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Annals of Aquaculture and Research

Research Article

A New Behavioral Phenotyping Strategy for Pacific Oyster (*Crassostrea gigas*) Larvae Reveals Cohort-Level Effects on Copper Toxicity Swimming Response

Megan Hall[3], Brad Foley[1], Emily Cheung[2], Mohammad Abbasi[1], and Nathan D. Churches[1]*

[1]*Molecular and Computational Biology, University of Southern California, USA*
[2]*Department of Biology, Skidmore College, USA*
[3]*Marine and Environmental Biology, University of Southern California, USA*

***Corresponding author**

Nathan Churches, Molecular and Computational Biology, University of Southern California, 1050 Child's Way, Los Angeles, CA, 90089, USA, Email: churches@usc.edu

Submitted: 09 September 2016

Accepted: 19 October 2016

Published: 21 October 2016

ISSN: 2379-0881

Keywords

- Copper
- Toxicity
- *Crassostrea gigas*
- Pacific oyster
- Behavior
- Larvae

Abstract

Copper is among the most studied marine metallotoxins. It is both heavily utilized in commercial applications (e.g. industrial discharges and antifouling hull coatings) and readily bioavailable in the water column. In bivalve mollusks, common responses to coppertoxicity include increased mortality rates and disruption of normal development, especially during early life history stages. Bivalve studies, however, focus primarily on physiological and morphological changes in conditional or field experiments, while behavior remains relatively unexplored. This study profiles the larval movement characteristics of 48 hour old larval Pacific oysters *Crassostrea gigas* (*C. gigas*) under increasing concentrations of Cu^{2+}. *C. gigas* full sibling families were subjected to a series of increasing Cu^{2+} concentrations in Filtered Sea Water (FSW), from 0 ppb to 36 ppb, for n=10 conditions. Across all trials, a negative correlation was observed with increasing Cu^{2+} loads and percent survival, normal morphological development, and average width. MovTrack, in-house developed tracking software, was used to quantitatively show that Cu^{2+} concentration and total movement of larvae are not dependently linked. A familial component of Cu^{2+} stress reaction was potentially observed, with some genetic lines showing significant differences in movement metrics, supporting the hypothesis that Cu^{2+} toxicity response may have a heritable component. This study provides evidence that previously documented physiological responses to Cu^{2+} toxicity are fundamentally a cellular response, rather than a synergistic effect of altered behavior and cell-level disruption. Finally, this work provides a proof of concept for MovTrack software as a reliable phenotyping strategy for quantitative measurement of marine larvae behavior.

ABBREVIATIONS

Cu^{2+}: Cupric Sulfate; ppb: Parts Per Billion; *C. gigas*: *Crassostrea gigas*

INTRODUCTION

Anthropogenic introduction of toxins in oceanic environments can have detrimental effects for marine life. Heavy metals are among the most studied marine toxins (metallotoxins), and have been shown to negatively affect survival, growth, stress protein pathways, and immune functions in several species [1-5]. Among the metallotoxins, copper (Cu, especially Cu^{2+}) has been extensively researched due to widespread introduction. As an example, San Diego Bay and the Port of Los Angeles have exceeded the EPA allowable Water Quality Criterion (WQC) of 4.8 parts per billion (ppb) in recent years [1,6-7]. The primary source of Cu contamination is antifouling hull paint, used by both civilian and naval vessels. Other sources included storm water runoff, rainfall, and municipal and industrial discharges [6]. This

study, and similar Cu pollutant load projects [4,8-12], clearly demonstrate the need to further understand the sources and effects of increased Cu load for both ecosystems and individual species.

Bivalves have often been the target of metallotoxicity investigations. In addition to extensive populations, many bivalves exhibit a broadcast spawning reproduction strategy and a planktonic life history stage, during which gametes and larval/embryonic stages are particularly vulnerable to ambient seawater conditions. Moreover, mollusks are commercially important, harvested for a total of more than 13.2 million metric tons in 2012 [13]. As such, there has been an interest in funding studies related to increasing wild harvest and aquacultural yields, and understanding toxicity response. Several studies have demonstrated the wide-ranging negative consequences that bioavailable Cu^{2+} has on bivalves and other mollusks [14-26]. Because the vast majority of aquaculture farms are in- or near-shore operations, understanding anthropogenic Cu loading effects to waterways is of great economic value.

The Pacific oyster, *Crassostrea gigas*, is among the most extensively cultured bivalves. For *C. gigas,* the percent normal development and percent survivorship has been used to approximate the concentration at which 50% of population experiences lethality (LC_{50}) when exposed to Cu^{2+}. For embryos and larvae, the LC_{50} has been estimated at 5-20 ppb, and upwards of 500 ppb for established adults [3,27-31]. Each of the studies on metallotoxicity in bivalves mentioned here, excluding environmental collection experiments, have similar strategies: to expose organisms to varying concentrations of agonist and observe physiological and morphological impacts. Reports on larval swimming behavior in the metallotoxin literature are scant. A single study reported that swimming behavior in *C. gigas* larvae showed overall increases when exposed to leachates of chemically treated timber at days 3 and 7 [32]. While informative, this study did not control for Cu concentration across experiments, and leachates included other toxins including chromium and arsenic. A second study mentioned erratic swimming behavior in *C. gigas* in relation to Cu load, but this was never quantified [28]. Previous work has hypothesized that swimming strategy and feeding may be linked in bivalve larvae, demonstrating a direct link to behavior and access to nutrients [33]. This brings about the hypothesis that a negatively synergistic effect of cellular responses and altered behavior (and therefore access to nutrients) is accountable for previously observed physiological changes, rather than cellular responses alone.

