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Consistency of morphological endpoints used to assess developmental timing in zebrafish (*Danio rerio*) across a temperature gradient

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ABSTRACT

Zebrafish (*Danio rerio*) are model organisms for testing developmental toxicity at the morphological level. In this study, influence of temperature (24.5–28.5° C) and silver nanoparticles on developmental staging, ear–eye distance, and ratio of ear–eye distance to inner ear diameter was investigated. As temperature decreased, all endpoints showed developmental delay, with differences between endpoints in amount and type of delay measured. Differences in developmental delay patterns were observed, with rate delays increasing over time when staging endpoints were utilized and rates remaining constant when using ear–eye measurements. Integrated predictive equations were created to normalize each endpoint for temperature. Influence of image rotation on ear–eye distance accuracy showed that more than 75% eye overlap during analysis is necessary to minimize error. Exposure to silver nanoparticles demonstrated a lack of consistency between developmental endpoints and highlighted the usefulness of a multi-endpoint approach when measuring changes to developmental timing.

1. Introduction

Zebrafish (*Danio rerio*) have long been used as a model organism for testing developmental toxicity at the morphological level. Ease of culture, prolific breeding, and rapid rate of development all contribute to the attractiveness of zebrafish as a vertebrate model. In addition, transparent embryos allow in vivo assessment of morphology [1,2] and developmental measurements such as head-trunk angle [3], ear–eye distance (EED) [4], and ratio of ear–eye distance to inner ear diameter (EED/IED) [5,6]. Environmental toxicity tests developed by organizations such as the Organisation for Economic Co-Operation and Development employ zebrafish in a variety of testing scenarios [7–9]. In particular, the use of zebrafish embryos in the FET (Fish Embryo Toxicity test) [8] has been recommended as a possible alternative to existing acute and life-cycle toxicity tests that require juvenile and adult fish [10,11]. Zebrafish have been utilized to investigate the developmental effects of various substances, including metals [12,13], nanoparticles [14,15], uranium [16], and endotoxins [17]. Furthermore, recent work by Padilla et al. [18] used zebrafish embryos to assess toxicity of more than 309 chemicals from the EPA's ToxCast™ chemical library.

Despite the large body of research utilizing zebrafish as a model system, there remains considerable variation in testing methods with respect to variables such as temperature, measurement tools, and vital timepoints [5,6,12,19–27]. One fundamental concern when utilizing exothermic animals is environmental temperature during exposure [28]. Developmental rate in zebrafish is directly affected by environmental temperature, with development slowing at lower temperatures [1,2,29–31]. These differences in developmental rate can become problematic if data is directly compared to other studies without first normalizing for temperature. To date, only one normalization technique for temperature variation exists and is limited to developmental staging endpoints [1].

Comparison of zebrafish morphology via developmental stage has been a well-utilized endpoint to measure effects on rate of development [1,2,5,29]. Though less common, EED and EED/IED have been used as alternatives to compare developmental rates [4–6] and offer the advantage of reduced subjectivity, since these endpoints are based on a quantifiable measurement rather than an observer's determination of morphological characteristics. However, one potential concern with the use of EED and EED/IED is the consistency of measurement; length, a 2-dimensional endpoint, is measured from a 3-dimensional image and could be considerably influenced by embryo position with respect to the image angle captured.

In order to determine consistency among developmental endpoints in this study, zebrafish embryos were exposed to silver nanoparticles. Silver nanoparticles are one of the most studied nanomaterials due to extensive commercial use [32,33]. The antimicrobial properties of silver nanoparticles are frequently employed in water purification systems, cosmetics,

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medicinal products and many consumer products, including washing machines and food containers [34]. Previous research has shown that silver nanoparticles cause impaired behavior [35,36], teratogenicity [4] and toxicity [37] in zebrafish. Silver nanoparticles have also been shown to penetrate the chorion of developing zebrafish embryos, leading to circulatory and morphological abnormalities [38].

In this study, we investigated the consistency of several common morphological endpoints of zebrafish development across a temperature gradient within their thermal tolerance range (24.5–28.5 °C) [1,28]. Additionally, we attempted to demonstrate the consistency and usefulness of each measurement endpoint by exposing fish to a potentially teratogenic compound, silver nanoparticles, and directly comparing the results. The specific objectives of this study were to (1) determine the influence of temperature on classic developmental endpoints (developmental stage, EED, EED/IED); (2) develop integrated predictive models for developmental staging, EED and EED/IED that take into account environmental temperature; (3) determine the influence of image position on EED and EED/IED measurements; (4) demonstrate the potential usefulness of conventional developmental endpoints (developmental stage, EED, EED/IED) on zebrafish embryos exposed to a potential teratogen, silver nanoparticles.

