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Authors

Adams, Lisa G
Gordon, Malcolm S
Buth, Donald G
et al.

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A Comparison of Isogenic Homozygous Clone and Wildtype Zebrafish (*Danio rerio*): Survival and Developmental Responses to Low pH Conditions

Lisa G. Adams,[1,*] Malcolm S. Gordon,[2] Donald G. Buth,[2] and Erica M. Hutchings[3]

Abstract

The value of bioassays as analytical methods for assessing the potency of particular stressors on live animal models depends on the precision of their results, which are greatly influenced by the choice of test subjects. The genetic makeup of experimental subjects varies, and, as such, so will their responses to the test environment. Genetic diversity of test populations may contribute to statistical variability; therefore, the use of genetically similar subjects may enhance the utility of bioassays. This study addresses the efficacy of using isogenic homozygous zebrafish (*Danio rerio*) as subjects for bioassays. Stress responses (acidic conditions) were compared during early development for gynogenetically produced isogenic homozygous line of zebrafish (C32) and wildtype (WT) zebrafish. Experiments evaluated early life stage milestones after exposure to low pH in water of a different electrolyte composition. Because the isogenic homozygous clonal (IHC) fish possessed far less genetic variability than the WT fish tested, it was predicted that the IHC fish would exhibit less variability in their response to stress. Although we found no significant differences in the variability between the responses of the IHC and WT fish, pH and water hardness level had a differential effect on the two groups. Simple strain differences may be the probable cause of the response differences to environmental stress. Factors that may affect stress response, such as heterogeneity, co-adapted gene complexes, and domestication, are discussed. Our findings and review of recent zebrafish literature stress the need for researchers to carefully consider breeding histories and trait characteristics for each potential test subject to maximize the sensitivity of the assay.

Keywords: ecotoxicology, environmental stress, bioassay, strains, isogenic, clone

Introduction

Bioassays play a key role in helping scientists understand how organisms respond to substances of concern. The value of the findings depends on the precision of their results, which are, in turn, affected by the genetic variability of the test population. Test subjects with greater genetic variability would be expected to exhibit more variation in their response compared with those showing genetic uniformity, posited by Adams.[1] This study addressed the question of whether the precision of bioassays could be improved by using genetically uniform fish. To answer this question, the stress responses of two strains of zebrafish, isogenic homozygous clones (IHC) and wildtype (WT), were compared.

The IHC subjects in this study were genetically uniform C32 zebrafish. Streisinger *et al.*[2] established the C32 clonal line of isogenic homozygous zebrafish by producing gynogenetic progeny from a single female gynogenetic homozygous diploid parent fish. The female eggs collected were fertilized by irradiated sperm, and diploidy was restored by the interruption of the first cleavage. These gynogenetic progeny shared an identical maternal genotype. Mating between cloned siblings (hormonally transformed males) maintained the clonal line of zebrafish, called C32. The progenitors of this clonal line were selected among other gynogenetic zebrafish based on their survival scores and lack of apparent abnormalities. The C32 line differs from inbred zebrafish lines due to DNA origin from one parent, length of

[1]Ecology, Evolution, and Organismal Biology, Kennesaw State University, Kennesaw, Georgia, USA.
[2]Ecology and Evolutionary Biology, University of California, Los Angeles, California, USA.
[3]Ecology, Evolution, and Organismal Biology, Kennesaw State University, Kennesaw, Georgia, USA.
*Current affiliation: Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California, USA.

time to develop (many generations vs. one event), and lack of inbreeding depression and evolving properties.[3]

This article presents data obtained in the early 1990s, and at that time, it was the first to examine survival and embryonic growth comparisons of IHC and WT larvae under relevant environmental stress.[1] Though it would have been ideal to compare several strains of laboratory-generated zebrafish, at the time this study was conducted, zebrafish research was in its infancy, and multiple strains did not yet exist. Before this date, studies focused on performance levels and physical characteristics of gynogenetic fish in the absence of stress, including comparing survival[2,4–7] and morphological features of gynogenetic and WT fish.[6,8] In this investigation, IHC and WT embryos were exposed to low pH, and their hatchability and growth rates were compared. The developing embryo or larva is considered to be sensitive to low-level changes in the environment,[9] so pH levels tested ranged from 3.5 to 5.0 (titrated with either HCl or H_2SO_4, to assess counterion effects) in water at two grades of hardness (Fig. 1). Controls at pH 7.8 (hard water) and at pH 7.3 (soft water) provided baseline levels. Hatchability and larval length (LL) were used as indicators of environmental stress.

Understanding the effects of low pH on fish continues to be an ecologically important topic. Acidic water is a common stressor for fish. The negative biological impact of the acidification of lakes and streams is widely recognized as a major environmental problems that can result in dramatic fish decline.[10–16] Ocean acidification due to increasing atmospheric carbon dioxide, which is associated with climate change, may have direct effects on marine life by impairing early development. Dissemination of our findings remains important and may contribute to a better understanding of the effects of low pH on early life stages, particularly as our study looked at two genetically different lines of zebrafish, one of which was experimentally made isogenic.

As zebrafish grow in popularity as a model organism, studying how strains vary in their sensitivity and variability of response to environmental stimuli remains important to the future of zebrafish research. Recent developmental, performance, and ecotoxicological studies on various strains of zebrafish were reviewed and contrasted with the findings of this comparative study. The literature demonstrates the importance of the identification of strain characteristics when selecting bioassay test subjects.

Materials and Methods

Subjects

The experiments described in this article were done in accordance with the University of California, Los Angeles's animal care regulations, under the ARC Protocol Number 20806702.

In 1991, the Institute of Neuroscience, University of Oregon provided our lab with 6 adult (3 male and 3 female) and 17 juvenile, C32 zebrafish that originated from a line of gynogenetic homozygous diploidfish.[2] The 23, C32 zebrafish were fifth, sixth, and seventh generation offspring of a gynogenetically induced C32 female in 1987 at the University of Oregon.[2] After the receipt of these fish, a breeding colony of C32 zebrafish was established at UCLA that served as the

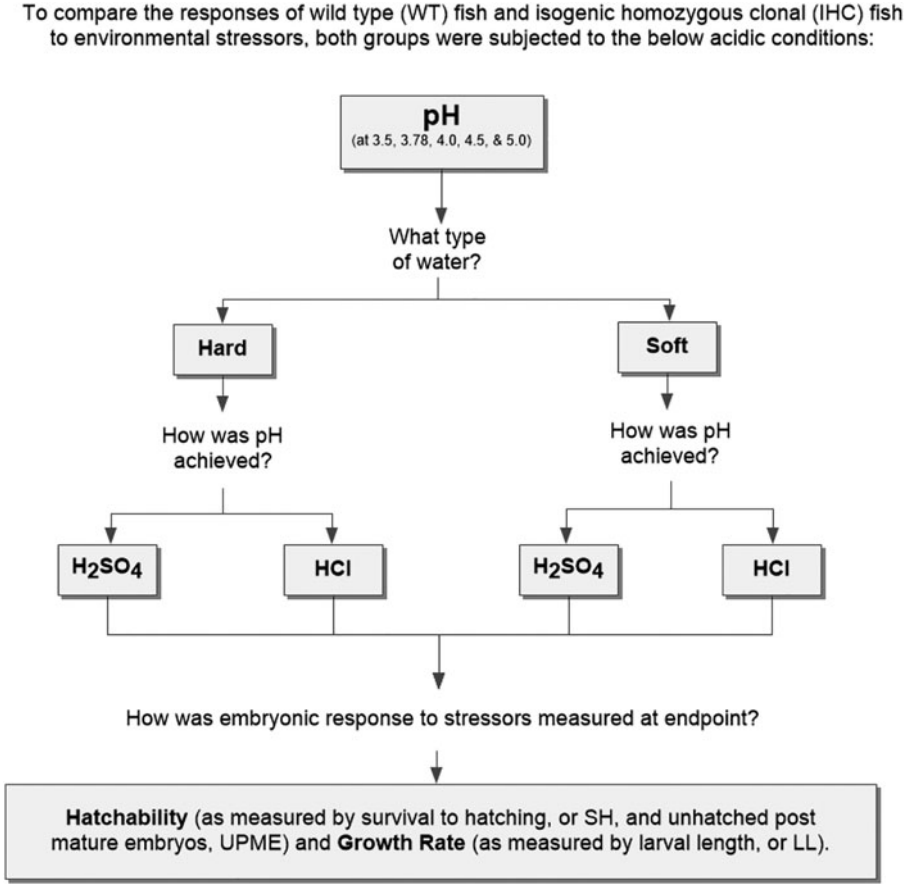

FIG. 1. Twenty two experiments consisting of combinations of H_2SO_4 and HCl in hard and soft water for pH 3.5, 3.78, 4.0, 4.5, 5.0 and a hard (pH 7.3) and soft water (7.8) control were run on embryos and larvae of IHC and WT fish comparing hatchability and LL. The SH and the number of UPME were used as indicators of hatchability. Each experiment had five replicate petri dishes for each fish type (IHC and WT), each with a sample size of 10 embryos. Experimental conditions (pH, water type, and acid type) are represented in *boxes*. IHC, isogenic homozygous clone; LL, larval length; SH, survival to hatching; UPME, unhatched postmature embryos; WT, wildtype.