Here, we address this knowledge gap by using new phenotyping strategy for marine bivalve larvae. In this study, a series of full sibling *C. gigas* families were created from a unique population of Southern California oysters, sourced from Carlsbad Aquafarms, USA. Each family (or cohort) was exposed to a series of increasing Cu^{2+} concentrations in Filtered Sea Water (FSW), from 0 ppb to 36 ppb, for a total of n=10 conditions. After 48 hours in condition, a quantitative measure of swimming behavior was recorded using an in-house developed computer tracking software, MovTrack. In parallel to quantitative behavioral measurements, phenotypic assays were performed to document survival, developmental pace and abnormalities, and growth rates. The purpose of this work is to i) further explore estimated LC_{50} values for an unstudied population of *C. gigas*, ii) understand between-and among-family variance in response to Cu load, and iii) demonstrate a new behavioral assay for bivalve larvae. The timing of this project is especially pertinent, as the US Environmental Protection Agency (EPA) is set to release new Ambient Water Quality Criteria for copper documents, currently in the public comment phase.

MATERIALS AND METHODS

Animals

Adult *C. gigas* samples were kindly donated from Carlsbad Aquafarms (Carlsbad, California, USA) on May 12th, 2016. Individuals collected were held at Wrigley Marine Science Center (WMSC, Catalina Island, California, USA) in flow-through tanks with raw seawater until time of experimentation, which was as late as July 29th, 2016. Animals were fed a mixture of *Isochrysis galbana* and/or *Tetraselmis spp.*ad libitum on a semi-daily basis to keep gravid.

Seawater and chemical preparation, trial conditions

Seawater at WMSC laboratories was sourced from the point at Big Fisherman's Cove (Catalina Island, California, USA). From this source, salt water is sent through a series of pleated reusable filters, down to 2µm to remove animals and other debris, and hereafter referred to as Filtered Sea Water (FSW). For Cu^{2+} trials, a 100 millimolar stock solution was prepared from solid state $CuSO_4$ crystals (EM Science, product number CX2203-1) mixed with Milli-Q filtered fresh water. From the stock solution, a 0.1 millimolar working solution was prepared using filtered Milli-Q fresh water. Experimental concentrations were then obtained by diluting $CuSO_4$ working solution in 1L FSW at appropriate ratios. A total of 7 trials were performed, each with a control group (0 ppb, i.e. FSW) and 6 experimental conditions. Three replicates per condition were performed, except for trial 7 in which six replicates were conducted for three conditions at the upper end of the Cu^{2+} spectrum (Table 1). Experimental conditions were as follows for trials 1-3 in ppb Cu: 0, 3, 6, 9, 12, 15, 18; trials 4-6 in Cu ppb: 0, 6, 12, 18, 24, 30, and 36; trial 7: 0, 24, 30, and 36 (Table 1). Trials 1-3 were initial experiments, which clearly did not capture the range in which 48 hpf larvae were viable, so trials 4-6 were performed with a greater range. Because some overlap occurred between trials 1-3 and 4-6, we performed a final trial (trial 7) to increase representation in the full dataset at higher copper concentrations.

Spawning and toxicity assay

Gametes were obtained by following the common aquaculture technique of strip-spawning gravid adult *C. gigas* [34]. Eggs and spermatozoa with the greatest number and maturity were selected for fertilization, which was determined by visual subjective analysis for roundness and motility, respectively. The creation of full-sib families was done by mixing mature gametes from a single male and single female in a beaker with approximately 250 mL FSW, such that the sperm: egg ratio was approximately 5:1. After one hour, fertilized eggs were rinsed through a 20 µm nylon sieve to reduce risk of polyspermy.

Table 1: Cu^{2+} effect results across seven isogenic *C. gigas* cohorts (previous page). Trials/cohorts are indicated in the furthest left column and boldly boxed. Each trial contains four metrics calculated in this study: average size (in µm), abnormal/normal estimate (ratio: number of abnormal counted over number of normal counted), percent survival (ratio: count surviving over initial population estimate), and adjusted average movement (proxy for how much a given volume of *C. gigas* larvae had moved per assay, see materials and methods: VIII. Statistical Analysis). Trial 7 was created to increase representation at higher copper concentrations for the full dataset.

