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BORON IS REQUIRED FOR ZEBRAFISH EMBRYOGENESIS

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Summary

Boron is the only element known to be essential for plants, but only circumstantial evidence for essentiality exists in animals. We report here that boron is essential for the embryonic development of zebrafish (*Danio rerio*). Zebrafish were maintained in water with a boron concentration of 0.1 μmol l^{-1} or supplemented to a concentration of 45 μmol l^{-1} using ultrapure boric acid. Both groups were fed boron-depleted brine shrimp. This procedure depleted the boron content of the blastulas from low-boron parents to only 5 % of the boron content of the blastulas from boron-supplemented parents. Sperm from low-boron males successfully fertilized eggs from low-boron females, but 92 % of the embryos died within 10 days. The early cleavage stage of development was the most sensitive to boron deficiency. Of the fertilized embryos, 46% did not live to complete the blastula stage. Repletion of low-boron embryos during the first hour after fertilization rescued them from death. These observations provide strong evidence that boron is essential for zebrafish development.

Key words: boron, fish, zebrafish, *Danio rerio*, cleavage stage, nutrient requirement, embryogenesis.

Introduction

For 75 years, boron has been known to be essential for vascular plant growth. The first unambiguous report, made by Warington (1923), demonstrated that boron stimulated the growth of broad beans. It is now known that boron deficiency is required for the reproduction and growth (Smyth and Dugger, 1981; Lovatt and Dugger, 1984) of all vascular plants. There is evidence for at least two different roles for the element, suggesting that the requirement for boron arose more than once during evolution. These roles include requirements for the cross-linking of cell wall polymers and for the structure and ion-transport properties of cellular membranes (Blevins and Lukaszewski, 1998). Despite repeated attempts to understand the primary function underlying the essentiality of boron, the molecular mechanism has not been identified.

The evaluation of boron essentiality in species other than vascular plants has been hampered by the difficulty of preparing food, deplete in the element, that interrupts the life cycle. Aquatic species represent an exception. The oceans are rich in boron, with a concentration of approximately 425 μmol l^{-1} (Bassett, 1990). Synthetic salt solutions have been prepared that mimic ocean conditions except for abnormally low boron concentrations. This has made it possible to demonstrate that boron is required for the growth by several marine species of diatoms (Lewin, 1966a,b) and algal flagellates (Lewin, 1966a). Cyanobacteria have also been shown to require boron for nitrogen fixation (Bonilla et al., 1990).

Several investigators have studied the importance of boron in human and animal nutrition. The ‘stressor model’ of Nielsen (1966) has been the most widely used design. In this model, a diet deficient or marginal in one or more nutrients such as Ca^{2+}, Cu^{2+}, Mg^{2+}, K^{+} or vitamin D is used as a nutritional stress to enhance the opportunity of observing a response to trace elements such as boron (Nielsen, 1996). Nielsen and his colleagues conducted three studies evaluating the effects of dietary boron supplementation in humans provided with diets containing marginal quantities of Mg^{2+} or Cu^{2+} (Nielsen, 1994). Under these conditions, boron significantly changed the blood chemistry of several important indicators of human health including blood glucose, steroid hormone, hemoglobin and platelet levels. Boron supplementation has been found to reduce the severity of vitamin D and calcium deficiencies in birds (Hunt, 1989) and rats (Dupre et al., 1994). Unambiguous boron-dependent growth in vertebrates has only been reported in fish. Boron has been shown to stimulate the growth of embryonic rainbow trout (*Oncorhynchus mykiss*) in a dose-dependent manner (Eckhert, 1998).

Embryonic fish offer several advantages as a model for evaluating the biological importance of boron. Eggs can be fertilized in ultrapure water, do not have to be fed and are capable of absorbing boron and several other essential elements from the water (Eddy and Talbot, 1985; Eckhert, 1998). One critical test of nutrient essentiality is whether an element is required for the development of viable offspring. This report presents the results of our evaluation of the relationship between boron and the embryonic development of zebrafish (*Danio rerio*).