source of the IHC (C32) embryos used in this comparative study.[1] Buth *et al.*[17] performed allozyme analysis on gene products of 38 presumptive loci by using gel electrophoresis on the offspring from the IHC and WT zebrafish colonies tested in these experiments, and their levels of heterozygosity were compared. WT zebrafish were acquired from local aquarium suppliers in Los Angeles, CA.[1]

Experimental procedures

IHC and WT zebrafish were kept in separate aerated 10-gallon aquaria. Each aquarium had its own filtration system. The aquaria were maintained at 28°C on a 14:10 light: dark cycle. Fish were fed brine shrimp and Tetra flakes. Stocking density was 10–20 fish per tank. Sexes were not separated and there were more females than males in each aquarium. Well-fed zebrafish spawn almost nightly, just before dawn, so to collect fertilized eggs, glass marbles were placed on the bottom of the aquaria the night before an experiment. The marbles covered the entire surface of the tank and protected the fertilized eggs from being eaten by the parent fish. Approximately 2 h after dawn (laboratory lights on), the water in each tank (IHC and WT) was siphoned through a net and the fertilized eggs were transported to holding containers. Each batch of IHC and WT fertilized eggs was the product of a spawning event involving multiple female and male zebrafish. Fertilized eggs were examined with a dissecting microscope. For all experimental conditions described next, IHC and WT embryos at the blastula stage[18] were randomly selected from the IHC and WT batches of fertilized eggs and transferred to petri dishes. Each petri dish contained 85 mL of treatment water (reconstituted water at the desired pH, acid type, and water hardness level). Treatment water was changed daily and collected periodically to test the pH level. The dishes were maintained in a shallow water bath at 28.5°C with a 14:10 light/dark (LD) cycle. Petri dishes were monitored daily, data were collected, and dead fish were removed. Death was determined at the 24 h stage by the appearance of a whitish-colored, degraded egg, or at later stages, by the absence of a heartbeat. Determination of a heartbeat was readily observable due to the transparency of the egg.[1]

Treatment water

Experiments were conducted in either hard or soft reconstituted water containing ACS-certified constituents. Hard water constituents were 192 mg/L $NaHCO_3$, 120 mg/L $CaSO_4 \cdot 2H_2O$, 120 mg/L $MgSO_4$, and 8 mg/L KCl. Soft water constituents were 48 mg/L $NaHCO_3$, 30 mg/L $CaSO_4 \cdot 2H_2O$, 30 mg/L $MgSO_4$, and 2 mg/L KCl.[19] Reconstituted water was mixed in 20-liter high-density polyethylene carboys of aerated deionized distilled water. The desired pH level for each experiment was achieved by using either H_2SO_4 or HCl (Fisher ACS grade). Treatment water was refrigerated and stored for up to 3 weeks until used. pH levels of both treatment water and waste water collected from daily rinses were monitored regularly and found to remain stable with time. pH levels were measured at ambient temperature by using a Fisher Accumet 910 meter and Ross (Orion) combination electrode (±0.01 pH).[1]

Experimental design

Control conditions. Four control experiments consisting of hard water were run on the embryos and larvae of IHC and WT fish comparing hatchability and LL. Survival to hatching (SH) and the number of unhatched postmature embryos (UPME) were used as indicators of hatchability. For each hard water experiment, there were five replicate petri dishes of both fish types (IHC and WT). Each replicate dish contained 10 embryos that were randomly selected from the batch of fertilized eggs collected the morning of the experiment, as described in the Experimental Procedures section. The fertilized eggs in each batch (IHC and WT) were the result of a spawning event involving multiple female and male zebrafish. The four hard water experiments were run on separate days, with a total of 40 petri dishes (20 IHC and 20 WT) with 400 zebrafish (200 IHC and 200 WT) being tested[1] (Fig. 1).

Stressful acidic conditions. Twenty experiments consisting of combinations of H_2SO_4 and HCl in hard and soft water for pH 3.5, 3.78, 4.0, 4.5, 5.0 and a hard (pH 7.3) and soft water (7.8) control were run on embryos and larvae of IHC and WT fish comparing hatchability and LL. SH and the number of UPME were used as indicators of hatchability. Every experiment had five replicate petri dishes for each fish type (IHC and WT), each with 10 embryos. Therefore, all replicates for each experiment under stressful conditions in this multifactor analysis of variance (ANOVA) were conducted on the same day to eliminate a possible source of variation associated with uncontrollable conditions that may vary daily. Each experiment involved 100 fish (50 IHC and 50 WT), with a total of 2000 fish being tested throughout the 20 comparisons for different conditions of pH, acid type, and water hardness level. In addition to the 20 experiments under stressful conditions, randomly one of the four repeated control experiments for hard water was selected as the control and a soft water control was added to this comparison. The order of the 21 experiments was randomized with regard to which pH and water type. Two of the 21 experiments were run simultaneously, based on the initial randomized order. The number of experiments running simultaneously was limited by the size of the circulating water bath where the petri dishes were housed.

Acid types were chosen due to the common usage of HCl in other laboratory studies involving fish[20] and the ecological relevance of H_2SO_4. H_2SO_4 is one of the two primary acids found in nature, nitric being the other acid. Both acids are naturally occurring and industrially caused. Experiments began at the embryonic blastula stage and terminated post-hatching, at 96 h. The duration of the experiment was chosen based on two factors: The first, zebrafish larvae begin to feed shortly after 96 h when raised at this temperature.[18] The addition of food introduces an unwanted competitive variable of food acquisition between larvae, which gives rise to growth rate variability.[21] The second factor was in accordance with standard bioassay protocol at the time of testing.[19,22]

Experimental endpoints

The number of living fish hatched at 96 h divided by the starting number of embryos (e.g., 8/10) determined SH. The number of living unhatched embryos still in their chorions at 96 h divided by the starting number of embryos (e.g., 8/10) determined UPME number. LC_{50} values traditionally represent the lethal concentration of a toxin where at least 50% of

the test population dies. For this study, LC_{50} values represent pH levels where at least 50% of the fish failed to hatch by the 96-h endpoint as set by the SH endpoint criteria. LC_{50} values can be derived by the SH Figures.[1]

The incubation times for IHC and WT fish reared at 28°C in reconstituted hard water (160–180 mg $CaCO_3$/L, pH 7.8) were 48 and 72 h, respectively. Incubation period is the duration of time between fertilization and 50% hatch.[9] At 96 h, the fish were observed for the final time. The fish were then anesthetized with FINQUEL, and LL measurements were made by using an ocular micrometer (±0.02 mm) and a dissecting microscope.[1]

Statistical analysis

Control experiments. A multi-factor ANOVA was used to compare the responses of IHC and WT fish and their precision. Fish type (IHC and WT) and experiment (four identical sets) were independent variables with % survival, % hatched, or LL as dependent variables. Survival and hatching data were arcsine transformed before analysis to meet the assumption of normal distribution[23] Variance ratio tests were used to test for homogeneity of variance. Ratios were calculated with the larger variance of either fish type (IHC and WT) as the numerator.[1,23]

To test whether IHC or WT fish varied more in their response, a Mann–Whitney test was performed on the standard deviations for each experiment and parameter. Regarding LL analysis, a separate ANOVA was run for each fish type (IHC and WT) and experiment to test for a dish affect. If no significant difference in LL was detected among the five dish replicates, the length measurements were pooled for each experiment to increase the sample size. A Mann–Whitney test was then performed on the standard deviations for the pooled LL values to determine whether IHC or WT fish varied more in their length. An overall alpha level of 0.05 was used as the significance level for both ANOVA and Mann–Whitney tests.[1]