		Parts Per Billion Copper									
Trial Number	**Metric**	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**24**	**30**	**36**
1	Average Size	62.41	55.91	55.48	55.82	55.90	56.59	55.40	-	-	-
	Abnormal/Normal Estimate	0.17	0.25	0.19	0.39	0.31	0.74	1.98	-	-	-
	Percent Survival	1.91	2.27	1.98	1.79	1.93	1.61	2.01	-	-	-
	Adjusted Average Movement	3.43 (n=2)	1.92	2.41	2.33	1.84 (n=2)	1.55	1.65	-	-	-
2	Average Size	74.68 (n=2)	66.98 (n=2)	59.80 (n=2)	51.33 (n=1)	58.45	53.43 (n=2)	N/A	-	-	-
	Percent Normal Development	0.11(n=2)	0.29	1.26 (n=2)	1.39 (n=2)	0.98	1.725 (n=2)	**12 (n=1)***	-	-	-
	Percent Survival	0.53	0.87	0.97	0.54	0.61	0.28	0.27	-	-	-
	Adjusted Average Movement	2.29 (n=2)	1.47 (n=2)	2.96 (n=2)	3.03 (n=1)	2.48	3.79 (n=2)	N/A	-	-	-
3	Average Size	56.60 (n=1)	55.52	50.63	48.96	49.17	47.80 (n=2)	48.18 (n=1)	-	-	-
	Percent Normal Development	0.12 (n=1)	0.52	0.88 (n=2)	3.63 (n=1)	0.94	N/A	**34 (n=1)***	-	-	-
	Percent Survival	1.98	1.84	1.51	1.00	1.53	0.44	0.47	-	-	-
	Adjusted Average Movement	1.54 (n=1)	2.66	3.80 (n=2)	2.26	3.43 (n=1)	4.57 (n=2)	1.62 (n=1)	-	-	-
4	Average Size	58.76	-	52.99	-	51.15	-	49.81	49.51	48.08	48.45
	Percent Normal Development	0.45		0.62		1.02		2.17	3.74	4.37	12.97
	Percent Survival	0.53		0.66		0.46		0.62	0.65	0.34	0.46
	Adjusted Average Movement	2.99 (n=2)	-	3.66	-	3.30	-	4.46 (n=2)	3.90	3.23	3.82 (n=2)
5	Average Size	65.63	-	60.83	-	56.51	-	51.48	49.75	41.83 (n=1)	45.17 (n=2)
	Percent Normal Development	0.43		0.47		1.52		2.04	3.11	7.33 (n=1)	8.31 (n=2)
	Percent Survival	0.48		0.80		0.43		0.36	0.27	0.02	0.20
	Adjusted Average Movement	1.30	-	1.07	-	2.41 (n=1)	-	1.94 (n=2)	2.98 (n=1)	N/A	2.10 (n=2)
6	Average Size	50.61	-	51.77	-	53.71	-	50.65	52.63	50.25	48.38
	Percent Normal Development	0.50 (n=2)		0.65 (n=2)		0.63		1.51	6.02	8.24	14.43 (n=2)
	Percent Survival	1.36		0.78		0.85		0.64	0.32	0.14	0.60
	Adjusted Average Movement	3.13	-	3.16 (n=2)	-	3.09	-	3.01 (n=2)	4.05 (n=2)	3.69 (n=1)	3.45 (n=2)
7**	Average Size	79.12 (n=3)	-	-	-	-	-	-	59.37	56.39	52.48
	Percent Normal Development	0.04 (n=3)	-	-	-	-	-	-	0.85	3.60	5.43
	Percent Survival	2.54 (n=3)	-	-	-	-	-	-	0.78	0.61	0.40
	Adjusted Average Movement	3.02 (n=3)	-	-	-	-	-	-	2.96	2.55	3.13
*= population considered 'crashed' beyond normal larval recognition, removed from subsequent PND analysis											
** n=6, unless otherwise noted											

After approximately 1.5-2 hours, fertilization counts were performed by concentrating egg/sperm solutions into approximately 20-30 mL of FSW in a 50 mL conical tube, which was gently bubbled from the lowest point to achieve homogeneity. Four 20 microliter aliquots of egg/sperm solution were counted on a Sedgewick rafter to obtain an accurate estimate of total fertilized egg concentration in the population. Evidence of fertilization was noted by observation of a polar body or cell cleavages, with most organisms achieving a 2-8 cell stage by the 2 hour mark.

Twenty-one 1L polycarbonate bottles were then filled with FSW and amended with a $CuSO_4$ working solution to achieve test concentrations of $CuSO_4$ ranging from 0ppb to 36ppb, as described above. Each experimental condition was performed in triplicate to control for batch effects. Bottles were stirred well and left to sit for up to one hour to allow for equilibration before stocking with *C. gigas*. Using the estimated fertilized embryo concentration, tanks were stocked at a density of 15 fertilized eggs ml^{-1}. Next, stocked trial bottles were placed in a Percival Intellus incubator at 25˚C on a 12:12 hour light:dark cycle for all trials, except for the first trial performed in which the temperature was set at 18C. Though the temperature was different for trail 1, the overall patterns of development and movement were not significantly different from later trials, so data was included in subsequent analysis (Table 1). The total time from the start of spawning to placement in an incubated experimental condition was less than three hours. At 48 hours post fertilization, bottles were randomly selected one at a time, and removed from the incubator to be processed for phenotyping (described below).

