Boron Stimulates Embryonic Trout Growth

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ABSTRACT  Boron is present in our soil, water and air. Cyanobacteria require it for nitrogen fixation, and vascular plants require it for the formation of cell walls and membranes. I report here how boron affects the growth of embryonic rainbow trout (Oncorhynchus mykiss). Fertilized ovum from the Mt. Whitney rainbow trout strain were incubated at (12.5°C) in Type 1 ASTM ultrapure grade water supplemented with boric acid (99.5% purity) during the 1995 and 1997 spawning seasons. Boron concentrations of the incubation solutions were determined by direct measurement using the curcumin procedure or inductively coupled plasma-mass spectrometry. In the 1995 study boron ranged from 1 to 936 μmol/L. Ca, Na and Mg salts were included in the incubation solutions to approximate concentrations in natural water. In the 1997 study fertilized eggs were incubated in ultrapure water supplemented with boric acid alone over a range from 2.2 to 90.6 μmol/L. The 1995 study used 144 embryos per B concentration and the 1997 study used 96 embryos per B concentration. Growth and teratogenicity were evaluated at the eye, hatch and 2-wk posthatch developmental stages. Boron stimulated growth in a dose-dependent manner in both studies (P < 0.001), and exposure was associated with an increase in B body concentration (P < 0.05). No teratogenic or microbicidal effects were apparent. These results are consistent with those expected of an element essential for vertebrate development. J. Nutr. 128: 2488–2493, 1998.

KEY WORDS: ● boron  ● rainbow trout  ● growth  ● embryo  ● development

The nutritional importance of boron has been difficult to assess because of its ubiquitous presence and the absence of a long-lived radioactive isotope to serve as a tracer. Boron is present in at least 150 different minerals of the earth's crust and is readily transported into soil, water and air (Basset 1990, Nemodruk and Karalova 1969). Agricultural soil boron concentrations range from 0.6 to 7.4 mM (Stevenson 1986). Water concentrations vary from 0.4 μmol/L in rain (Fogg and Duce 1985, Libes 1992), to 1.7 μmol/L in low boron fresh water rivers and lakes and 420 μmol/L in the ocean (Basset 1990). Boron is carried in the lower atmosphere both in particulate and gaseous phases (Anderson, 1994). The particulate burden of the troposphere is 0.4 × 10^10 g over land and 0.19 × 10^10 g over the oceans. The gaseous burden is 4.5 × 10^10 g over land and 22 × 10^10 g over the oceans. The cycling of these enormous amounts of boron in the environment indicate that early life forms could have developed a biochemical dependency on the element with minimal restriction to geographical distribution.

A few species of bacteria and eukaryotes have been shown to require the element (Loomis and Durst 1992). Cyanobacteria require boron for the formation of heterocysts, the site of nitrogen fixation. They do not require boron if grown in the presence of fixed nitrogen. In eukaryotes, boron has been shown to be essential for vascular plants, diatoms and some marine algal flagellates. Poor yields, arising from the cultivation of crops in low boron soil, led to the discovery that broad beans required boron for growth (Warington 1923). It is now known that boron is essential for all vascular plants (Loomis and Durst 1992). Boron has not been shown to be essential for fungi, green algae or animals. The potential for a boron requirement in archaea, the third domain of life, has not been evaluated.

In contrast to its essentiality in vascular plants, boron has been reported to be toxic to some aquatic eukaryotic species (Butterwick et al. 1989). Teratogenesis and reduced hatching were observed when batches of fertilized rainbow trout eggs were exposed to concentrations as low as 0.8 μmol/L (Black et al. 1993). This concentration is substantially below the median boron concentrations of many wild trout streams (U.S. Geological Surveys 1969, 1978, 1979 and 1980). The water boron concentrations of the major trout hatcheries in California range from 2 to 9.2 μmol/L (Bingham 1982).

The fact that trout grow and develop normally in boron plentiful natural waters stimulated my interest to reexamine the effect of boron on rainbow trout development. In this report I present evidence that boron is required for maximum embryonic growth.