Materials and methods

Zebrafish and food

Adult wild-type *Danio rerio* were obtained from Scientific Hatcheries (Huntington Beach, CA, USA). Fish were maintained in molded acrylic aquaria housed in urethane-sealed light boxes illuminated by fluorescent lamps (General Electric, Cool White, 20 W) controlled on a 12 h:12 h light:dark cycle. Male and female fish were maintained in separate aquaria. Tank water was purified to ASTM type 1 grade quality and had a resistivity greater than 18.0 MΩ. This was accomplished by passing reverse-osmosis-treated water through Q-Gard 1 (MSP000850) and Quantum I deionization cartridges (Millipore). Fish were fed brine shrimp (*Artemia salina*) grown in a modified brine solution (Provasoli and D'Agostino, 1969) prepared using ultrapure salts and water. The brine solution contained: NaCl (Sigma S-7653; 99.5 %), 418 mmol l^{-1}; $MgSO_4 \cdot 7H_2O$ (Sigma, M-5921, 98 %), 24 mmol l^{-1}; $MgCl_2 \cdot 6H_2O$ (Sigma, M-2670, 99 %), 20 mmol l^{-1}; $CaCl_2 \cdot 2H_2O$ (Sigma, C-5080, 99 %), 15 mmol l^{-1}; KCl (Sigma, P-333, 99 %), 8 mmol l^{-1}; Na_2EDTA (Aldrich, E2,629-0, 98 %), 694 nmol l^{-1}; $MnCl_2$ (JT Baker, 2540, 99.2 %), 95 nmol l^{-1}; $ZnCl_2$ (Mallinckrodt, 8780, 97 %), 11 nmol l^{-1}; $CoCl_2$ (Mallinckrodt, 4532, 99.59 %), 2 nmol l^{-1}; $NaBrO_3$ (Baker and Adamson, 2217, 99.5 %), 663 nmol l^{-1}; LiCl (JT Baker, 2370, 99.2 %), 47 nmol l^{-1}; $Na_2MoO_4 \cdot 2H_2O$ (JT Baker, 3764, 101 %), 21 nmol l^{-1}; KI (Baker and Adamson, 2120, 99.74 %), 1 nmol l^{-1}; and $FeCl_3 \cdot 6H_2O$ (Mallinchrodt, 5029, 99.85 %), 2 nmol l^{-1}. The brine shrimp were harvested and washed with ultrapure water containing ascorbic acid (0.3 mmol l^{-1}) and DL-α-tocopherol acetate (0.1 mmol l^{-1}) and frozen for later use or used directly as feed. The protocol for animal use was reviewed and approved by the Chancellor's Animal Research Committee, Office for Protection of Research Subjects of the University of California, Los Angeles, USA.

Boron exposure

Wild-type zebrafish were assigned to one of two treatments. One group was placed in aquaria filled with ultrapure water. This was designated the low-boron group. The commercial tropical fish industry in southern California relies on the Los Angeles Metropolitan Water District for their water supply. The second group was placed in aquaria filled with ultrapure water to which boric acid, H_3BO_3 (Fisher Scientific, Fair Lawn, NJ, USA; Cat. No. A-74, purity 99.5 %), had been added to achieve a final boron concentration comparable with that supplied by the district. Aquaria held 37 l, and 3–4 l was replaced during daily cleaning. In repletion experiments, zygotes from low-boron parents were transferred to boron-supplemented water. Boron exposure was assessed by analyzing water and food samples. Both groups were exposed to their respective media for 6 months before spawning.

Breeding

Male and female fish selected for breeding were transferred to polycarbonate breeding tanks equipped with polyethylene mesh bottoms (Westerfield, 1995). Fertilized eggs were removed from breeding tanks and transferred to polycarbonate cups containing the same boron concentrations as those of their parents, except when low-boron zygotes were repleted with boron.

Morphological evaluation

Fish embryos were evaluated at 70× magnification using an Olympus (Melville, NY, USA) SZH10 research stereo microscope. Death was determined in embryos before the appearance of heart contractions by the presence of denatured yolk albumen. The absence of cardiac function was used as the indicator of death in later stage embryos.