Environmentally stressful experiments with varying pH levels in hard and soft water. The effects of pH on SH, UPME, and LL were compared between fish type (IHC and WT) and water type (hard and soft) for each acid type (H_2SO_4 and HCl) by using a two-factor ANOVA. The effects of acid type and pH were compared between fish types in hard and soft water by using a three-factor ANOVA when permissible, which was determined by the absence of a significant interaction between the three independent variables of fish type, acid type, and pH. If a three-way interaction was detected for the acid comparison, a two-factor ANOVA was performed for each fish type and water type. Hard and soft water control experiments were not considered stressful conditions and were therefore not included in the low pH analysis, but they were analyzed separately. SH and UPME data were arcsine transformed before analysis to meet the assumption of normal distribution.[23] Variance ratio tests were used to test for homogeneity of variance. Ratios were calculated with the larger variance of either IHC or WT fish as the numerator.[23] A Mann–Whitney test was performed on the experimental standard deviations for SH, UPME, and LL data to test whether IHC and WT fish varied more in their responses. An overall alpha level of 0.05 was used as the significance level for the ANOVA and Mann–Whitney tests. Coefficients of variation were calculated on the pooled LL data for the five replicates per experiment.[1]

Results

Allozyme comparisons between IHC and WT fish

Of the 41 loci surveyed in WT fish, 35 were monoallelic (homozygous) whereas six loci were polyallelic (sAcoh-A, Gpi-A, Mpi-A, Pep-B, Pep-D, and Pgdh-A) in the WT fish. Thirty-seven of the 38 loci examined in clonal IHC fish were monoallelic. One locus, sMdh-A, was diallelic in IHC fish. One of the two alleles present at this locus in IHC fish was present in WT fish. For the assayed loci, Buth *et al.*[17] reported heterozygosity levels for IHC and WT fish at H = 0.012 and H = 0.052, respectively. Genotype arrays and electrophoretic results for IHC and WT zebrafish were presented and discussed in Buth *et al.*[17]

Survival to hatching

Hard and soft water controls (without acid). For the hard water control experiments that were repeated four times, WT fish has a significantly higher survival rate compared with the IHC fish at 24 h ($p = 0.007$), 48 h ($p = 0.006$), 72 h ($p = 0.02$), and 96 h ($p = 0.05$) (Table 1). At 96 h, all fish were hatched. There was not a significant difference between experiments for either type of fish. A Mann–Whitney test revealed no significant difference between the variation in SH responses of IHC and WT fish. For the comparison between hard and soft water SH responses in IHC and WT fish, there was a significant difference ($p = 0.002$) in SH between IHC and WT fish, with WT fish having a higher SH than IHC fish. In hard water, 74% (1.34 SD) of WT fish hatched by the 96-h endpoint, whereas 64% (1.67 SD) of IHC fish hatched by this time. In soft water, 78% (0.84 SD) of WT fish and 70% (0.71 SD) of IHC fish hatched. Water hardness did not significantly affect SH. There were no significant differences in the

Table 1. Survival Rates for Isogenic Homozygous Clone and Wildtype Zebrafish in Hard Treatment Water

Hard water experiments	*Fish type*	*% Survival 24 h*	*% Survival 48 h*	*% Survival 72 h*	*% Survival 96 h*
1	IHC	68 (8.4)	68 (8.4)	68 (8.4)	66 (8.9)
	WT	86 (11.4)	86 (11.4)	82 (8.4)	76 (11.4)
2	IHC	74 (8.9)	70 (12.2)	68 (13.0)	68 (13.0)
	WT	90 (7.1)	88 (4.5)	84 (5.5)	82 (8.4)
3	IHC	66 (18.2)	66 (18.2)	66 (18.2)	64 (16.7)
	WT	74 (13.4)	74 (13.4)	74 (13.4)	74 (13.4)
4	IHC	84 (8.9)	84 (8.9)	84 (8.9)	84 (8.9)
	WT	82 (8.4)	82 (8.4)	82 (8.4)	82 (8.4)

Survival data were collected every 24 h for the duration of the 96-h experiment. Each experiment consisted of 5 replicates of 10 zebrafish per petri dish for both IHC and WT zebrafish. Percent survival was measured as the number of fish alive divided by the starting number of embryos (e.g., 8/10). The mean and standard deviation (within parentheses) for each time period were based on five replicates per experiment. The four sets of experiments had identical conditions. A Mann–Whitney test revealed no significant difference between the variation in responses by IHC and WT fish.

IHC, isogenic homozygous clone; WT, wildtype.

variability in SH responses between SH responses of the IHC and WT fish for the control experiments.[1]

Stressful acidic conditions. Comparing hatching success in response to environmental stressful conditions, a multifactor ANOVA was performed with fish (IHC and WT), acid (H_2SO_4 and HCl), pH (3.5 to 5), and water (hard and soft), as factors. Table 2 reports degrees of freedom, sum of squares, mean of square, F tests, and *p*-values. Based on the presence or absence of interactions between the four factors, the appropriate numbered factor ANOVA was used for each comparison and reported next.

H_2SO_4 treatments. The WT fish scored significantly higher in SH than IHC fish ($p=0.002$) in hard water/H_2SO_4 conditions (Fig. 2). pH level significantly affected SH ($p=0.0001$). The LC_{50} value for WT fish was pH 3.5, whereas the SH curve was more complicated for IHC fish in that there were two pH values with less than 50% of IHC fish surviving to hatching. These two pH values for IHC fish were pH 4.0 and 3.5, whereas pH 3.78 had 66% hatching. The IHC and WT fish differed significantly in SH in response to pH ($p=0.002$). The IHC fish surpassed WT fish in SH at pH 3.5 and 3.78, whereas at 4.0, 4.5, and 5.0 WT fish exceeded that of the IHC fish. The SH responses for pH 4.0 differed greatly between IHC and WT fish. The IHC fish dropped in their SH scores at pH 4.0, whereas WT fish peaked in their SH curve. The WT fish scored significantly higher in SH than IHC fish ($p=0.01$) in soft water/H_2SO_4 conditions (Fig. 2). pH level significantly affected SH ($p=0.0001$). The LC_{50} values for both IHC and WT were at pH 3.78. The SH response differed significantly between IHC and WT fish in response to pH ($p=0.01$). The IHC fish surpassed WT fish in SH at pH 3.5 and 3.78, whereas WT fish exceeded in SH at pH 4.0, 4.5, and 5.0. This crossover in SH curves for IHC and WT fish resembled the hard water analysis. Water hardness did not significantly affect SH for IHC fish in H_2SO_4 conditions. pH significantly affected SH ($p=0.001$). The LC_{50} values for IHC fish in hard water were at pH 4.0 and 3.5 (see above) and pH 4.0 for soft water. The SH values for hard and soft water differed significantly with pH ($p=0.004$). Hard water favored SH at the lower pH values of 3.5 and 3.78, and soft

Table 2. Multifactor Analysis of Variance Performed on Survival Rates at the 96-h Endpoint Comparing Fish (Isogenic Homozygous Clone and Wildtype), Acid (H_2SO_4 and HCl), pH (3.5 to 5), and Water (Hard and Soft), as Factors

ANOVA table for a five-factor repeated-measures ANOVA

Source	*Degrees of freedom*	*Sum of squares*	*Mean square*	F-*test*	p-*Value*
Fish (A)	1	11.984	11.984	64.095	0.0001
Acid (B)	1	0.05	0.05	0.266	0.6071
AB	1	0.09	0.09	0.482	0.4887
pH level (C)	4	40.701	10.175	54.419	0.0001
AC	4	7.595	1.899	10.155	0.0001
BC	4	0.938	0.235	1.254	0.2903
ABC	4	0.111	0.028	0.148	0.9635
H_2O (D)	1	3.834	3.834	20.503	0.0001
AD	1	0.606	0.606	3.239	0.738
BD	1	0.007	0.007	0.038	0.8456
ABD	1	0.731	0.731	3.911	0.0497
CD	4	12.579	3.145	16.819	0.0001
ACD	4	3.221	0.805	4.306	0.0025
BCD	4	2.627	0.657	3.513	0.0089
ABCD	4	0.486	0.121	0.65	0.628
Subjects w. groups	160	29.917	0.187		
Repeated measure (E)	3	0.117	0.039	14.28	0.0001
AE	3	0.027	0.009	3.362	0.0186
BE	3	0.003	0.001	0.318	0.8125
ABE	3	0.006	0.002	0.702	0.5511
CE	12	0.115	0.01	3.052	0.0001
ACE	12	0.03	0.003	0.932	0.5144
BCE	12	0.051	0.004	1.547	0.1038
ABCE	12	0.066	0.006	2.022	0.0209
DE	3	0.029	0.01	3.496	0.015
ADE	3	0.006	0.002	0.685	0.5613
BDE	3	0.015	0.005	1.808	0.1449
ABDE	3	0.013	0.004	1.585	0.192
CDE	12	0.038	0.003	1.173	0.2997
ACDE	12	0.027	0.002	0.825	0.6246
BCDE	12	0.056	0.005	1.701	0.0635
ABCDE	12	0.028	0.002	0.86	0.5884
E × subjects w. groups	480	1.309	0.003		

The table reports degrees of freedom, sum of squares, mean square, F tests, and *p*-values for each comparison.
ANOVA, analysis of variance.