MATERIALS AND METHODS

Fish. Oncorhynchus mykiss (rainbow trout) zygotes were obtained from the Mt. Whitney California State Fish Hatchery in two studies. The water boron concentration in the runs of the hatchery was 1.3 ± 0.1 μmol/L. Brood stock were fed commercial diets. The first study took place in 1995 and utilized 2-γ-old brood stock. The males and

females were artificially spawned in the morning. After hardening, fertilized eggs (zygotes) were transported to Los Angeles and transferred to wells of polystyrene culture plates with 15.49 mm bottom diameter (Cat. No. Falcon 3047, Becton Dickinson, Franklin Lakes, NJ) filled with incubation solution. Posthatch larvae were maintained in wells with a bottom diameter of 35.00 mm (Cat. No. Falcon 3046, Becton Dickinson, Franklin Lakes, NJ). A second study took place in 1997. Trout eggs and semen from 3-yr-old brood stock were mixed in ultrapure water for 1 min. The fertilized eggs were washed in ultrapure water and immediately transferred to polyethylene jars containing grinded boron concentrations. The zygotes were transported to Los Angeles and transferred to wells of polystyrene cell culture plates containing identical incubation solutions. Culture plates were maintained in a forced air aquaculture incubator (12.5°C) in both studies. Embryos were checked daily and solutions changed once each week. If dead embryos were observed, they were immediately removed and the incubation solution replaced. 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.

**Incubation solutions.** Fertilized eggs were incubated in water, pH 7.2–7.4, which had been purged through a Milli-Q-System to The American Society for Testing and Material (ASTM) ultrapure Type 1 grade, with a resistivity of >18.0 MΩ m. Boron was added as H₂BO₃ (Cat. No. A-74, purity 99.9%, Fisher Scientific, Fair Lawn, NJ). Boron was added to incubation solutions by transferring weighed aliquots from a stock solution containing 9.25 mmol B/L to pre-washed new polyethylene containers containing preweighed quantities of salt solution in the 1995 study and ultrapure water in the 1997 study. In solutions containing >92.5 μmol B/L, the addition of NaHCO₃ was necessary to maintain the pH between 7.2 and 7.4 in the incubation wells. Boron concentrations reported refer to the amount of boron in incubation solutions in use as determined by direct analysis.

In the 1995 study salts were added to the ultrapure water to obtain an incubation solution that approximated the quality of California’s Merced River. It contained Ca (1.54 mmol/L), Mg (1.44 mmol/L), Na (8.18 mmol/L), Cl (5.87 mmol/L), sulfate (2.49 mmol/L) and bicarbonate (3.20 mmol/L) (Hamilton and Buhl 1990). The following salts were used: CaSO₄ · 2 H₂O (Cat. No. C 3771, purity 99.0%, Sigma Chemical, St. Louis MO), CaCl₂ · 2 H₂O (Cat. No. C 5080, purity 99.0%, Sigma), MgSO₄ · 7 H₂O (Cat. No.M-5921, purity 98%, Sigma), NaCl (Sigma-S-7653, purity 99.5%) and NaHCO₃ (Cat. No. S 2331–500, purity 100%, Fisher Scientific, Fair Lawn, NJ).

No salts were added in the 1997 study and ultrapure water was filtered through a 0.22 μm filter to reduce the opportunity for microbiological contamination.

**Boron analysis.** The concentration of boron in the incubation solutions was determined by direct measurement. In the 1995 study the curcumin procedure was utilized (Standard Methods 1985). The detection limit was 1 μmol/L. In the second study samples were analyzed for ¹⁰⁷B and ¹⁰⁸B content by inductively coupled plasma-mass spectrometry using a VG PlasmaQuad II (VG Elemental, Danvers, MA). An internal standard of ¹⁰⁷B was added in the form of boric acid (purity 99.9%). 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 so as to achieve a high level of precision between ultrapure water blanks. The detection limit for aqueous samples was 9 nmol B/L based on three times the standard deviation of five replicate ultrapure water samples. Spike recovery of ¹⁰⁷B was 100.7%. The detection limit was within the range reported by others, using as a matrix ultrapure water with a resistivity > 17 meq/h (Malhotra et al. 1996).