Boron analysis

The concentration of boron was determined by inductively coupled plasma-mass spectrometry (ICP-MS). An internal standard of ^{10}B was added in the form of boric acid (purity 99.9 %). Samples were analyzed for ^{10}B and ^{11}B content by using a VG PlasmaQuad II (VG Elemental, Danvers, MA, USA). The instrument was fully optimized for boron analysis. This was accomplished by cleaning the cones, quartz spray chamber and nebulizer assembly before analysis to reduce noise in order to achieve a high level of precision between ultrapure water blanks. The detection limit for boron in aqueous samples was 9 nmol l^{-1} based on three times the standard deviation of five replicate ultrapure water samples. Spike recovery of ^{10}B was 100.7 %. The detection limit was within the range reported by others using a matrix of ultrapure water with a resistivity greater than 17 MΩ (Malhotra et al., 1996).

Boron levels in aqueous samples were determined directly. Biological tissues were decomposed prior to analysis by microwaving (CEM microwave, model MDS-2000) in ultrapure HNO_3 within preconditioned Teflon digestion flasks (CEM advanced composite vessels). The detection limit for boron in biological samples was 46 nmol l^{-1}.

Statistical analyses

Data were analyzed using a statistical software package (Systat for Windows, SPSS, Inc., Chicago, IL, USA). The effects of boron were evaluated by using a one-way analysis of variance (ANOVA) or a *t*-test. If heterogeneous variance was evident, data were evaluated using the Kruskal–Wallis one-way ANOVA on Ranks and Dunn's multiple-comparison test (Dixon and Massey, 1983). Values are presented as means ± S.E.M. $P<0.05$ was considered statistically significant.

Results

The boron concentrations of the water and food used in our evaluation are given in Table 1. The water purification and supplementation produced aquarium water with a 450-fold difference in boron concentration between the low-boron and boron-supplemented groups. The water in the tanks of the low-

Table 1. *Boron concentrations of water, food and zebrafish blastulas*

Materials	Boron concentration (μmol l^{-1})	(μmol kg^{-1})	nmol per blastula
Water			
Los Angeles Metropolitan District tap	46.2±1.6		
Low-boron aquarium*	0.1±0		
Boron-supplemented aquarium‡	44.9±1.7		
Food			
Brine solution for rearing shrimp	2.5±0.4		
Dried shrimp eggs		6423±656	
Shrimp reared in brine and used as food		97±18	
Commercial fish food for fry (Tetra Min)		544±24	
Tissue concentrations			
Depleted group			3.1±0.7
Supplemented group			59.0±18.0

All values represent the mean ± S.E.M. of 6–9 samples.

Boron was determined by inductively coupled plasma-mass spectrometry.

*Metropolitan water that enters the UCLA Center for the Health Sciences building and undergoes pretreatment by reverse osmosis and deionization before distribution to laboratories. This water was passed through Millipore deionization cartridges MSP000850, Q-Gard 1 and Quantum IX to reduce boron concentrations further.

‡Ultrapure boric acid was used as the source for boron supplementation.

boron and boron-supplemented groups contained 0.1±0 μmol l^{-1} and 44.9±1.7 μmol l^{-1} boron, respectively. These values represent the mean of nine samples taken below the surface of the aquarium water during the course of the experiment. Fish and uneaten shrimp were present in the aquarium at the time of sampling. Zebrafish usually occupied the entire water column and, when fed, swarmed to the areas where brine shrimp dropped through the water column. They did not usually bottom-feed. This behavior prevented the suspension of bound boron in the brine shrimp from entering the water column. Uneaten brine shrimp were removed from the bottom of the tanks by vacuuming.

Rearing brine shrimp in low-boron brine solution (2.5±0.4 μmol B l^{-1}) reduced the amount of boron present in food. Dried brine shrimp eggs contained 6423±656 μmol B kg^{-1}. Adult shrimp, which were harvested from low-boron salt solution, washed with ultrapure water and used as food, contained 97±18 μmol B kg^{-1}. This was 5.6 times lower than the boron concentration of commercial fish food (Tetra Min) (544±24 μmol B kg^{-1}).