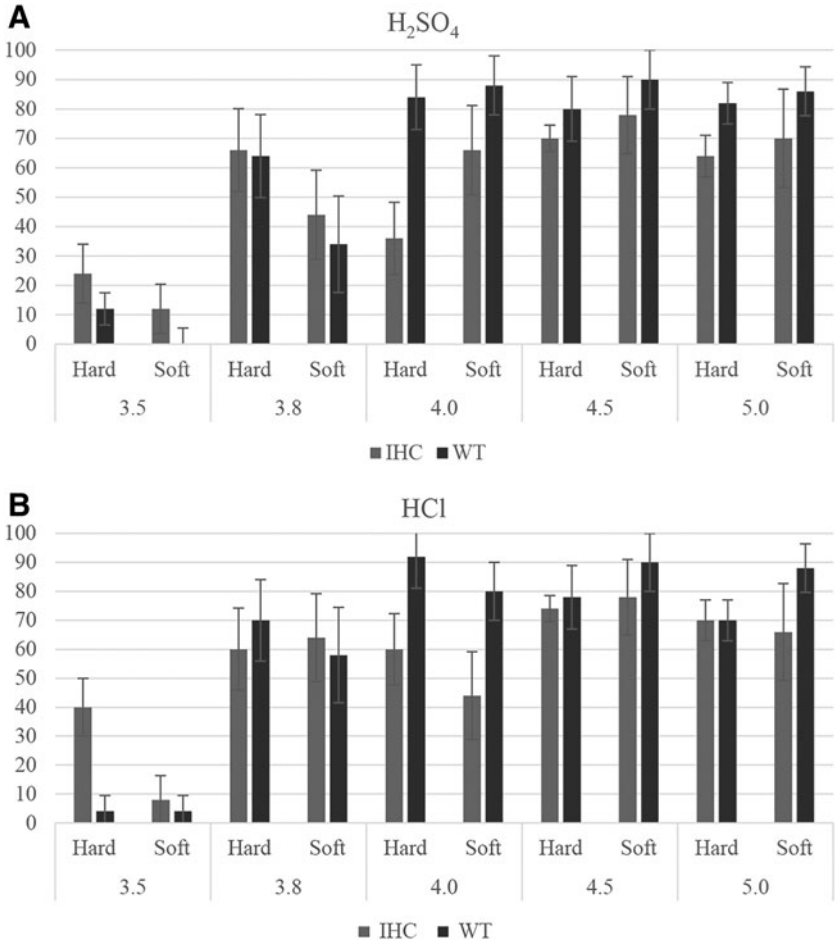

FIG. 2. The SH for IHC and WT zebrafish at the 96-h endpoint in H_2SO_4 **(A)** and HCl **(B)** for hard and soft waters as a function of pH. The mean SH for IHC and WT zebrafish control experiments were 64% and 74% for hard water and 70% and 78% for soft water, respectively. Each point represents the mean of 5 replicates, each containing 10 embryos at the blastula stage at time zero. Bars represent the standard deviation of the mean.

water favored SH at the higher pH values of 4.0, 4.5, and 5.0 (Fig. 2). Water hardness did not significantly affect SH for WT fish in H_2SO_4 conditions. pH significantly affected SH ($p = 0.0001$). The LC_{50}s values for WT fish in hard water were pH 3.5 and 3.78 for soft water conditions. The SH values for WT fish in hard and soft water differed with pH, although not significantly, as was found in the IHC analysis. Hard water favored SH at pH 3.5 and 3.78, and soft water favored SH at higher pH treatments of 4.0, 4.5, and 5.0 (Fig. 2). There were no significant differences in the variability between SH responses of the IHC and WT fish for the H_2SO_4 treatments.[1]

HCl treatments. Overall, WT fish scored significantly higher overall in SH than IHC fish ($p = 0.05$) in hard water/HCl conditions (Fig. 2). pH significantly affected SH ($p = 0.0001$). The LC_{50} values for both WT and IHC fish were at pH 3.5. The IHC and WT fish significantly differed in SH in response to pH ($p = 0.001$). The IHC fish surpassed WT fish in SH at pH 3.5, whereas WT fish exceeded that of IHC fish for the remaining pH levels, with WT fish peaking at pH 4.0. The WT fish scored significantly higher overall in SH than IHC fish ($p = 0.06$) in soft water/HCl conditions (Fig. 2). pH significantly affected SH ($p = 0.0001$). The LC_{50} value for WT fish was pH 3.5, whereas IHC fish had two pH levels where at least 50% of the fish did not hatch: pH 4.0 and 3.5; whereas at pH 3.78, 64% of the fish hatched. The SH in response to pH differed significantly ($p = 0.009$) between IHC and WT fish. The IHC fish surpassed WT fish in SH at pH 3.5 and 3.78; however, at pH 4.0 and 4.5, the SH of the WT fish exceeded that of IHC fish. The SH curve for IHC fish dipped at pH 4.0. Water hardness did not significantly affect SH for IHC fish in HCl conditions, but pH significantly affected SH ($p = 0.001$). The LC_{50} value for IHC fish in hard water was pH 3.5, whereas in soft water, LC_{50} values were recorded for pH 3.5 and 4.0 (see above). The SH values for hard and soft water differed significantly with pH ($p = 0.03$); therefore, no consistent trend was observed (Fig. 2). Water hardness did not significantly affect SH for WT fish in HCl conditions, but pH significantly affected SH ($p = 0.0001$). For both hard and soft water, the LC_{50} values were at pH 3.5 for WT fish. The SH values for hard and soft water differed significantly ($p = 0.002$) with pH. Hard water favored SH at pH 3.78 and 4.0, whereas soft water favored SH at pH 4.5 and 5.0 (Fig. 2). There were no significant differences in the variability between SH responses of the IHC and WT fish for the HCl treatments.[1]

Acid comparisons. Acid type did not significantly affect SH in either hard or soft water. The SH values for H_2SO_4 and HCl acids varied significantly with pH in soft water ($p = 0.007$); however, no consistent trend was observed. The IHC and WT fish did not significantly differ in their variances associated with SH.[1]

Unhatched postmature embryos

Hard and soft water controls (without acid). Hatching success was 100% for both IHC and WT fish in hard and soft water controls. The IHC and WT fish did not significantly differ in their variances associated with UPME.[1]

Stressful acidic conditions

H_2SO_4 treatments. The IHC fish had significantly fewer UPME than WT fish ($p=0.0005$) in hard water/H_2SO_4 (Table 3). pH significantly affected UPME ($p=0.0001$). The number of UPME significantly differed between IHC and WT fish in response to pH ($p=0.0001$). The number of UPME was lower for IHC fish at the lower pH range of pH 3.5, 3.78, and 4.0; whereas the number of UPME was lower for WT at pH 4.5 and 5.0. Hatching success was 100% for both IHC and WT fish in soft water/H_2SO_4 conditions. Water hardness significantly affected the number of UPME for both IHC ($p=0.002$) and WT ($p=0.0001$). Hatching success was 100% for both fish types in soft water and less than that in hard water. pH did not significantly affect the number of UPME for IHC fish but did affect WT fish ($p=0.0001$). The numbers of UPME for WT fish found in hard and soft water differed significantly ($p=0.001$) with pH. The numbers of UPME were higher at pH 3.5, 3.78, and 4.0 in hard water; whereas at 4.5 and 5.0, 100% of WT fish hatched. There were no significant differences in the variability between UPME responses of the IHC and WT fish for the H_2SO_4 treatments.[1]

Table 3. Unhatched Postmature Embryos for Isogenic Homozygous Clone and Wildtype Zebrafish at the 96-h Endpoint for H_2SO_4 and HCl Conditions

			% Unhatched postmature embryos	
			Acid type	
Water type	*pH*	*Fish type*	*H_2SO_4*	*HCl*
Hard	3.50	IHC	10 (12.2)	12 (11.0)
		WT	50 (20.0)	62 (23.9)
	3.78	IHC	0 (0.0)	0 (0.0)
		WT	10 (7.1)	2 (4.5)
	4.00	IHC	8 (4.5)	2 (4.5)
		WT	10 (7.1)	0 (0.0)
	4.50	IHC	2 (4.5)	0 (4.5)
		WT	0 (0.0)	0 (0.0)
	5.00	IHC	2 (4.5)	2 (4.5)
		WT	0 (0.0)	0 (0.0)
Soft	3.50	IHC	0 (0.0)	2 (4.5)
		WT	0 (0.0)	0 (0.0)
	3.78	IHC	0 (0.0)	0 (0.0)
		WT	0 (0.0)	0 (0.0)
	4.00	IHC	0 (0.0)	0 (0.0)
		WT	0 (0.0)	0 (0.0)
	4.50	IHC	0 (0.0)	2 (4.5)
		WT	0 (0.0)	0 (0.0)
	5.00	IHC	0 (0.0)	0 (0.0)
		WT	0 (0.0)	2 (4.5)

Percent UPME was measured as the number of fish still in their chorion at the 96-h endpoint divided by the starting number of embryos (e.g., 8/10). Hatching success was 100% (0 UPME) for IHC and WT fish in both hard and soft water control experiments. The mean and standard deviation (within parentheses) were based on five replicates, each containing 10 embryos at the blastula stage at time zero.