Percent survival counts

After the 48 hour mark, the contents of each experimental trial were filtered through a nylon mesh sieve (20 μm) to collect larvae, rinsed with FSW, and then concentrated into approximately 30 mL FSW in separate 50 mL conical tubes. Concentrated larvae samples were gently bubbled and four aliquots were counted on a Sedgewick rafter to estimate a remaining larvae count for each experimental condition. Final counts were compared to initial egg and fertilization stocking concentrations to estimate percent survival.

Movement phenotyping assays

At the 48 hour mark, after performing the percent survival counts, a 2 ml movement assay solution was prepared by mixing room temperature FSW and larvae isolates at appropriate volumes to achieve a concentration of 250 animals ml^{-1}. (Video assays were not performed in the cases where significant die-offs made it infeasible to collect a total of 500 animals. These populations were considered 'crashed'). Movement assay solutions were then mixed in a single well of a 3x2 Falcon Polystyrene Microplate, which was placed under an Olympus SZ-PT dissecting microscope with Techniquip 150W Fiber Optic Illuminator at 20x total magnification for recording. Consistency between trials, and avoidance of capturing 'petri-edge movement effects', was achieved by measuring and marking the center point of the dish and focusing the camera on that point consistently among all trials. Thus, the edge of the petri dish was not in view during video assays, and larvae could swim in and out of frame during any given assay. This magnification strategy was chosen based on MovTrack software feasibility control studies, described below. Temperature was recorded for the first two trials using an Onset Hobo thermocoupler K-Type device. Temperature fluctuations within control trials were not found to fluctuate more than 0.15C, and so were disregarded as a covariate.

Videos were recorded using a Canon Rebel Ti5 camera with 1920x1080 pixel resolution at 29 frames per second (fps). A single five-minute video was recorded for each replicate.

Morphology & percent normal development

In addition to movement, we recorded other phenotypes common to metallotoxicity literature, including morphology and percent normal development. After video recording, remaining concentrated larval samples were used to determine average width using an EpiScope Microscope at 40x total magnification. Ethanol was added to aliquots from larval concentrates to reduce motility, and a series of still images were taken and measured using the built in Olympus DP2-BSW XV Imaging Processing Software. A single width measurement was conducted on surviving larvae by using the Arbitrary Line Measurement Tool, which gives a measurement in micrometers, and spanning the longest possible axis for each individual larvae. A target of 50 total measurements were taken per assay replicate on the first 50 animals encountered, and was achieved for each replicate unless otherwise noted in table 1. To calculate proportion normal development, the same photos were used, and at least 50 larvae per experimental trial were noted as either normally or abnormally developed, based on shape (roundness, presence of velum, D-hinge, etc.). Ratios were calculated by dividing normal by abnormal counts for each replicate, and were log transformed, for linear regression.

Movtrack software

To track the movement of bivalve larvae, we used MovTrack, a tool developed in our lab to allow for high-throughput analysis of behavior from video-recorded organisms. MovTrack is implemented in Matlab, and produces summaries of animal movement from video input. MovTrack allows for the adjustment of settings such as luminance thresholding and frame-rate resolution because different experimental setups will produce videos that vary in such features.

A brief description of optimization of this software for this study follows. Because MovTrack was originally developed for land based assays to track fruit flies, *Drosophila spp,* the software had to be tested for aquatic applications. In order to do this, California mussels, *Mytilus californianus,* were spawned out using common "heat shock" methods. Larvae were reared using standard bivalve hatchery techniques essentially similar to the ones described herein, then concentrated and counted at the 48-hour mark, to a final concentration of 500 animals ml^{-1}. We optimized for volume and magnification and found that a volume of 2 ml of this concentrate was sufficient to cover the sides of the petri well in our 3x2 Falcon plates and provide adequate volume for larvae to swim and not be 'edge-kept' by the meniscus of the water body. We also determined that the best magnification range for data was between 12.5 and 20x magnification, in

terms of minimizing within-video movement variance across time. During this period, we optimized the cutoff threshold for object detection by eye, at 40 intensity units (based on the 8-bit grayscale intensity spectrum 0-255). Finally, we also optimized the frame sub-sampling rate, and found manually that a rate of one frame per second allowed for efficient detection of real movement (instead of noise).

To test the sensitivity of the software to fluctuations in larval movement activity, the authors then performed a video assay using *M. californianus* 48 hour larvae at 12.5x magnification using a starting (control) concentration of 500 animals ml^{-1} in 2 ml total FSW, and serially diluting the assay solution at 5, 10, 15, 25, 50 and 75%. From these control assays, it was clear that MovTrack was detecting movement profiles declining at the same rate as the dilution scheme, and that a target of 250 total animal's ml^{-1} would be sufficient for assays herein.