Fish larvae were washed three times in ultrapure water prior to drying. Whole dried fish larvae were delineated by microwaving (CEM Microwave Model MDS-2000) in ultrapure HNO₃ within preconditioned Teflon digestion flasks (CEM Advanced Composite Vessels) for analysis. The detection limit for biological samples was 46 nmol B/L.

**Growth.** Lengths were obtained 5 wk after fertilization when embryos hatched by emerging from their chorionic membranes (Fig. 1A and B). This was done to assess the effect of boron before significant post-hatch exposure. The same fish were measured again after 2 wk. For clarity I have referred to newly hatch embryos as embryos and 2 wk post-hatch fish as larvae. Live larvae remained separated for long periods allowing them to be measured within the cell culture wells. This made it possible to obtain measurements without handling or contamination. Measurements were taken from head to tail after first orienting, at 10× magnification, the culture plate to align the longitudinal body axis with a straight edged ruler mounted on the stereo microscope stage. The ruler was visible without distortion through the optical quality polycarbonate bottom of the cell culture well. In the 1995 study measurements were taken under 10× magnification to the nearest 0.1 mm. In the 1997 study longer aquatic measurements were obtained under 70× magnification using an Olympus (Melville, NY) SZH10 Research Stereo Microscope.

**Morphological evaluation.** The teratological evaluation screened for death, percentage of embryos to reach the eye stage and hatch, and gross morphological deformities and abnormalities in swimming behavior. The eye stage is the period during development when embryonic eyes become visible without the aid of magnification. This stage is used as a marker at breeding hatcheries to assess embryo development.

**Quantification of microbiological contamination.** Microbiological contamination was quantified using indirect turbidity and direct counts. Five samples were obtained from each 24-well cell culture plate. These were taken from each corner and the middle (second row, third cell). Turbidity was measured at 600 nm using a Hewlett Packard Model 8451A Diode Array Spectrophotometer (Palo Alto, CA) according to the procedure of Lech and Brent (1994). An Improved Neubauer Hemacytometer (Reichert, Buffalo, NY) was used for direct counting according to the manufacturer’s directions.

**Statistical analysis.** Data were analyzed with a statistical software package (SigmaStat for Windows). The effects of boron were evaluated using one way ANOVA or 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). Data are presented as means ± SEM. A P value < 0.05 was considered statistically significant. The mortality data for coinubated embryos were transformed using arcsin transformation prior to evaluation by paired t test.

**RESULTS**

Boron stimulated growth in both studies. In the 1995 study, boron exposure during the combined embryonic and early larval stages caused a dose dependent increase in body length (P < 0.001) (Fig. 2). Growth stimulation was most pronounced at low concentrations. As boron increased from 2 to 10 μmol/L, growth increased 8% but the response was less pronounced at higher concentrations. This observation was replicated in the 1997 study in the absence of Ca, Mg and Na salts (P < 0.001) (Fig. 3A). When boron was increased from 2 to 4 μmol B/L, growth during the embryonic stage increased 6.5%. A smaller but still significant growth differential of 2.5% was still apparent in the larval stage of development 2 wk post-hatch (P < 0.001) (Fig. 3B, Fig. 1C). Thus boron’s ability to stimulate growth was more pronounced during the embryonic period before hatch. Length was not measured at the eye stage because embryos were curved over their yolk sacs and could not be aligned with a straight edged rule (Fig. 1A). Similarly neither hatched embryos nor larvae that were curved were measured. The rate of rejection for curvature was 1.2% with no apparent differences between groups.

Boron body concentrations were 65% higher in larvae exposed to 9.1 μmol B/L than those exposed to 2.2 μmol B/L (P < 0.05) (Fig. 4). No morphological or swimming defects were observed in embryos or hatched larvae exposed to boron at levels up to 925 μmol B/L.

The boron concentrations as measured by ICP-MS were higher than expected at the lower end of the dose response.
curve in the 1997 study. This resulted from a breakthrough of boron from the ion exchange purification cartridge which raised the ultrapure water from a nondetectable level (sensitivity 0.1 μmol/L) to 2 μmol/L. Boron is the first element to break through ultrapure water purification systems (Malhotra et al. 1996) and it occurs before a significant drop in resistivity. Our procedure has been to prepare all solutions for a single study at one time. This protects the study from fluctuations in boron concentration.