UPME, unhatched postmature embryos.

HCl treatments. As in the H_2SO_4 analyses, IHC fish had significantly fewer UPME than WT fish in hard water ($p=0001$; Table 3). pH significantly affected the number of UPME ($p=0.0001$). The number of UPME significantly differed for IHC and WT fish in response to pH ($p=0.0001$). As in the H_2SO_4 analysis, there were fewer UPME in the lower pH range of pH 3.5 and 3.78 for IHC fish, whereas 100% of WT fish hatched at pH 4.0, 4.5, and 5.0. For soft water HCl treatments, there were no significant differences in the numbers of UPME between fish types, nor did pH significantly affect the numbers of UPME. One hundred percent of WT fish hatched at all pH levels tested, except 5.0, whereas 100% of IHC fish hatched at all pH levels, except 3.5 and 4.5. Water hardness significantly affected the number of UPME for IHC ($p=0.04$) and WT fish ($p=0.0001$). Soft water had fewer UPME than hard water treatments. pH significantly affected the numbers of UPME for IHC ($p=0.02$) and WT fish ($p=0.0001$). For WT fish, the numbers of UPME for hard and soft water significantly differed with pH ($p=0.0001$), as soft water had fewer UPME at pH 3.5 and 3.78, no difference at pH 4.0 and 4.5, and at pH 5.0, hard water had fewer UPME than soft water. There were no significant differences in the variability between UPME responses of the IHC and WT fish for the HCl treatments.[1]

Acid comparisons. Acid type did not significantly affect UPME number in hard water or soft water nor did it vary with fish type. The IHC and WT fish did not significantly differ in their variances associated with UPME.[1]

Larval length

Hard and soft water controls (without acid). The IHC and WT fish differed significantly in LL ($p=0.0001$). There were no significant differences between experiments for either fish type. However, LL for IHC and WT fish varied with respect to experiment ($p=0.0016$), with WT larvae larger than IHC fish in three of the four experiments. In seven of the eight analyses, LL did not significantly differ between dish replicates. The WT fish in experiment #1 was the exception ($p=0.04$). Regarding the analysis for length differences, experiment #1 was also the exception with IHC fish significantly larger than WT fish, whereas the reverse was true for the other three experiments (Table 4). Control WT larvae were significantly larger than control IHC larvae ($p=0.0001$). Water hardness significantly affected LL (Table 4). Soft water favored growth ($p=0.03$). The mean LL value for WT fish reared in hard water was 3.89 and 3.72 mm for IHC fish. In soft water, the mean LL value for WT was 3.92 and 3.80 mm for IHC fish. There were no significant differences in the variability between LL responses of the IHC and WT fish for the control experiments.[1]

Stressful acidic conditions. For H_2SO_4 and HCl, both hard and soft water acidic experiments at pH 3.5 were excluded from the following analyses due to unacceptably small experimental sample sizes of 10 or fewer fish.

Table 4. Larval Length in Millimeter for Isogenic Homozygous Clone and Wildtype Zebrafish at the 96-h Endpoint in Hard Treatment Water

Hard water experiments	*Fish type*	*LL 96 h (mm)*	*CV*	*Sample size*	*Range (mm)*
1	IHC	3.81 (0.12)	3.06	33	0.50 (3.50–3.99)
	WT	3.86 (0.13)	2.45	37	0.50 (3.61–4.11)
2	IHC	3.81 (0.12)	3.23	34	0.53 (3.50–4.03)
	WT	3.71 (0.16)	4.28	40	0.88 (3.12–3.99)
3	IHC	3.72 (0.12)	3.19	32	0.61 (3.35–3.96)
	WT	3.90 (0.15)	3.87	37	0.57 (3.61–4.18)
4	IHC	3.75 (0.14)	3.71	42	0.61 (3.35–3.96)
	WT	3.90 (0.13)	3.24	41	0.50 (3.65–4.15)

The mean, standard deviation (within parentheses), and CV were calculated on pooled data (sample size shown) disregarding dish replicates. The minimum and maximum values for the range are in parentheses. The four sets of experiments had identical conditions. A Mann–Whitney test revealed no significant difference between the variation in responses by IHC and WT fish.

CV, coefficient of variation; LL, larval length.

Table 5. Larval Length in Millimeter for Isogenic Homozygous Clone and Wildtype Zebrafish at the 96-h Endpoint for Hard and Soft Water Conditions in H_2SO, HCl, and Control Conditions

Acid type	*Water type*	*pH*	*Fish type*	*LL (mm)*	*CV*	*Sample size*	*Range (mm)*
H_2SO_4	Hard	3.78	IHC	3.54 (0.13)	3.72	32	0.57 (3.23–3.80)
			WT	3.63 (0.16)	4.30	32	0.65 (3.34–3.99)
		4.00	IHC	3.54 (0.17)	4.79	18	0.65 (3.12–3.76)
			WT	3.57 (0.09)	2.39	42	0.42 (3.31–3.72)
		4.50	IHC	3.68 (0.09)	2.40	35	0.38 (3.50–3.88)
			WT	3.82 (0.13)	3.32	39	0.57 (3.46–4.03)
		5.00	IHC	3.75 (0.17)	4.47	32	0.57 (3.38–3.95)
			WT	3.60 (0.18)	4.91	41	1.03 (2.77–3.80)
	Soft	3.78	IHC	3.43 (0.18)	2.34	22	0.80 (2.85–3.65)
			WT	3.51 (0.12)	3.53	16	0.46 (3.23–3.69)
		4.00	IHC	3.54 (0.12)	3.37	33	0.53 (3.15–3.69)
			WT	3.53 (0.11)	2.97	44	0.49 (3.19–3.69)
		4.50	IHC	3.65 (0.11)	2.94	39	0.49 (3.34–3.84)
			WT	3.79 (0.11)	2.97	45	0.49 (3.57–4.07)
		5.00	IHC	3.68 (0.13)	3.57	35	0.68 (3.31–3.99)
			WT	3.86 (0.12)	3.08	44	0.57 (3.46–4.03)
HCl	Hard	3.78	IHC	3.55 (0.17)	4.82	30	0.80 (2.00–3.80)
			WT	3.58 (0.11)	2.93	35	0.42 (3.34–3.76)
		4.00	IHC	3.46 (0.25)	7.43	30	1.14 (2.28–3.69)
			WT	3.60 (0.10)	2.73	46	0.53 (3.27–3.80)
		4.50	IHC	3.63 (0.15)	4.23	35	0.72 (3.15–3.88)
			WT	3.43 (0.21)	6.21	38	1.14 (2.62–3.76)
		5.00	IHC	3.76 (0.12)	3.06	36	0.53 (3.42–3.95)
			WT	3.91 (0.11)	2.78	34	0.61 (3.53–4.14)
	Soft	3.78	IHC	3.52 (0.10)	2.70	32	0.34 (3.38–3.72)
			WT	3.52 (0.16)	4.58	29	0.65 (3.23–3.88)
		4.00	IHC	3.58 (0.15)	4.13	23	0.53 (3.27–3.80)
			WT	3.50 (0.15)	4.19	37	0.53 (3.23–3.76)
		4.50	IHC	3.61 (0.10)	2.75	38	0.38 (3.42–3.80)
			WT	3.78 (0.12)	3.23	45	0.42 (3.57–3.99)
		5.00	IHC	3.74 (0.10)	2.63	32	0.46 (3.50–3.95)
			WT	3.89 (0.12)	3.09	44	0.57 (3.49–4.07)
Without Acid (Control)	Hard	7.80	IHC	3.72 (0.12)	3.19	32	0.61 (3.34–3.95)
			WT	3.89 (0.15)	3.87	37	0.7 (3.60–4.18)
	Soft	7.30	IHC	3.8 (0.15)	3.96	35	0.61 (3.38–3.99)
			WT	3.91 (0.13)	3.36	39	0.49 (3.49–4.07)

A Mann–Whitney test revealed no significant difference between the variation in responses by IHC and WT fish. The mean, standard deviation (within parentheses), and coefficient of variation were calculated on pooled data (sample size shown) disregarding dish replicates. The minimum and maximum values for the range are in parentheses. A Mann–Whitney test revealed no significant difference between the variation in responses by IHC and WT fish.

