Impact of Water Hardness on Zebrafish Early Stage Development

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Aim: To investigate the effect of altered water hardness on zebrafish embryonic development and survival rates from point of fertilisation until day three post fertilisation.

Intro

Water Parameters [1,2]		
Parameter	**Suggestions**	**Comments**
Temperature	24 – 30 (°C)	28.5 (°C)
pH	7 - 8	7 - 8
Conductivity	400 - 1200 µS	Highly variable
Dissolved Oxygen	Saturation point – 7.8mg/l	Unknown, but likely 7.8mg/l
Nitrogenous Waste **NH_3** **Nitrites** **Nitrates**	 0 0 0 – 100ppm	 <0.02 ppm considered toxic <0.01 ppm possibly tolerated less toxic than nitrites and NH3
Water Hardeness (General Hardness)	75 – 220mg/L	As defined for freshwater fish

Table 1: Water parameters commonly used for zebrafish.

As ectothermic poikilotherms, zebrafish are significantly more reliant on environment for bodily function than endothermic homeotherms such as man and mice.

As the environment in which zebrafish live, many water quality parameters are measured regularly.

Water quality is the number one factor in the health and welfare of zebrafish (Table 1). All water has a hardness level – those low in minerals are referred to as soft water and are alkaline in nature. Those high in minerals are referred to as hard water and are more acidic in nature. Zebrafish are considered a hard water species.

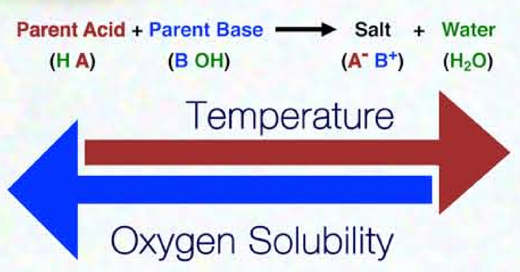


Fig 1: Water quality is highly dependent on its components; each can influence and change another.

Water chemistry is a fairly complex thing as all water parameters are interconnected (Fig.1). All water quality measures are interdependent on one another.

Methods

We undertook five separate trials to determine the effect of various components of water hardness on the survival rate, growth and development of early stage zebrafish embryos (table 2); we then placed fertilised one-cell embryos into each mix and kept them there until four days post fertilisation (dpf) to assess survival and developmental rates (fig. 2).

Fig. 2 left: water of five different water hardnesses were made. Right: embryos that were placed into the different mixes.

The Five Different Water Hardness Trials
Trial 1 Altering ratios of TH cations - Ca^{2+}, Mg^{2+}, maintaining TH at 200 mg/L; maintaining KH at 50 mg/L in a ratio of 1:1
Trial 2 Altering the levels of TH but always maintaining a ratio of cations at 1:1; maintaining KH at 50 mg/l in a ratio of 1:1
Trial 3 Altering KH levels but maintaining a ratio of 1:1; maintaining TH at a 0:0 ratio of Ca^{2+}: Mg^{2+}
Trial 4 No KH; Altering ratios of TH cations - Ca^{2+}, Mg^{2+} maintaining at 200 mg/L
Trial 5 Increased the TH to 900 mg/L whilst also altering ratios of cations of Ca^{2+}: Mg^{2+}; maintaining KH at 50 mg/L

Table 2: the different mixes used for the five water hardess trials

Differences Between Total Hardness, Non-Carbonate Hardness and Carbonate Hardness		
Total Hardness (TH) *(sum of multivalent cations)*	Non-Carbonate Hardness (NCH)	Carbonate Hardness (KH)
Cations Ca^{2+} Mg^{2+}	Combination of non-carbonate anions and monovalent cations	Anions H_2CO_3 HCO_3^- CO_3^{2-}
Acid	The mixture of which determines stronger acid or alkaline	Alkaline

Table 3: Water hardness has three component parts: total hardness, carbonate hardness and non-carbonate hardness. In this trial we excluded non-carbonate hardness as it is usually included in as a component of total hardness.

Total hardness is the sum of multivalent cations, (commonly magnesium and calcium). We used potassium bicarbonate and sodium bicarbonate to create carbonate hardness (carbonates, bicarbonates and carbonic acid in solution). In each trial we used different levels of total and carbonate hardness and in some varied the ratios of calcium and magnesium in the total hardness. We used magnesium sulphate heptahydrate and calcium sulphate dihydrate.

Results

Trial 1: Percentage survival at 0 and 4 dpf. High levels of mild heart odema in the Ca^{2+} free trial. (Data not shown)

Trial 2: Percentage survival at 0 and 4 dpf. High levels of debris on chorions at 1200 mg/L TH. No survival at 2 dpf at 0 mg/L TH.(Data not shown)

Trial 3: Percentage survival at 1, 2 and 4 dpf. High levels debris in water and on chorions at 400 mg/L KH. (Table 4)

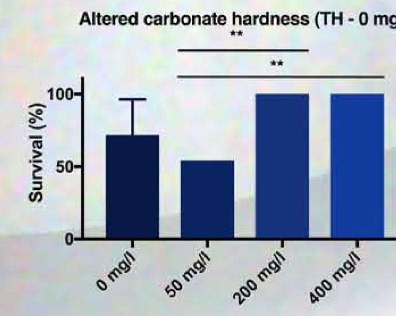


Table 4: Survival was significantly different between 50 mg/L and 200 and 400 mg/L. ** Indicates $p < 0.01$. (One-way ANOVA with Tukey's HSD post-test). Data are shown as mean ± SD.