The effect of the low-boron exposure on the proportion of zebrafish offspring surviving to day 10 post-fertilization is shown in Fig. 1. During this period, 92% of the low-boron embryos died (Fig. 1). A second experiment evaluated death during the first 24 h post-fertilization. Of these embryos, 46% died within 4 h of fertilization compared with only 2% of the controls (P<0.001) (Fig. 2). This time frame included the zygote period (one-cell stage), the cleavage period in which six cycles of cell division resulted in the formation of a 64-cell blastodisc positioned on the animal pole, and part of the blastula period (Kimmel et al., 1995).

Blastulas from both groups were analyzed for their boron content to determine whether the differences in boron exposure had a measurable effect on the embryos. Low-boron blastulas were found to contain only 5% of the boron of supplemented blastulas (Table 1). Thus, the treatments had a direct and measurable effect on the amount of boron available for development. Low-boron zygotes began to die during the one-cell stage. Blebbing of the cell and yolk-sac membranes marked the early stages of death (Fig. 3B). This was followed by extrusion of cytoplasm and yolk-sac contents.

We then undertook repletion studies to determine whether

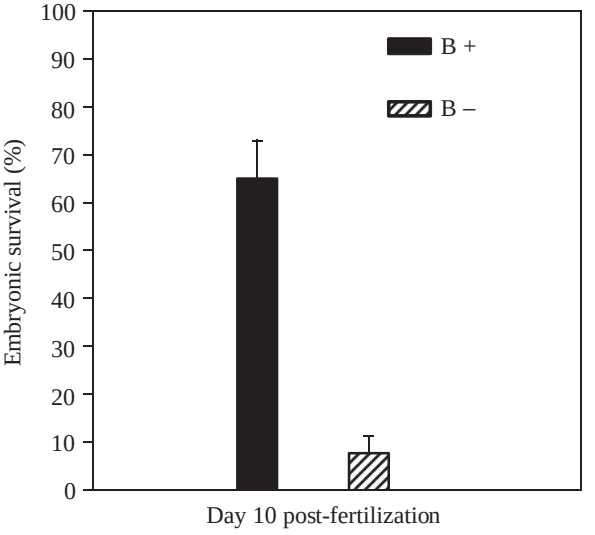

Fig. 1. Survival rate of zebrafish 10 days post-fertilization. Offspring of parents from the low-boron group (B–) had a significantly reduced survival rate (P<0.001) compared with the control group (B+). Columns represent the results of six experiments using an average of 91±15 eggs per experiment. Each column represents the mean + S.E.M.

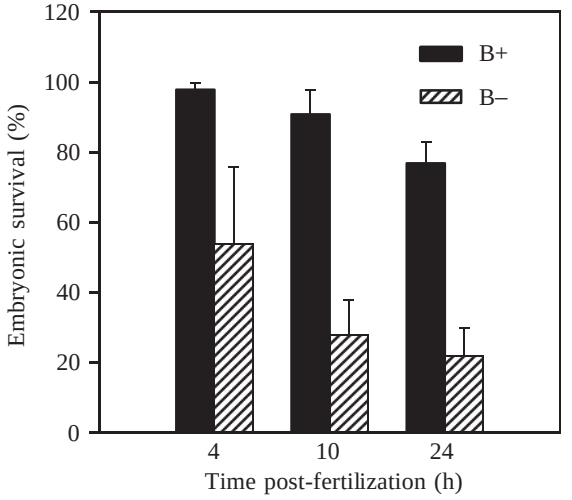

Fig. 2. Survival of zebrafish embryos 4, 10 and 24 h post-fertilization. Offspring of parents from the low-boron group (B–) had a significantly reduced survival rate (P<0.001) compared with the control group (B+). Columns represent the results of six experiments using an average of 90±27 eggs per experiment. Each column represents the mean + S.E.M.

zygotes from low-boron parents could be rescued by exposure to boron. For this experiment, the low-boron embryos collected from matings were transferred immediately after fertilization to either low-boron or boron-supplemented water. Transferring zygotes to boron-supplemented water rescued the low-boron zygotes from death (Fig. 4). After supplementation, nearly 98 % of the embryos survived the first 24 h compared with only 19 % of the unsupplemented low-boron group. The repleted group remained viable until 3 days post-fertilization.