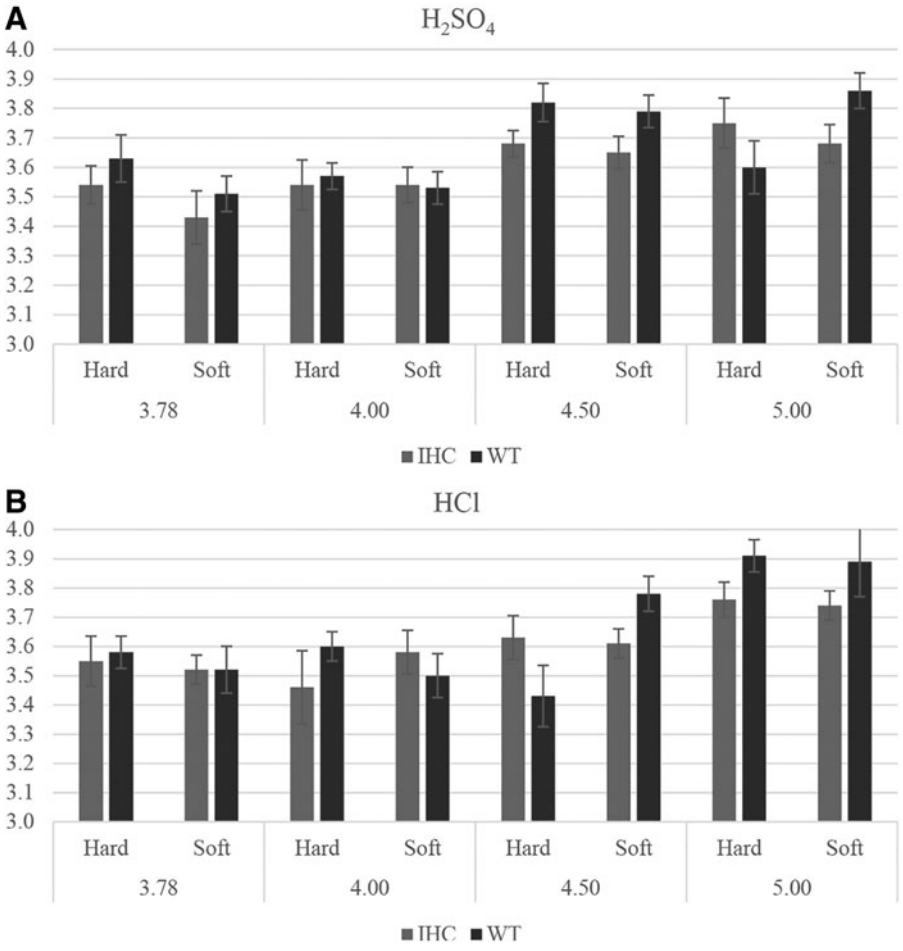

FIG. 3. LL in mm at the 96-h endpoint for IHC and WT zebrafish in H_2SO_4 **(A)** and HCl **(B)** for hard and soft waters as a function of pH. The mean LL values for IHC and WT zebrafish were 3.72 and 3.89 mm in hard water and 3.80 and 3.92 mm in soft water. Symbols as in Figure 2.

H_2SO_4 treatments. The LL did not significantly differ for IHC and WT fish in hard water, but pH did significantly affect LL ($p=0.0001$); further, IHC and WT significantly differed in LL in response to pH ($p=0.0001$; Table 5 and Fig. 3). For IHC fish, LL increased with increasing pH levels, whereas the LL curve for WT fish was more erratic and peaked at pH 4.5. In soft water, WT fish were significantly larger than IHC fish ($p=0.0001$), and pH significantly affected LL ($p=0.0001$). IHC and WT fish significantly differed in LL in response to pH ($p=0.013$). The IHC fish were slightly larger than WT fish at pH 4.0, whereas at other pH values WT fish were larger (Fig. 3). The LL increased with increasing pH levels for both IHC and WT fish in soft water. Water hardness significantly affected LL for IHC fish, with hard water favoring growth ($p=0.01$) (Fig. 3). pH also significantly affected LL in IHC fish ($p=0.0001$), but for WT fish, water hardness did not significantly affect LL. pH significantly affected LL ($p=0.0001$) in WT fish. The WT larvae reared in hard and soft water differed significantly in LL with pH ($p=0.0001$). The trend observed for WT fish reared in soft water portrayed an increase in LL with increasing pH levels, whereas WT fish reared in hard water responded erratically to pH (Fig. 3). There were no significant differences in the variability between LL responses of the IHC and WT fish for the H_2SO_4 treatments.[1]

HCl treatments. The LL values for IHC and WT fish did not differ significantly in hard water/HCl, but pH was significantly affected by LL ($p=0.0001$). The IHC and WT significantly differed in LL in response to pH ($p=0.0001$; Table 5 and Fig. 3) in that IHC fish were larger than WT fish at pH 4.5, but WT fish were larger at other pH values. The LL increased with increasing pH levels for both fish, except at pH 4.0 for IHC and pH 4.5 for WT fish. Similar to the hard water/HCl analysis, there were no significant differences in LL values for the two fish types; however, pH significantly affected LL ($p=0.0001$) and WT significantly differed in LL in response to pH ($p=0.0001$). The IHC fish were larger than WT fish at pH 4.0 (Fig. 3), whereas, at other pH values, WT fish were larger. Water hardness significantly affected LL for IHC fish in HCl ($p=0.007$), and larvae reared in soft water grew larger than fish in hard water. pH significantly affected LL ($p=0.0001$). The LL of IHC fish reared in hard and soft water differed significantly with pH ($p=0.003$), and IHC fish reared in hard water experienced a slight dip in LL at pH 4.0, whereas IHC fish in soft water increased in LL with increasing pH, reaching a maximum at pH 4.5 (Fig. 3). Water hardness significantly affected LL for WT fish as well ($p=0.047$), and WT fish treated in soft water were longer than those in hard water. pH significantly affected LL ($p=0.0001$). The LL of WT fish reared in hard and soft water differed significantly with pH ($p=0.0001$). A slight dip in LL occurred at pH 4.0 in soft water and was less apparent than the dip observed at pH 4.5 in hard water (Fig. 3). There were no significant differences in the variability between SH responses of the IHC and WT fish for the HCl treatments.[1]

Acid comparisons. In hard water, acid type did not significantly affect LL in neither IHC fish nor WT fish; however, LL in H_2SO_4 and HCl differed significantly with pH in hard water for the WT fish ($p = 0.0002$; Fig. 3). The LL significantly differed between the two acid treatments for IHC fish in soft water ($p = 0.0001$). The IHC fish treated in HCl waters were longer than those in H_2SO_4waters (Fig. 3), and similar to soft water results, acid type did not significantly affect LL for WT fish in soft waters. The IHC and WT fish did not significantly differ in their variances associated with LL, and these measurements were pooled among five replicates per experiment and then presented in Table 5.[1]

Discussion

Variability of performance measured in IHC and WT zebrafish

We did not find a significant difference in the variability between responses of the IHC and WT fish. The argument behind the original hypothesis that predicted the response variability to stress would be less in the isogenic zebrafish than the WT zebrafish and it was based on the rationale that IHC possessed less allelic variability than the WT zebrafish. Although IHC zebrafish did not vary less in their response compared with WT, our results are noteworthy with regard to the assertion that heterogeneity affects early life stage fitness. A homogeneity and heterogeneity comparison of these lines of fish for hatching success and growth indirectly addresses the developmental homeostasis theory that heterogeneity promotes developmental stability,[24] an indicator of fitness. The argument states that individuals possessing variation in their genome have a greater chance of responding successfully to change in the environment.[25] This predicts that individuals with more genetic variation in their genome, demonstrating a so-called hybrid vigor, should successfully respond to environmental disturbances[24] more so than those with less genetic variability. Consistent with the heterosis assertion, one would expect IHC to be subordinate to the WT line. Our findings, however, showed that the C32 line, which lacked heterogeneity, fared better than WT under some of the low pH conditions.