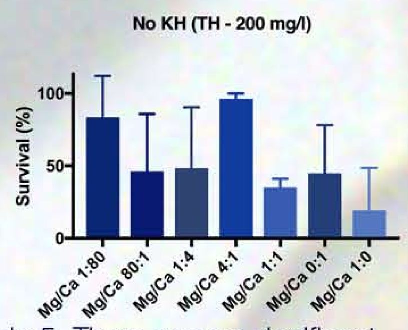
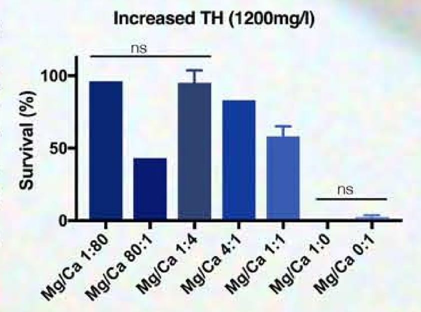


Table 5: There was no significant difference in survival between the seven different Mg/Ca ratios. (One-way ANOVA). Data are shown as mean ± SD.

Trial 4: Percentage survival at 1, 2 and 3 dpf. Low survival at 2 dpf in both Ca^{2+} and Mg free samples.Varying levels and types of embryo abnormalities over all other samples. (Table 5)

Trial 5: Percentage survival at 1, 2 and 3 dpf. Low or no survival at 2 dpf in both Ca^{2+} and Mg free samples. Varying levels and types of embryo abnormalities over all other samples.High levels of debris in water and on chorions in all samples. (Table 6)

Table 6: Survival was significantly different between most all the ratios. NS Indicates no significant difference.(One-way ANOVA with Tukey's HSD post-test). Data are shown as mean ± SD.



Discussion

Calcium and magnesium are essential for development, growth and survival. Both calcium and magnesium are required for bone growth, blood clotting and other metabolic reactions. These trials appear to confirm that both are imperative for development – the trials in which one or the other was missing resulted in statistically significant low survival rates. Somewhat strangely when no TH or KH was present (therefore water with no minerals in) the embryos still developed to 3 dpf; perhaps this is because the developing embryo is encased and protected from the water by the chorion and reliant on the yolk for nutrition. Older fish would not be able to survive in this very pure water.

When calcium and magnesium levels become too high they precipitate out of water and formed crystals (fig. 3), which was seen, especially in trial 5. As well as forming crystals in the water, it also clung to the chorions and gave high levels of embryo deformities, suggesting it was penetrating the chorion and damaging the embryo (fig 8).