Statistical analysis

Statistical analysis was performed using the built in linear model functions in R i386 3.3.1, using the average of size, percent survival, or movement as response variables [35]. Cu parts per billion or dummy variables assigned to replicate number was used as explanatory variables. Size correction for total movement was performed by employing equation 1:

$$M_{Ai} = \frac{\sum(M_{ri})}{M_{ni}} \Bigg/ \left[\frac{\sum(S_{ri})}{S_{ni}}\right]^2 \quad (1)$$

Where $\boldsymbol{M_{Ai}}$ is the adjusted movement for trial '***i***', M_{ri} is the raw movement per second output (measured in pixels) from MovTrack software for trial '***i***', $\boldsymbol{M_{ni}}$ is the total count '***n***' of $\boldsymbol{M_r}$ for trial '***i***', $\boldsymbol{S_{ri}}$ is the raw size measurement (width, measured in microns) for trial '***i***', and $\boldsymbol{S_{ni}}$ is the total count '***n***' of size measurements for trial '***i***'. Thus, the output is in units of average pixel change per square micrometer (pix/uM^2), or in other words how much a given volume of *C. gigas* had moved per assay.

In order to estimate LC_{50} at the 48 hour mark, we standardized survivorship estimates by first generating a proportion of survival at each concentration compared to the control concentration (0 ppb) for that cohort. Because the survivorship curve was nonlinear, we log transformed these proportion survival counts, and performed a linear regression of log survival for all replicates across changes in Cu^{2+} concentration. The LC_{50} is the point at which the predicted survivorship is ln (2)=0.69 lower than the intercept. Our estimated LC_{50} using this method was at 10.66 ppb.

RESULTS

Full-sibling family generation

Each of the 7 *C. gigas* families produced underwent some scenario of increasing Cu^{2+} toxicity exposure, as is described in methods. Percentages for families achieving D-hinge stages, surviving but not reaching D-hinge, and populations 'crashed' are shown in figure (1C).

Survival, LC_{50} estimates, growth, and percent normal development

Cu^{2+} greatly influenced the chances of a population 'crashing' in experimental conditions, and severely delayed or impeded normal development to the stages observed here. Figure (1A) shows a common pattern seen under the microscope. Control

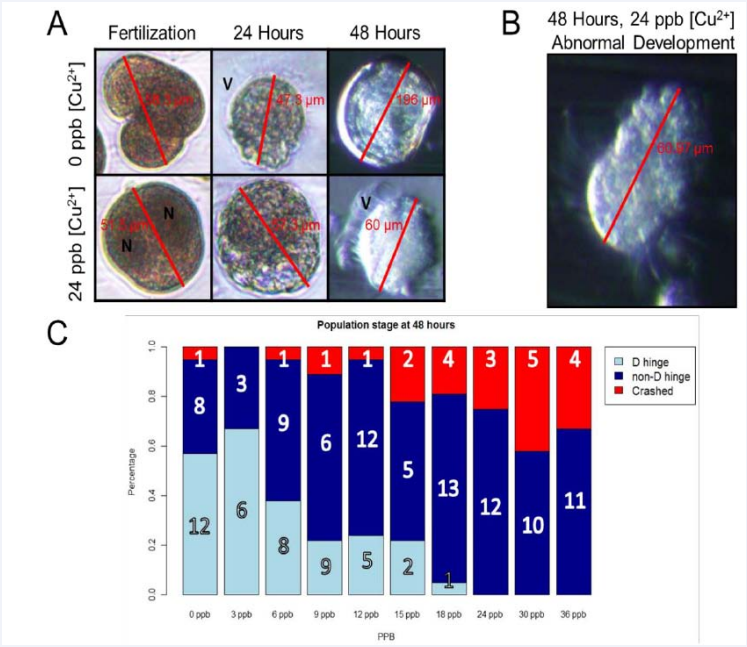

Figure 1 Cu^{2+} Effects on the Development of *C. gigas* Larvae: A) Representative photos of Cu^{2+} trials for *C. gigas* larvae. Rows demonstrate an increase in Cu^{2+} load, while the columns give 24 hour time points. The red lines indicate size. V = velum organ, N = Cell Nuclei. B) Representative photo for abnormal development. Compare to furthest right column in A. Notice strange structures, aberrant cells, and an elongated body frame. Red line represents size. C) Population stages as a function of Cu^{2+} ppb, given as a percentage of total trials. Numbers in white are total replicates (*n*) in a given category for that particular stage.