Researchers use inbred fish when addressing the heterozygosity hypothesis, which introduce unwanted inbreeding depression. McCune *et al.*[26] recorded lower mortality rates for inbred zebrafish compared with outbred zebrafish. Their inbred line was created from multiple sib pairings from wild-caught parent fish. Brown *et al.*[27,28] reported that inbred lines of zebrafish were more sensitive to sex determination factors than those of WT lines, as measured by sex ratio studies, and differed significantly in response to endocrine disruptors. Shinya *et al.*[29] created a highly homozygous line of zebrafish called the IM strain through 16 generations of full sibling matings, dropping their heterozygosity levels from 62% to 5%. Unless an established clonal line is used in these comparisons, as was in our case, it is difficult to separate out the effects of inbreeding depression on the inbred strain response to a given substance or performance.

Early studies[30–32] argued that stabilization of development is due to the presence of coadapted gene complexes that have been selected over time, with disruption to coadapted genomes resulting in phenotypic variation and decreased fitness.[33–35] Clarke[36] reviewed evidence for the heterosis and the coadapted gene complex arguments, and it was concluded that more studies support the genomic co-adaptation hypothesis than the heterozygosity theory. Besides observing inbreeding depression, Monson and Sadler[37] reported cases of outbreeding depression in zebrafish lines that were crossed, attributing it to the disruption of co-adapted gene complexes in the hybrid. The integrity of the genome itself should theoretically possess co-adapted gene complexes that fit the environment and therefore affect performance levels in naturally selected situations.

This study offered a unique approach in that the IHC line of fish tested was not acquired from mating closely related fish, but through a gynogenetic origin[2] selecting for traits and characteristics that favored laboratory conditions. As previously noted, the allozyme analysis performed on the IHC and WT fish was completed a few years after the experiments were conducted. Although our initial assumption was that our cloned fish were completely isogenic, at least one locus was found to be heterozygous. Still, the level of homogeneity for the IHC fish far exceeded that of the WT fish with heterozygosity scores of $H = 0.012$ and $H = 0.046$, respectively; further, other cyprinid fish heterozygosity values were close to that of our WT fish with $H = 0.052$.[17] The origin of the unexpected diallelic locus (sMdh-A) found in IHC zebrafish was first attributed to mutation by Buth *et al.*[17] However, later analysis[38] of 223 loci in the C32 line, referred to as IHC in this study, found that 91% of their genome was homozygous and attributed the detected heterozygosity due to strain contamination. Further studies performed by Guryev *et al.*[39] supported the conclusion of Nechiporuk *et al.*[38] that the limited heterozygosity detected in C32 was inherited by a common ancestor, and they detected polymorphic loci for 11% of the C32 loci assayed. Another inbred strain, SJD, also showed polymorphisms for 9% of their loci. Allozyme differences similar to those identified between WT and IHC zebrafish by Buth *et al.*[17] have been associated with differential survival in *Peociliopsis monacha* by Vrijenhoek *et al.*[40] It was not clear as to whether the allozymes themselves were the targets of selection or whether they were simply linked to chromosomal regions that contain the elements being selected.[40]

Survival findings, measured by hatchability. Under nonstressful conditions, WT fish were significantly more successful in hatching than IHC for both water hardness conditions. Lower survival scores have been noted for other gynogenetic fish, generally attributed to two factors. The first is due to the gynogenetic technique itself, often called the treatment effect.[41] The fish used in this study were many generations removed from the parental descendent of the C32 line, and therefore, treatment effect cannot be considered a factor in this study. Reduced fitness of isogenic homozygous fish can also be due to the fixation of a sub-lethal allele as a result of the cloning process.[41] However, as previously discussed, the C32 line was selected for favorable laboratory conditions, and the lower hatching scores may just be due to strain differences among zebrafish.

Sublethal effects at low pH were evident in hatchability of IHC and WT fish. The IHC and WT fish differed in their sensitivity, but not in the variation of the observations. The IHC hatchability was greater than WT at lower pH values, whereas WT fish hatched more successfully than IHC fish at

the upper end of the 3.5–5 pH range. Overall, WT fish had higher SH scores than IHC fish; however, IHC and WT fish significantly differed in their responses to pH. For both acids and water types, IHC fish had higher SH scores for the lower pH treatments than WT fish, whereas at the higher pH levels, WT fish had higher scores. In other words, IHC fish hatched more successfully than WT fish in the most stressful conditions. This finding was also observed for UPME number, another index used to determine hatchability. The IHC fish had better hatching success in hard water, as indicated by lower UPME numbers, than WT fish in hard water for both acid types. The IHC and WT fish differed in UPME number in response to pH. The IHC fish hatched more successfully at lower pH treatments than WT fish, whereas WT fish hatched more successfully than IHC fish at higher pH values. This interaction was similar to the SH analysis. Under nonstressful conditions, IHC fish hatched earlier than WT fish, which may account for the significantly higher hatching success observed under stressful conditions of low pH. The C32 IHC may have been more successful in hatching in these lower pH's due to their screening selection as a laboratory fish.[2] Gjedrem and Rosseland[15] reported that acid tolerance, as measured by survival, is a heritable trait among salmonids; therefore, the differences in sensitivity observed between the IHC and WT in our study may be due to simple strain differences among zebrafish, as opposed to varying levels of heterozygosity between the two tested strains in this study.

Low pH had no effect on UPME number, though it negatively affected SH, with the general trend of increasing SH scores with increasing pH level. The SH curves reached a maximum at pH 4.5 for most combinations of acid and water conditions. This trend was observed for most combinations of water and acid types except for fish in soft water/HCl and hard water/H_2SO_4 conditions. The WT fish experienced a sharper dip in their SH curve between pH 4.0 and 5.0 for hard water/HCl than was expressed in other acid and water combinations.

Water hardness did not significantly affect SH for either fish type. Further, SH in hard and soft water significantly varied with pH. Hard water acid treatments were less toxic at the lower pH range of 3.5, 3.78, and 4.0 (some conditions), whereas soft water acid treatments were less toxic at higher pH levels of pH 4.0 (some conditions), 4.5, and 5.0. This trend was observed for both fish types and most combinations of water and acid type.

Our observations are in part consistent with those of Graham and Wood,[20] who found hard water less toxic than soft water to rainbow trout (*Oncorhynchus mykiss*) for pH 3.0–4.6 titrated with H_2SO_4. Our results for IHC and WT zebrafish embryos corroborate these findings at pH 3.5 and 3.8. However, a crossover occurred between the two water hardness types for both IHC and WT fish, with soft water favoring survival at pH 4.0, 4.5, and 5.0. Carrick[42] found that water hardness only affected hatching in acidic waters at pH levels of 4.0 and below for salmonids. Our results agree with Carrick's[42] findings of hard water decreasing acid toxicity only at the very low pH values of 4.0 and below.

The ameliorating effect of hard water on acid toxicity is attributed to the ionic effects of Ca^{2+}.[43] This may explain the finding that hard water favored SH at lower pH values but it does not explain the observation that soft waters were less toxic than hard waters at higher pH treatments. Graham and Wood[20] observed a similar crossover for HCl toxicity curves in the rainbow trout. They found that at low pH values of 3.0 and 3.2, hard water is significantly less toxic than soft water, but at higher pH levels of 3.8 and above, soft water is significantly less toxic than hard water.[20]

Hatchability is another index of survival, although it focuses on the act of hatching, and was measured by UPME. One would expect a lower UPME number with a higher SH score, unless the embryo died before the 96-h endpoint. Water hardness had an opposite effect on UPME number compared with the SH results. Soft water significantly favored hatching in terms of UPME number for both IHC and WT fish. Hatching success was 100% for soft water/H_2SO_4 conditions, whereas UPME number was significant in hard water. The opposing effect of water hardness on SH was seen in UPME number for WT fish in HCl conditions, where soft water favored hatching at lower pH levels, whereas hard water favored hatching at pH 5.0.

The finding of fewer UPMEs reared in soft water is counterintuitive when considering the supposedly protective effects of Ca^{2+} in hard water. The lack of Ca^{2+} and stress of the low pH may be a synergism, resulting in early mortality of weaker embryos that would have been unable to hatch by the 96-h endpoint and thus counted as UPME. An alternative explanation, and one that supports the higher SH scores under the same conditions, is that zebrafish tend to be soft water inhabitants,[21] which may explain their survival and hatching preferences for waters with lower mineral content. Boisen *et al.*[44] reported zebrafish to be tolerant to water with extremely low ion concentrations due to their affinity and ability to uptake Na^+ and Cl- in very soft waters, thus maintaining osmoregulation. Despite this ability, in nature, zebrafish have been reported to inhabit waters that vary greatly in mineral content.[44,45]

Life history stage varies in sensitivity to the ameliorating effects of hard water on acid toxicity. Trojnar[46] found that water hardness did not affect hatchability at low pH in the white sucker (*Catostomus commersoni*) and brook trout (*Salvelinus fontinalis*). However, water hardness did affect survival to swim-up stage in white suckers. Some of the conflicting results reported for acid tolerance may be attributed to the different developmental stages tested or due to strain differences discussed next.