Boron did not change the frequency of embryos that survived to the eye or hatch stages. Approximately 78% survived to the eye stage and 60% survived to hatch in the 1995 study (Table 1). The cause of death was due to infection by the fungus *Saprolegnia*. This water mold is endogenous to the waters of trout hatcheries and is a major cause of mortality and morbidity of brood stock and embryos. Fungal mycelial growth on the fish surface is stimulated by sex hormone production of brood stock during spawning season (Hernandez-Hernandez 1995, Willoughby 1978). Hatcheries manage this infection by treating fertilized eggs for 10 min with free iodine to kill the fungus. However, the exposure to iodine would have compromised the present study and was not used.

The mortality due to microbial contamination was exacerbated in the 1995 study by the coincubation of pairs of fertilized eggs. The mortality risk for one embryo out of a pair was 12%. However, after one died the risk of death to the remaining embryo more than doubled (29%). The increased risk of death to an embryo following the death of a companion was significant (*P*, 0.0001). In the 1997 study embryos were incubated in separate wells to eliminate this problem. This

![FIGURE 2](https://academic.oup.com/jn/article-abstract/128/12/2488/4724239) Lengths of rainbow trout larvae in the 1995 study at 2 wk after hatching following incubation for 7 wk in solutions containing different boron concentrations. Each point represents the mean ± SEM, n = 144. Growth was dependent on boron concentration (*P* < 0.001). *Lengths of larvae exposed to boron concentrations of 11.4 μmol B/L and above were significantly greater (*P* < 0.05) than those exposed to 0.9 and 1.9 μmol B/L.

![FIGURE 1](https://academic.oup.com/jn/article-abstract/128/12/2488/4724239) Rainbow trout embryos from the Mt. Whitney strain incubated in polystyrene cell culture wells with a diameter of 15.49 mm. (A) Trout embryo within its chorionic membrane prior to hatching. (B) Trout embryo hatched (5 wk after fertilization) from the confines of its chorionic membrane (upper right). (C) Trout larvae 2 wk post-hatch (7 wk after fertilization) with attached yolk sacs. The upper larva was exposed to 9.1 μmol B/L and the lower to 2.2 μmol B/L.
procedural change was associated with an increase in the rate of survival at hatch of 58% to a mean value of 95.6% (Table 2). The contamination in this study was minimal and not affected by boron as seen by the low indirect optical density and direct counts. A value of 0.1 OD unit at 600nm is \(10^{11}\) organisms per liter (Lech and Brent 1994). The direct counting procedure validated the optical density readings. The values obtained by direct counting gave the impression of a high degree of variability compared to the optical density reading. This was a result of the threshold effect of counting samples with very low concentrations of microorganisms (Ball 1997). The difference between an average count of zero or one organism in five large squares counted in an Improved Neubauer Hemacytometer is 0 and \(5 \times 10^7\)/L.

**DISCUSSION**

A cultured trout embryo is shown in Figure 1A surrounded by its chorionic membrane. The emergence of the embryo from the chorionic sac, at hatch, marks the end of the embryonic stage and beginning of the larval stage (Fig. 1B). The yolk sac represents the major source of nutrients in both the embryonic and larval stages. The sac is slowly adsorbed over the next 6-wk period, forcing the young fish to become dependent on an external food supply.

Boron stimulated embryonic and larval growth in both studies. This observation was replicated in both the presence and absence of supplemental Ca, Na, Mg, Cl, and SO\(_4\). The potential that the observed growth was an indirect effect of boron acting as a microbicidal agent was ruled out in the 1997 study.