2. Materials and methods

2.1. Zebrafish culture

Zebrafish of various phenotypes were obtained from local sources, separated by gender, and cultured in 10-gallon aquaria containing system water. System water consisted of deionized water treated with Instant Ocean at 0.06 g/l, pH 7.0 ± 0.2, and temperature of 28.5 ± 0.5 °C. Fish were kept on a 14:10 h light/dark cycle. Fish were fed twice daily with TetraMin flakes and supplemented with brine shrimp. All experiments were approved by and carried out in accordance with guidelines of the Institutional Animal Care and Use Committee at Middle Tennessee State University.

2.2. Egg collection

Matings were staged twice weekly in separate holding tanks with perforated floors. Embryos were collected, washed with a 0.05% bleach solution, and serially rinsed to eliminate fungal growth. Collected embryos were screened to ensure fertilization and approximate age.

2.3. Time-lapse sequence capture

Prior to filming, 10 cm Petri dishes with a layer of agarose gel and 2 mm hemispherical wells were prepared and covered with system water. Three independent image sequences were captured at each temperature for embryos developing at 24.5 °C, 26.5 °C, 28.5 °C and 28.5 °C +silver nanoparticles. For each sequence, one newly fertilized embryo (<2 h post fertilization, or hpf) was collected using previously described techniques (see Section 2.2) and incubated at the desired temperature (±0.1 °C) in an environmental chamber (Sheldon Manufacturing, Model 2015, Cornelius, OR). Embryos remained incubated and were filmed using an Infinity-1 camera (Lumenera Corporation, Ontario, Canada) mounted to an inverted microscope (Nikon, Eclipse TS100, Japan) at a magnification of 20× and a frame capture rate of 1 frame/min for 36 h. Image sequences were analyzed with NIS-Elements software (Melville, NY, USA).

2.4. Developmental staging analysis

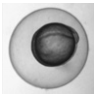
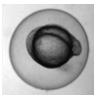
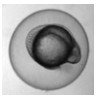
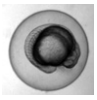
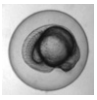
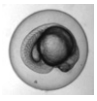
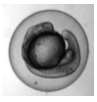
Thirteen morphological milestones were identified based on easily recognizable developmental characteristics (Table 1) between 2 and 30 h post fertilization.

Every image recorded was time-stamped and average time in hpf was determined for each milestone at each temperature. Average time to achieve each milestone at each temperature (mean of three independent image sequences) was used to determine relative rate of development. A slope of 1.0 was assigned to developmental data collected at 28.5 °C and the *y*-intercept assigned a value of zero. Developmental data at 24.5 °C and 26.5 °C were then assigned the same arbitrary developmental stage value (*y*-axis) as the 28.5 °C slope.

2.5. Developmental measurements

Measurements of EED and EED/IED were calculated from 20 to 23 time points in each time-lapse sequence using NIS-Elements software. These time points were chosen randomly from the period beginning with appearance of otic vesicle and sequence end. Linear regression analysis was performed to determine the existence of a significant linear relationship at each temperature for developmental stage, EED, and EED/IED ($\alpha = 0.05$). Analysis of covariance (covariate of time and fixed factor of temperature) was performed to determine if significant differences in developmental stage, EED, and EED/IED existed between zebrafish exposed to different temperatures. For each endpoint a slope comparison was first performed, and if a significant difference was observed ($\alpha = 0.05$) *y*-intercepts were compared for differences ($\alpha = 0.05$). *Y*-intercept analysis was not performed on developmental stage data since the *y*-intercept was forced through the origin of the graph. Linear regression analysis was performed on all silver treatments, as well as comparison

Table 1
Description of morphological milestones.

Milestone	Description	Still image
256 cells	Mass of 256 cells on yolk sac	
Sphere	Cell mass compacts to form a sphere including yolk sac; cell mass appears to have a flat surface at junction with yolk sac	
30% epiboly	Cell mass forms a cap that envelops ~30% of yolk sac	
Shield	Cell mass cap that envelops ~50 of yolk sac	
80% epiboly	Cell mass cap that envelops ~80 of yolk sac	
Budding	Cell mass appears as a ring around yolk sac; head and tail buds emerge at poles of embryo	
4 somites	Four clearly defined somites on trunk when viewed from the side	
10 somites	10 clearly defined somites on trunk when viewed from the side	
13 somites	13 clearly defined somites on trunk when viewed from the side	
Movement	First discernible muscle contraction	
17 somites	17 clearly defined somites on trunk when viewed from the side	
22 somites	22 clearly defined somites on trunk when viewed from the side	
Pigmentation	Appearance of 20 pigment spots on head and trunk