Chorionase is an enzyme that is required for hatching and is inhibited by low pH, which retards the incubation period.[9] Low pH has also been found to delay hatching in the fathead minnow,[47] the zebrafish,[48] salmonids (*Salmo salar* and *Salmo trutta*) below pH 4.5,[42] white sucker (*C. commersoni*), walleye (*Sander vitreum vitreum*), desert pupfish (*Cyprinodon macularius*), roach (*Rutilus rutilus*), and yellow perch (*Perea flavescens*).[9] Low pH also negatively affects the strength of the chorion. The implications of a soft shell are significant for substrate spawning fish, such as salmonids. When these soft-shelled eggs are exposed to movement, they easily break, killing the embryos.[9]

We observed that acid titration type (i.e., counter ion, either chloride or sulfate) did not significantly affect hatchability of zebrafish in either hard or soft water. This finding differs from Graham and Wood's[20] finding that below pH 3.8, H_2SO_4 was less toxic to rainbow trout than HCl in both hard

and soft water. Above pH 3.8, H_2SO_4 was less toxic than HCl in hard water, whereas in soft water, H_2SO_4 was significantly more toxic than HCl.[20]

Jellyman and Harding[49] found that fish do not survive in highly acidic streams and lakes with a pH of 3 to 3.5 based on lab studies suggesting anthropogenic causes. Zahangir *et al.*[50] discussed the secondary stress response to low pH in zebrafish, noting that young fish are especially sensitive to pH values below 5. In a recent review of zebrafish as a model for physiological response to low pH, Kwong *et al.*[51] summarized how freshwater fish successfully regulate ionic homeostasis and tolerate change in acid-base conditions. Andrade *et al.*[52] found that zebrafish embryos exposed to pH values below 3.5 experienced 100% mortality due to bradycardia and pericardial edema.

Growth findings, measured by LL. We observed that under nonstressful conditions, WT larva were larger than IHC in both hardness water types, suggesting faster growth rates. Contrary to these controls, IHC and WT did not differ in LL for most acidic/hardness conditions tested, except for soft water/H_2SO_4 titrations where WT were larger than IHC larvae.

Our data demonstrated that pH adversely affected LL in all acidic/hardness conditions in both IHC and WT, corroborating Rosenthal and Alderdice,[53] who found a reduction in LL in marine fish due to low pH. In our study, WT LL varied with pH in hard water titrated by both acids. Mount[47] reported similar results for the fathead minnow. Our IHC LL data displayed a trend of increased LL with increasing pH. Meyer *et al.*[54] observed differences in growth rates among metabolically stressed zebrafish with the most ''wild'' strains, such as Wild India Kolkata (WIK), exhibiting a mitigated response that was more expected of a strain possessing greater genetic variation.

We observed that the effects of water hardness on LL depended on the type of acid used for titration. For H_2SO_4 experiments, IHC LL values in hard water were greater than those in soft water. There were no detectable differences in LL of WT due to water hardness with H_2SO_4 titrations. In HCl experiments, LL from both IHC and WT in soft water were longer than those in hard water with an interaction between water type and pH detected. The soft water/HCl findings were similar to those of the control findings, where soft water favored growth.

Curiously, in our study, hard water favored survival at the lowest pH range, whereas at the higher pH range, soft water favored survival. Zebrafish are often found inhabiting waters of lower mineral content,[21] explaining why soft water favored growth and survival in nonstress conditions. In conditions of stress, such as low pH, toxicity may be ameliorated by the higher Ca^{2+} in hard water, especially at the lower pH (i.e., <4.0). The type of acid used for titration affected LL. Fish treated in soft water/HCl were longer than those in soft water/H_2SO_4. Graham and Wood[20] reported that HCl was less toxic to rainbow trout than H_2SO_4 in soft water for pH levels above 3.8. This difference in counter ion toxicity between HCl and H_2SO_4 may account for our observations. Although reduced LL is not considered to lower fitness, it does correlate with the abnormal size and shape of the yolk sac, which is indicative of impaired development.[9] Abnormal yolk sac size and shape were observed in this study, but no statistics were gathered.

As for the SH data, some LL response curves contained minor fluctuations that lacked correspondence between fish type (IHC and WT), experimental conditions (pH, acid, and water hardness level), and parameter (SH and LL). No explanations account for their occurrence, and these minor fluctuations are considered artifactual.

Importance of identifying characteristics of zebrafish strains when selecting test subjects. As popularity in the use of zebrafish as a model organism increases, so has the number of defined strains that vary in genetic makeup and have greater genetic drift from ancestral populations in India.[28,45,55,56] Generally, domesticated fish, including zebrafish, have been selected for characteristics that make them more suitable for handling in lab and aquaculture settings. Favorable traits include: higher surface affinity, lower startle reflex, and higher growth rates,[3] reflecting genetic components that favor those environments.

Coe *et al.*[55] discuss zebrafish strain differences and how they vary in their ecotoxicologic response to substances, advising caution when comparing performance endpoints without considering allelic differences. After examining multiple strains, they reported that wild zebrafish possessed far more allelic richness than those commonly used in ecotoxicological testing. They observed a decrease in heterozygosity over time for one line of fish, reporting that WIK zebrafish exhibited an allelic richness baseline value of 5.478 that decreased to 3.473 after 1 year of breeding.

Brown *et al.*[27] found that WIK zebrafish are far more susceptible to endocrine disrupters than related inbred fish, supporting the idea that strains vary in their susceptibility to environmental stress. Brown *et al.*[57] compared outbred and inbred zebrafish in a Fish Sexual Development Test and found that the level of inbreeding increases a skewed sex ratio in developing fish. Outbred fish develop reproductively faster than inbred fish, representing a more sensitive test subject than inbred fish. Similar findings of behavior ontology among zebrafish strains are documented by Lange *et al.*,[58] suggesting a challenge of distinguishing environmental versus genetic influences on developmental traits, such as locomotion. Bhat *et al.*[59] reported that some behaviors are influenced more by context than genetics, making it more difficult to address the importance of heterogeneity to fitness. Differences in ethanol sensitivity between TU and WIK strains were attributed to genetic factors[60] but observed only under acute exposure. Zhang *et al.*[61] also reported differences in drug sensitivity of AB and TU strains. Although many researchers select homogeneous strains, Gao *et al.*[62] tested three types of WT strains of zebrafish (AB, TL, and TLAB) and found that each varied in their light-induced locomotor response. The TL and AB strains of zebrafish also differ in their behavior to varied environments.[63] Wright *et al.*[64] detected differences in predator defense behaviors (i.e., shoaling and boldness) between WT-unspecified and AB strains, suggesting that domestication relaxes the typical selective pressures imposed in the wild that result in behavioral and presumably genetic strain differences.

To maximize the sensitivity and accuracy of assays, the traits of a strain's genome should be considered before

measuring physiological or behavioral endpoints. Each strain may be unique in its response and not truly representative of fish in the wild, suggesting the need to provide details of the established line, perhaps comparing with homozygous or heterozygous isogenic lines as a basis of reference. Familiarity with the characteristics of each strain is encouraged,[27,37,54,55,58,62,63] and the researcher will benefit from careful consideration of unique attributes before strain selection.[54] In addition, the more genetic and breeding descriptors of test subjects that are made available when reporting experimental results, the greater the likelihood of advancing our understanding of using zebrafish as an animal model.

At the time this study was performed, zebrafish research was in its infancy and multiple strains of laboratory-generated zebrafish did not exist; therefore, the comparison in this study was limited to two strains that varied by their level of heterogeneity. Currently, that is not the case, and thankfully researchers have many strains to choose from when selecting a test subject for a bioassay screening. To ensure the test subjects' response to a condition or substance is sensitive and representative of the species as a whole, researches need to consider their options and either screen many strains, or better yet, include the Wild India Kolkata (WIK) strain that is considered to resemble ancestral populations of zebrafish. In conclusion, although we found no significant differences in the variability between the hatching and growth responses of the IHC and WT fish, pH and water hardness level had a differential effect on the two groups. Simple strain differences between IHC and WT fish, as opposed to varying levels of heterozygosity, may be the probable cause of the response differences to environmental stress. Our findings and review of recent zebrafish literature stress the need for researchers to carefully consider breeding histories and trait characteristics for each potential test subject to maximize the sensitivity of the assay.

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Address correspondence to:
Lisa G. Adams, PhD
Associate Chair, SIO Department
Scripps Institution of Oceanography
University of California, San Diego
La Jolla, CA 92093
USA

E-mail: lgadams@ucsd.edu