, Rüther W, Busse B, Koehne T, Steiner M, et al. High fluoride and low calcium levels in drinking water is associated with low bone mass, reduced bone quality and fragility fractures in sheep. Osteoporos Int 2014;25:1891-903.
8 Wang YN, Xiao KQ, Liu JL, Dallner G, Guan ZZ. Effect of long term fluoride exposure on lipid composition in rat liver. Toxicology 2000;146:161-9.
9 Shivashankara AR, Shankara YMS, Rao SH, Bhat PG. A clinical and biochemical study of chronic fluoride toxicity in children of Kheru Thanda of Gulbarga district, Karnataka, India. Fluoride 2000;33:66-73.
10 Miao Q, Xu M, Liu B, You B. *In vivo* and *in vitro* study on the effect of excessive fluoride on type I collagen of rats. Wei Sheng Yan Liu/J Hygiene Res 2002;31:145-7.
11 Everett ET. Fluoride's effects on the formation of teeth and bones, and the influence of genetics. J Dent Res 2001;90:552-60.
12 Goh EH, Neff AW. Effects of fluoride on *Xenopus* embryo development. Food Chem Toxicol 2003;41:1501-8.
13 Long YG, Wang YN, Chen J, Jiang SF, Nordberg A, Guan ZZ. Chronic fluoride toxicity decreases the number of nicotinic acetylcholine receptors in rat brain. Neurotoxicol Teratol 2002;24:751-7.
14 Mousny M, Banse X, Wise L, Everett ET, Hancock R, Vieth R, et al. The genetic influence on bone susceptibility to fluoride. Bone 2006;39:1283-9.
15 Williams AJ, Robson H, Kester MH, van Leeuwen JP, Shalet SM, Visser TJ, Williams GR. Iodothyronine deiodinase enzyme activities in bone. Bone 2008;43:126-34.
16 Kerney R, Wassersug R, Hall BK. Skeletal advance and arrest in giant non-metamorphosing African clawed frog tadpoles (*Xenopus laevis*: Daudin). J Anat 2010;216:132-43.
17 Haas A. Larval and metamorphic skeletal development in the fast-developing frog *Pyxicephalus adspersus* (Anura, Ranidae). Zoomorphology 1999;119:23-35.

18 Brown DD, Cai L. Amphibian metamorphosis. Dev Biol 2007;306:20-33.
19 Zhao HF, Chai LH, Wang HY. Effects of fluoride on metamorphosis, thyroid and skeletal development in Bufo gargarizans tadpoles. Ecotoxicology 2013;22:1123-32.
20 Gosner KL. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 1960;16:183-90.
21 Taylor WR, van Dyke GC. Revised procedures for staining and clearing small fishes and other vertebrates for bone and cartilage study. Cybium 1985;9:107-11.
22 Duellman WE, Trueb L. Biology of amphibians. Baltimore, USA: Johns Hopkins University; 1994.
23 Bridges CM. Long-term effects of pesticide exposure at various life stages of the southern leopard frog (*Rana sphenocephala*). Arch Environ Contam Toxicol 2000;39:91-6.
24 Boone MD, Bridges CM, Rothermel BB. Growth and development of larval green frogs (*Rana clamitans*) exposed to multiple doses of an insecticide. Oecologia 2001;129:518-24.
25 Fordham CL, Tessari JD, Ramsdell HS, Keefe TJ. Effects of malathion on survival, growth, development, and equilibrium posture on bullfrog tadpoles (*Rana catesbeiana*). Environ Toxicol Chem 2001;20:179-84.
26 Greulich K, Pflugmacher S. Differences in susceptibility of various life stages of amphibians to pesticide exposure. Aquat Toxicol 2003;65:329-36.
27 Rohr JR, Elskus AA, Shepherd BS, Crowley PH, McCarthy TM, Niedzwiecki JH, et al. Lethal and sublethal effects of atrazine, carbaryl, endosulfan, and octylphenol on the streamside salamander, *Ambystoma barbouri*. Environ Toxicol Chem 2003;22:2385-92.
28 Matsuda SS, Silva TL, Buzalaf MA, Rodrigues AC, de Oliveira RC. Differential effects of fluoride during osteoblasts mineralization in C57BL/6J and C3H/HeJ inbred strains of mice. Biol Trace Elem Res 2014;161:123-9.
29 Yan X, Hao X, Nie Q, Feng C, Wang H, Sun Z, Niu R, Wang J. Effects of fluoride on the ultrastructure and expression of Type I collagen in rat hard tissue. Chemosphere 2015;128:36-41.