Discussion

The essential elements for plants include H, B, C, N, O, Na, Mg, P, S, Cl, K, Ca, Mn, Fe, Co, Cu, Zn and Mo. With the exception of boron, all these elements have been shown to be essential for animals. The aim of the present study was to restrict the exposure of adult zebrafish to boron to assess whether it was critical for the development of their offspring. This aim was achieved, and in this report we extend the knowledge of boron essentiality to include the zebrafish.

Our previous studies, using embryonic rainbow trout, identified boron as a growth stimulator (Eckhert, 1998). Trout zygotes were transferred to a series of boron concentrations for incubation during the period of embryogenesis. The length of embryos, when they hatched, was used as the indicator of biological response. Embryonic growth was stimulated by boron in a dose-dependent manner, with the greatest effect occurring at boron concentrations between 2.2 and 4.4 μmol l^{-1}.

The trout brood stock used for these studies had been exposed to exogenous boron in feed and water for up to 3 years before spawning. Our inability to control the boron exposure of adults during germ-cell maturation limited experiments in this model to the effect of boron during the post-fertilization period. The zebrafish model offered an opportunity to restrict boron exposure over an extended period before fertilization.

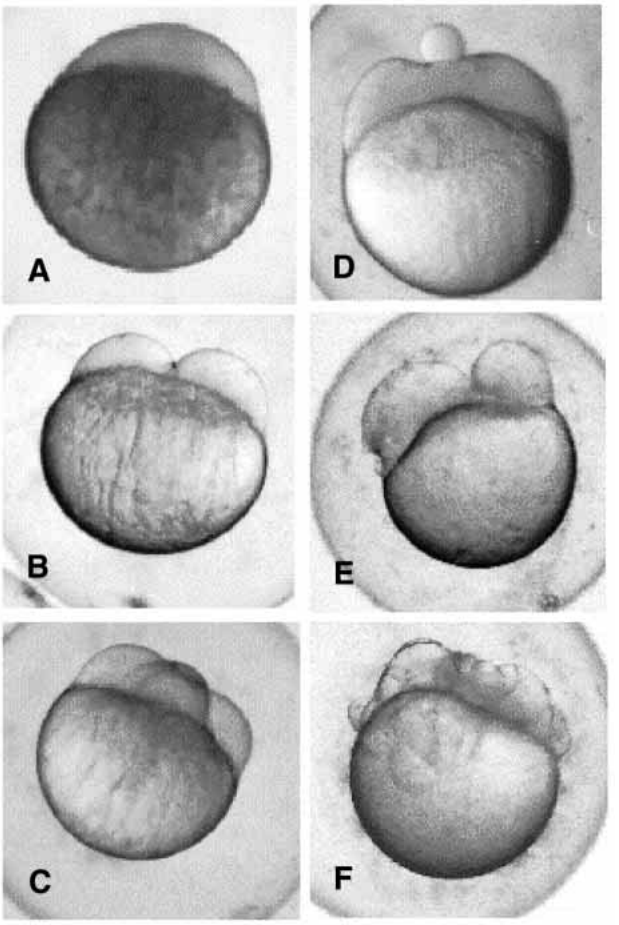

Fig. 3. Zebrafish embryos during the early cleavage stage of development. Boron-supplemented embryos (A–C) exhibited normal morphology; (A) one-cell stage; (B) two-cell stage; (C) four-cell stage. Boron-depleted embryos (D–F) exhibited membrane blebbing and asymmetrical cleavage; (D) one-cell stage with apical blebbing from the cell membrane; (E) two-cell stage with unequal cell division and blebbing; (F) four-cell stage exhibiting asymmetrical cleavage and blebbing on embryonic and yolk-sac membranes.