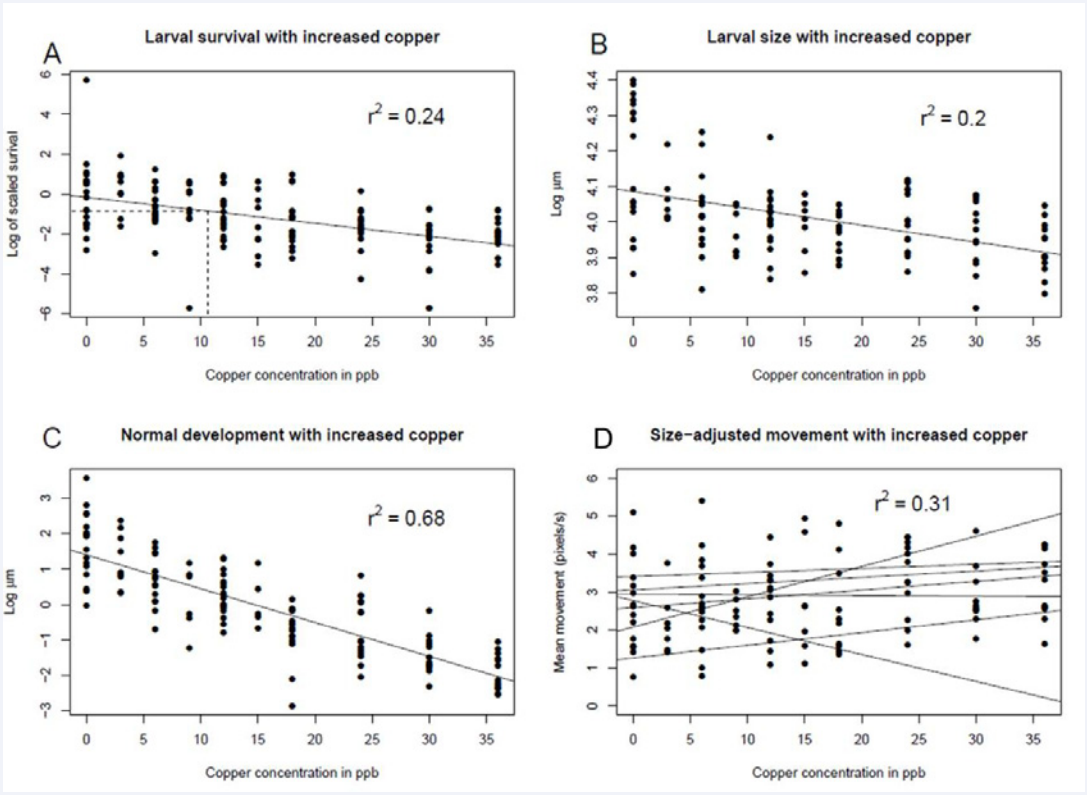

Figure 2 Percent Survival, Lethal LC_{50} Estimates, Size Effects, Percent Normal Development, and Size-adjusted Movement for *C. gigas* Larvae Exposed to Cu^{2+}": All graphs here are data taken at the 48 hour post fertilization mark. A) Percent survival across all trials as a function of increasing Cu^{2+} parts per billion. Linear model: est -0.065, t=-6.86, adj r^2 = 0.240, P=1.9e-10. LC_{50} is estimated from this linear model at 10.66 ppb. B) Average size effects for *C. gigas* larvae exposed to Cu^{2+}. Each point represents an average width, in micrometers, taken from n>50 measurements from a replicate of the condition described on the x-axis in ppb Cu^{2+}. Linear model: est -0.005, t=-5.63, adj r^2 = 0.193, P=1.1e-7 C) Abnormal to normal development ratios for *C. gigas* larvae exposed to Cu^{2+}. Note that abnormal development begins to skew at values significantly less than the estimated LC_{50} of 10.66 ppbestimated in A, at approximately 6 ppb Cu^{2+} here. Linear model: est -0.095, t=-16.17, adj r^2= 0.678, P=2.2e-16. D) Movement as a function of increased Cu^{2+} load. All movement data together (black circles) with increasing copper concentration was significant (Linear model: est: 0.0186, t = 2.19, r^2=0.03396 P=0.0307), however models that considering family effects and other variables were not significant. Each line represents a linear model for genetically distinct families (cohorts) generated in these experiments. Note the different responses (slopes) among the family lines.

populations exhibited a round shape and developed velum through to the D-hinge stage, often reached by the 48 hour mark. Experimental condition larvae often showed slower, abnormal development, with many obvious cellular division disruptions. Higher Cu^{2+} concentration populations showed many abnormally developed larvae that were evidently living and swimming, but which rarely or never achieved D-hinge morphology at 48 hours (Figure 1B).

Survival counts across all trials showed a highly significant decline with increasing Cu^{2+} load (Figure 2A, est -0.065, t=-6.86, adj r^2 = 0.240, P=1.9e-10). Considered individually, all families displayed similar average survivorship curves to the collective data: increasing Cu^{2+} negatively affects survival, sometimes significantly among a family (Table 1, Supplemental Table 1). The LC_{50} dose across all populations was determined to be approximately 10.66 ppb (Figure 2B).

Average size at the 48 hour post fertilization mark was negatively correlated with increasing Cu^{2+} concentration (Figure 2B, est -0.005, t=-5.63, adj r^2 = 0.193, P=1.1e-7). Within family, growth rates were sometimes not statistically significant, though trends were always toward decreasing size (Table 1, Figure 2C).

There was a pronounced decrease in the ratio of normally developed larvae with increasing Cu^{2+} concentrations (Figure 2C, est -0.095, t=-16.17, adj r^2= 0.678, P=2.2e-16).