The results of the present studies are in agreement with the reports by Thompson et al. (1976) and Hamilton and Buhl (1990) that boron is not toxic to fish of the genus *Oncorhynchus* at concentrations < 925 \(\mu\)mol/L. Batches of embryos are frequently used in toxicological studies of developing fish. The present study demonstrates that the close contact of embryos adds a confounding variable. A comparison of the death rates of pairs of coincubated embryos showed they were not independent. Following the death of one embryo of a pair, the second was more than twice as likely to die. This occurred despite the daily removal of dead embryos and exchange with fresh incubation solution. The increased risk of mortality following the death of a companion embryo may have resulted from its exposure to metabolites or infectious microbiological agents feeding on the dead or dying companion. Cross toxicity and infection were controlled for in the 1997 study by the separation of embryos into individual incubation wells and the filtration of water through a 0.22 \(\mu\)m filter. The failure to remove dead embryos for up to 15 days may have contributed to the apparent toxicity of boron at 7–8 \(\mu\)mol B/L reported by
Survival to hatch stage 53%
9.1 100 95
6 100 90
2.9 100 94
4.2 100 90
5.9 100 86
9.1 100 67
90.6 100 42

Others (Black et al. 1993). The use of batches of embryos may also have masked the opportunity to observe boron’s growth promoting effect.

The role of boron in maintaining the optimum health of human bone and joint tissue has been an area of interest for over four decades (Newnham 1994). In 1987 Nielsen and his colleagues reported that boron supplementation of postmenopausal women had a beneficial effect on bone mineral metabolism (Nielsen et al. 1987). Supplements containing 3.0 mg B/d for 119 days reduced urinary excretion of calcium and magnesium and elevated serum 17B-estradiol and testosterone particularly when magnesium intakes were low. Nielsen extended these findings in a second study that showed that boron depletion was associated with both lower plasma-ionized calcium and serum 25-hydroxycholecalciferol concentrations, and higher serum calcitonin and osteocalcin concentrations (Nielsen et al. 1990).

The use of combined nutritional deficiencies has been used in animal studies to further elucidate the role of boron in mineral metabolism. Using vitamin D-deficient chicks, Hunt (1989) reported that boron supplementation reduced narrow sprout distorsion of the proximal tibial epiphysial plate and (1989) reported that boron supplementation reduced marrow mineral metabolism. Using vitamin D-deficient chicks, Hunt (1996) determined these findings in a second study that showed that boron depletion was associated with both lower plasma-ionized calcium and serum 25-hydroxycholecalciferol concentrations, and higher serum calcitonin and osteocalcin concentrations (Nielsen et al. 1990).

The use of combined nutritional deficiencies has been used in animal studies to further elucidate the role of boron in mineral metabolism. Using vitamin D-deficient chicks, Hunt (1989) reported that boron supplementation reduced narrow sprout distorsion of the proximal tibial epiphysial plate and elevated the number of osteoclasts. Supplemental boron increased plasma calcium and magnesium concentrations and inhibited cartilage calcification in chicks deprived of both vitamin D and magnesium. In vitamin D-deficient rats, Hegsted, Dupre and their colleagues observed that boron supplementation enhanced the absorption and balance of calcium and phosphorus (Dupre et al. 1994, Hegsted et al. 1991).

Numerous hypotheses have been put forward to explain the effects of boron on animals and humans (Blevins and Łukaszewski 1994, Hunt 1996, Loomis and Durst 1992). Some hypotheses include modulation of extracellular and intracellular calcium transport in thrombin-activated platelets, and regulation of enzymes, for example, pyridine and flavin oxidoreductases and serine proteases (Nielsen 1996). Most of these hypotheses focus on putative roles in maintaining the health of fully differentiated or adult animals.

In contrast to the work done by others on the health-promoting effects of boron, the present work was initiated to determine if organ culture techniques could be applied to environmental studies of aquatic embryos. The observation that boron promoted growth was unexpected and also surprising, since it occurred during a period of development before the formation of bone. The procedures used in the present study were close to ideal for identifying the growth-promoting effect of boron. The use of trout embryos with nutrient-laden yolk sacs eliminated the need for complex diets but afforded the opportunity for absorption of elements from the incubation solution (Eddy and Talbot 1985). The use of ultrapure water and high-grade boric acid and salts minimized influences from unwanted elements. Aeration was accomplished by incubating embryos in cell culture plates maintained in a forced air incubator. This eliminated the need for a complex aeration system with its associated pumps and contaminants.