In the present study, zebrafish brood stock in both low-boron and boron-supplemented groups were fed brine shrimp with substantially lower boron concentrations than commercially available tropical fish diets (Table 1). The same ultrapure water was provided to both groups, with the exception that boric acid was added to the water of the supplemented group. The level of boron supplementation (45 μmol l^{-1}) was set to approximate the concentration of boron in the drinking water supply of Los Angeles (46 μmol l^{-1}). The addition of boric acid, a Lewis acid, did not change the pH of the water. The impact of supplemental boric acid on the ionic strength of the water would have been minor compared with the contribution from the low-boron brine shrimp fed to both groups. In some experiments (not shown), electrophoresis-grade boric acid (FisherBiotech, BP168-1, Lot 944011) rather than ultrapure boric acid was

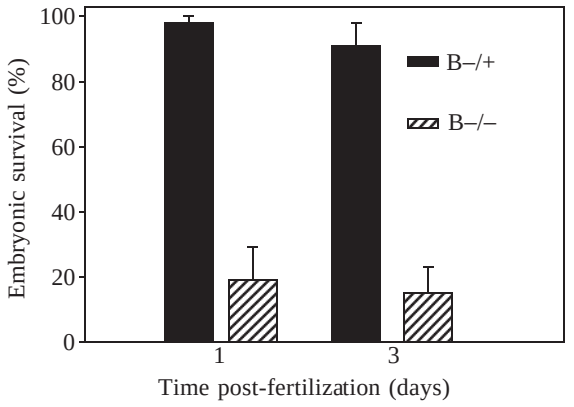

Fig. 4. Survival rates at 1 and 3 days of offspring from low-boron zebrafish with (B–/+) or without (B–/–) boron repletion in the first hour post-fertilization. Boron significantly increased survival rate (P<0.001). Each column represents the mean + S.E.M. By day 3, zebrafish have absorbed their yolk sac.

used as the supplement to determine whether the response was uniquely associated with the source of boron. Both sources of boric acid produced the same results.

This protocol was followed for 6 months before mating. Both groups were then spawned, and the resulting blastulas were evaluated for boron content. The mean boron content of the blastulas of the low-boron group was only 5% of that of the supplemented group. This confirmed that the experimental procedures established disparate boron concentrations in the embryonic cells of the two treatment groups. Associated with this difference in boron concentration was a dramatic difference in the survival of embryos.

Boron deficiency caused developmental disruption and death. The period most vulnerable to boron deficiency was during the cleavage stage when the cells undergo rapid division. Nearly half the zygotes died before completing blastulation. The first manifestation of boron deficiency was blebbing of the cell and yolk membranes (Fig. 3). This was followed by cytoplasmic and yolk extrusion. Repletion with boron shortly after fertilization rescued these embryos from death (Fig. 4).

The membrane disruption observed during zebrafish development was consistent with observations of membrane alterations reported in boron-deprived cyanobacteria and vascular plants. Cyanobacteria require a fixed nitrogen source when they are grown under limiting boron conditions. In these species, a specialized organelle, the heterocyst, encloses nitrogenase in a glycolipid membrane, protecting it from oxygen. The formation of heterocysts requires boron (Bonilla et al., 1989). Boron deficiency in plants causes alterations in both the composition and function of membranes (Belver and Donaire, 1983). The membranes of deficient sunflower seedlings exhibit lower concentrations of unsaturated fatty acids and higher concentrations of neutral lipids (Kimmel et al., 1995). In sunflower root cells, boron deficiency leads to hyperpolarization and an increased permeability to K^+ (Schon et al., 1990). A review of the changes in plant membrane structure and function during boron deficiency can be found elsewhere (Blevins and Lukaszewski, 1998).

The present results provide no information concerning the primary mechanism underlying cell death induced by boron deficiency. However, the success of the zebrafish model provides an opportunity to design experiments that can elucidate the function of boron in vertebrates. The observation that boron is essential during the zygote period of development is important, for it narrows the period in which future experiments will need to focus. The observation that the primary defect is reversible at the one-cell stage suggests that boron is required for one or more basic mechanism(s) of cell biology rather than a mechanism unique to embryogenesis. It also identifies boron repletion as a tool to screen for boron-responsive molecular events that occur during the most sensitive period of development.

In summary, the data presented here demonstrate that boron is essential for early zebrafish development. The study identifies the stage between fertilization and blastulation as the most sensitive period. The first indication of a deficiency is membrane blebbing followed by extrusion of cytoplasm and yolk. Zebrafish now join plants as organisms in which boron is known to be essential but in which the exact functions of boron are unknown.

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