Larval swimming behavior

Larval swimming behavior responded differently depending on which group was tested. Five out of the seven families tested returned a positive slope when fitted with a linear model, while the other two returned negative slope values. When all of the movement data is considered, normalized for size, and fitted with a movement-by-parts per billion Cu^{2+} linear model, a significant result is found (Linear model: est=0.0186, df=107, t=2.19, P= 0.0307). However, increasing the complexity of the models to include any combination of the variables family, percent normal development, and survival - and their interactions - reduced or eliminated significant correlations between swimming behavior and increasing Cu^{2+} concentrations. A Bayesian Information Criteria (BIC) test across all possible models showed that family by movement was the simplest model to explain swimming behavior. Put differently, in our tests Cu^{2+} concentration is not

a reliable predictor of overall movement, and there may be a familial component of Cu response in larval swimming behavior at 48 hours.

DISCUSSION AND FUTURE DIRECTIONS

Copper effects on morphology

The data presented here is consistent with previous literature in regards to copper effects on normal development, percent survival, and average size [1,3,14-30]. Copper likely disrupts cellular pathways, leading to very strange and retarded developmental patterns, as seen in figure (1). We calculated an LC_{50} of 10.66 ppb, which is within the range of previous literature [3,27-31]. This number is, however, very likely higher than a total lifespan estimate of LC_{50} as the assays herein terminated at the 48 hour mark, and many families were considered 'crashed' and so were not able to contribute to LC_{50} calculations (9 total families at or under 10% survival; 22 total families without sufficient animals to record videos). If an attempt was made to rear the *C. gigas* families to full term (i.e. to the juvenile/adult stages), the LC_{50} would likely shift toward the lower end of the parts per billion spectra tested here. It is likely that animals that survived to the 48 hour mark in this study in conditions less than our estimated LC_{50} value (<10.66 ppb) were still significantly developmentally delayed or completely stunted and would exhibit increased mortality rates at later larval stages than controls. This hypothesis is supported by figure (1C), which show an obvious impact on development beginning at 3-6 ppb Cu^{2+} in terms of stocks 'crashed', and by figure (2C), which demonstrates that abnormal development is more frequently documented than normal development starting at approximately 6-9 ppb. Put succinctly, this study focused on a narrow portion of the *C. gigas* life stage, which is the case in most (if not all) Cu^{2+} toxicity literature, leaving little room for accurately predicting long term trends for exposed populations. Future studies should therefore consider extending the rearing process to later time-points to fully understand Cu^{2+} induced developmental and environmental defects.

Movement effects

BIC tests showed that cohort alone, a proxy for genetically distinct families, was the best model to explain effects on movement profiles recorded in this study, and that Cu^{2+} concentration was not a good predictor of larval movement after considering family effects. One possible explanation for this result is that families respond differently to Cu^{2+} toxicity due to genetic heritage. Families produced in this study were isogenic, and no gametic contamination was observed or expected for any one cohort. Lineage based responses for this study are conjecture, because ambient seawater conditions were not tested prior to trials, and it is not possible to control for even gamete maturity across individual oysters. However, it is clear that cohorts responded differently to copper exposure, when each of the slopes are observed for individual family response (Figure 2D). In 5 out of 7 trials, cohorts responded with slightly-to-heavily increased movement as copper concentration increased, while 2 of 7 showed significantly negative reduction in movement (Figure 2D, Table 1). This result may also be a function of differing baseline movement among cohorts, which again points to a possible genetic influence in general movement. Decoupling of genetic influence on general movement versus that caused by Cu^{2+} concentration is not possible with this data set. However, 6/7 trials showed changes in movement from control trials, suggesting that Cu^{2+} is the main factor mediating these trends.

As larvae, *C. gigas* do not have shells until later stages, and so may employ swimming to stressful environmental stimuli. For example, bivalves are known to exhibit chemically mediated behavioral responses including predator avoidance and settling strategies [36-38], and respond behaviorally to other environmental conditions such as wave action during larval stages [39]. The fact that family, and not Cu^{2+} concentration, was the simplest model to explain the movement data was therefore somewhat surprising, especially considering the extensive literature documenting Cu^{2+} toxicity on physiology. It is possible that Cu^{2+} does not affect the development of the velum's ciliation or activity of the cilia, and is therefore not affecting the movement profiles evenly across all families tested here. One factor that may be different among Cu^{2+} exposed groups is the direction of swimming motion, as abnormally developed larvae seem to swim in more erratic patterns. This may be a function of hydrodynamics due to strange body shape, but direction of swimming motion remains quantitatively unverified here. More trials across different groups of Pacific oysters, as well as more granular analysis of movement profiles involving direction and angular velocity, would perhaps demonstrate differences in swimming behavior at increased Cu^{2+} concentrations.