In summary, the present study demonstrates that boron stimulates embryonic growth both in the presence and absence of several known essential elements. The present data do not confer any information as to how boron stimulates growth. It is possible that it is related to the ability of boron to bind to cellular cis-diols such as ribose. Boron binding might confer changes to membrane glycoproteins or improve the kinetics of nucleotide transport. If boron is essential for these or other essential cellular processes, there is likely to be a transport protein to protect it from free association with diols. Boron transporters would be expected to be expressed when its concentration was growth limiting. Such a mechanism has been reported for silicon in diatoms (Hildebrand 1997). Hildebrand

| Table 1 |

Effect of supplemental boron on trout survival in 1995 Mt. Whitney study³

<table>
<thead>
<tr>
<th>Boron exposure, μmol/L</th>
<th>0.9</th>
<th>1.9</th>
<th>11.4</th>
<th>37.5</th>
<th>103.7</th>
<th>936.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye stage survival</td>
<td>76 ± 4</td>
<td>84 ± 4</td>
<td>76 ± 4</td>
<td>85 ± 4</td>
<td>77 ± 4</td>
<td>69 ± 4</td>
</tr>
<tr>
<td>Survival to hatch stage</td>
<td>53 ± 5</td>
<td>53 ± 6</td>
<td>65 ± 5</td>
<td>66 ± 5</td>
<td>65 ± 5</td>
<td>56 ± 5</td>
</tr>
</tbody>
</table>

³ Fertilized eggs were incubated two per well. Embryos hatched 5 wk post-fertilization. Values are presented as mean ± SEM, n = 144. Boron had no significant impact (P > 0.05) on the proportion of embryos that survived to the developmental stages of eye and hatch.

| Table 2 |

Relationship between mortality, microbial contamination and boron concentrations in the 1997 Mt. Whitney Study³

<table>
<thead>
<tr>
<th>Treatment groups, B, μmol/L</th>
<th>Survival at eye stage, %</th>
<th>Survival at hatch stage, %</th>
<th>Microbiological contamination of incubation solutions</th>
<th>OD600 nm</th>
<th>count × 10⁷/L (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2</td>
<td>100</td>
<td>97 ± 3</td>
<td>0.01</td>
<td>0.01</td>
<td>15 ± 14 (0–62)</td>
</tr>
<tr>
<td>2.5</td>
<td>100</td>
<td>92 ± 5</td>
<td>0.01</td>
<td>0.01</td>
<td>100 ± 54 (0–312)</td>
</tr>
<tr>
<td>2.9</td>
<td>100</td>
<td>96 ± 4</td>
<td>0.01</td>
<td>0.01</td>
<td>25 ± 15 (0–62)</td>
</tr>
<tr>
<td>4.2</td>
<td>100</td>
<td>90 ± 4</td>
<td>0.01</td>
<td>0.01</td>
<td>0 ± 0 (0)</td>
</tr>
<tr>
<td>5.9</td>
<td>100</td>
<td>96 ± 4</td>
<td>0.01</td>
<td>0.01</td>
<td>25 ± 15 (0–62)</td>
</tr>
<tr>
<td>9.1</td>
<td>100</td>
<td>95 ± 7</td>
<td>0.01</td>
<td>0.01</td>
<td>50 ± 23 (0–125)</td>
</tr>
<tr>
<td>90.6</td>
<td>100</td>
<td>98 ± 2</td>
<td>0.01</td>
<td>0.01</td>
<td>15 ± 14 (0–62)</td>
</tr>
</tbody>
</table>

³ Fertilized eggs were incubated one per well. Values are presented as mean ± SEM, n = 96.
and his colleagues have identified five different silicon transporters that are expressed in *Cylindrotheca fusiformis* when this element becomes growth limiting. The sequence identity of the carboxy-terminal ends of these transporters is low, suggesting that this region is responsible for targeting and transporting silicon to different cellular compartments. It is not inconceivable that such a mechanism also exists for boron in vertebrates. In conclusion this report identifies the embryonic fish as a model that could be used to search for boron transport proteins in vertebrates.

**LITERATURE CITED**