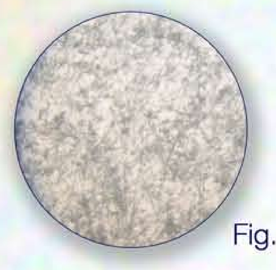


Fig. 3: crystals precipitating out of in the trials water

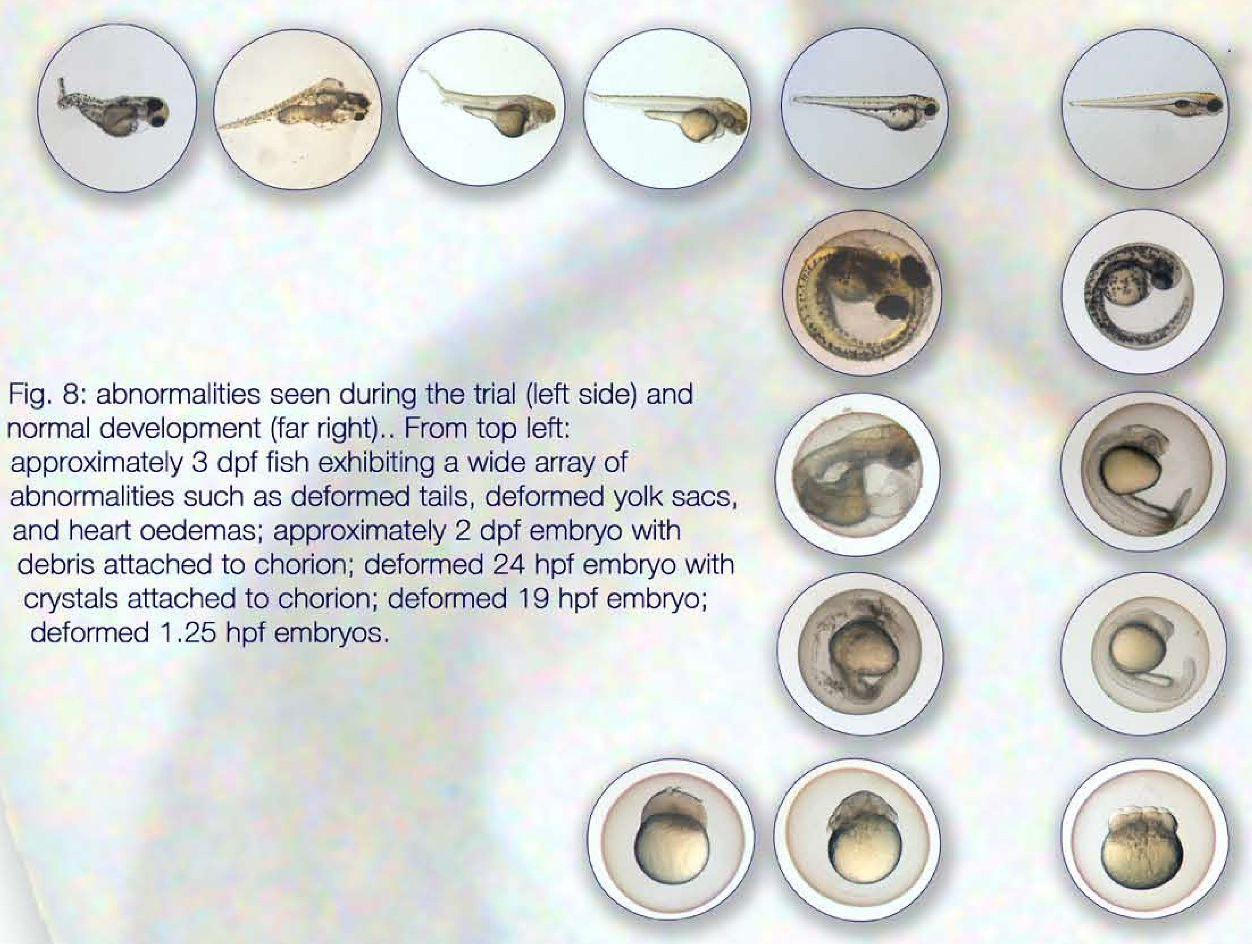


Fig. 8: abnormalities seen during the trial (left side) and normal development (far right).. From top left: approximately 3 dpf fish exhibiting a wide array of abnormalities such as deformed tails, deformed yolk sacs, and heart oedemas; approximately 2 dpf embryo with debris attached to chorion; deformed 24 hpf embryo with crystals attached to chorion; deformed 19 hpf embryo; deformed 1.25 hpf embryos.

1 1:80 89	1 8 0:1 27	1 1:4 28 ab	1 4:1 71	1 1:1 62	1 Ca0 40 ab	1 Mg0 79	1 1:20 75 ab	1 20:1 75 ab
2 1200 77 debris	2 600 96	2 300 95	2 0 1 ab					
3 0 43 unhat 6.5	3 50 54 debris 7.75	3 200 100 debris 8	3 400 100 debris 9					
4 1:80 50 unhat 6.5	4 80:1 69 pigles 7	4 1:4 68 ab 6.5	4 4:1 92 ab 7	4 1:1 29 ab 6.5	4 0:1 6 ab 7	4 1:0 1 ab 6		
5 1:80 96 debris 9	5 80:1 43 pigles 7.5	5 1:4 85 debris ab 9	5 4:1 83 debris 7.5	5 1:1 53 debris ab 8	5 1:0 0 ab 7.5	5 0:1 1 ab 9		

Table 7 Legend: *First row* indicates trial number. *Second row* indicates the ratio of magnesium:calcium or in trials 2 and 3 amount of carbonate hardness used. *Third row* indicates final survival rates. *Fourth row* indicates the following: *debris*–water debris; *unhat*–unhatched embryos; *ab*- embryonic abnormalities; *pigles*–pigmentless embryos. *Fifth row* indicates pH of the water in trial 3,4,5 (not tested in trial 1,2)

When the results were compiled (table 7), better survival rates with no abnormalities or precipitation were observed in trial 2, suggesting that slightly higher TH levels may increase embryo viability.

The orange square indicates normal water hardness conditions run at UCL. The red squares indicate water hardness conditions that potentially should be rejected on the grounds of debris in the water, pH of the water, and abnormalities in the embryos. The green squares need further investigation, and were chosen because no embryonic abnormalities were found, nor debris in the water and had an overall high final survival rate.

Further Work

From the overlaid table, we were left with two potential candidates for further investigating. The results suggest that raising the levels of TH may improve embryo survival,

An increase in total hardness would have to be tested on adult fish to establish if there were any repercussions to them. This seems unlikely as anecdotal evidence suggests that water hardness levels are generally held at higher levels in the USA.

References:
1. Lawrence, C. (2007). "The husbandry of zebrafish (Danio rerio): A review." Aquaculture 269(1-4): 1-20.
2. Alestrom, P (2013) Zebrafish: housing, husbandry, and health monitoring recommendations (unpublished)

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