MovTrack software application

One of the objectives of this work was to apply in-house developed quantitative imaging software (MovTrack) in a novel aquatic setting. These experiments were successful in implementing MovTrack, which the authors believe has much potential for further application movie quantitation experiments. For future users of MovTrack, a few parameters which need to be considered depending on the experiment are discussed here. First, the video frames are subsampled at set intervals, by an amount that the user has specified. A short sampling interval will be appropriate if animals are moving rapidly, while a longer interval risks reaching saturation (i.e. all animals will move at a greater-than-body-length in each interval). If animals are moving slowly, however, a longer interval is more appropriate, as it will reduce noise (for instance from light fluctuation, or minor jitter). In our study, we found that we were able to detect sufficient movement changes among treatments using a resolution of 1 frame per second.

The difference in pixel intensity between pairs of consecutive sampled frames is then calculated as the "difference frame". This difference frame is the measure of change between time points. The difference frame is then transformed to an 8-bit gray-scale image, and thresholded to filter out minor changes in frames not caused by movement of organisms (such as light fluctuations, or minor changes in non-focal features). It is best to maximize difference in light intensity between foreground (target) and background, within the experimental setup, to facilitate threshold selection. We applied a threshold of 40 (based on the

8-bit grayscale intensity spectrum 0-255).

The threshold is then applied to the pixel intensities of the grayscale image, to convert grayscale to binary "different" or "not different" bins. The measure of movement between two consecutive frames is then calculated by summing over all the pixels in the thresholded difference frame. By repeating this calculation across the video, the user produces a time series estimate of movement. Considering these parameters, essentially any video that is standardized for camera position and contains movement at least one pixel large can be quantified. MovTrack has many other applications, including path tracking of individuals, which will be described in future publications (Abbasi et al., in prep).

Implications

Bivalves employ a non- or partially-lecithotrophic free swimming trochophore and veliger life history. These organisms may not be able to decouple swimming and feeding during these early stages, as the velum functions as both a swimming and feeding organ. If *C. gigas* larvae stop swimming, it is unclear if they are able to continue feeding effectively. Adult bivalves are known to filter particulate matter in their gills and eject unwanted material as pseudofeces, but whether or not larvae are capable of this behavior is also unknown [37]. The fact that movement was not shown here to be affected similarly across all trials during Cu^{2+} stress, but survival and percent normal development all showed similar trajectories, indicates that feeding behavior (and therefore increased or decreased access to nutrients) is likely not a factor in the mode of lethality for Cu^{2+} toxicity. Thus, our hypothesis that a negatively synergistic effect of cellular responses and altered behavior (and therefore access to nutrients) is accountable for previously observed physiological changes, rather than cellular responses alone, is not supported here.

Though our original hypothesis was not supported, genetically mediated response to Cu^{2+} load may have been observed in this study. Clear differences in growth curves, percent survival, and developmental delay were evident between the families (though all showed similar directional trends). Swimming behavior was different among families, with some showing a generally increased profile with increasing Cu^{2+} concentrations, and others showing a decrease (Figure 2D). If cohort level differences in swimming behavior belie genetically mediated cellular level response to Cu^{2+} toxicity, there are implications for aquaculture selective breeding efforts and ecological toxicity responses.

Field studies may be able to confirm genetically based differences in toxicity response; sites with high Cu loads which continue to support adult *C. gigas* and other bivalves may reveal metallo-tolerant genotypes and phenotypes. The *C. gigas* used for this study were sourced from the Pacific Northwest and planted at the Agua Hediona site by Carlsbad Aquafarms approximately 25 years ago, with some subsequent broodstock supplementation since. More research into Cu^{2+} hydro-geo-bio dynamics of Agua Hidiona would be required before comparative analysis would be possible for environmental selection parameters. On the other hand, the Agua Hidiona population presents a stock that has almost certainly been selected for higher temperature tolerance, considering this population is one of the southern-most sites at which *C. gigas* is commercially harvested in the United States. Comparisons between this population and *C. gigas* grown in the US Pacific Northwest and Canadian West coast may provide an interesting comparative group in both metallotoxicity, warming earth scenarios, and any additive effects of these phenomenon.

CONCLUSION

This work reaffirms the rich body of scientific literature demonstrating negative Cu^{2+} toxicity effects in bivalves, and adds a new behavioral phenotyping strategy via use of MovTrack software. Future work in this area would benefit from a focus on full term rearing of exposed larvae, understanding the effects of multi-generational exposure to large Cu^{2+} load (whether reared in a hatchery or observed in the wild), and testing of more Cu^{2+} sensitive species.

ACKNOWLEDGEMENTS

This work was supported by USC's Research Experience for Undergraduates program at the Wrigley Institute for Environmental Studies, and the Waitt Foundation. Many thanks to Prof. Sergey Nuzhdin for mentorship on this project; Helen Foley for feedback on statistical models; our lab technician Dave Anderson, MS; Dr. Nadya Struppul; Thomas Grimm, Carlsbad Aquafarms CEO and President for *C. gigas* broodstock; and the Wrigley Institute for Environmental Studies staff for facilities support.

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Cite this article

Hall M, Foley B, Cheung E, Abbasi M, Churches ND (2016) A New Behavioral Phenotyping Strategy for Pacific Oyster (Crassostrea gigas) Larvae Reveals Cohort-Level Effects on Copper Toxicity Swimming Response. Ann Aquac Res 3(3): 1025.