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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.

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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^{TM} 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	

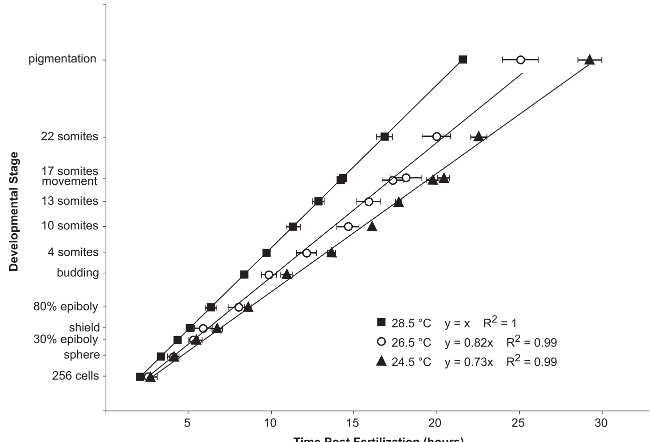

Fig. 1. Time to achievement of morphological milestones. As a baseline rate of development, time to achievement at 28.5 °C was plotted and assigned a slope of 1. $n = 3$ for each milestone at each temperature. Data for each temperature derived from three independent image sequences. Data shown as mean ± SE.

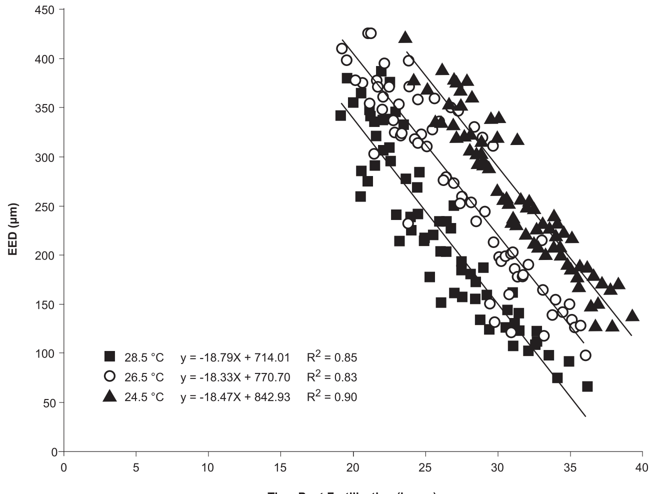

Fig. 2. Ear–eye distance measurement of developing zebrafish. $n = 65$–69 for each temperature; data for each temperature derived from three independent image sequences.

to 28.5 °C data for differences in developmental stage, EED, and EED/IED using an ANCOVA ($\alpha = 0.05$; covariate of time and fixed factor of silver or no silver).

2.6. Predictive equations

Predictive equations for the influence of temperature on developmental stage, EED and EED/IED measurements were calculated using linear regression modeling. For developmental stage calculations the slope differences between each temperature were averaged and normalized to 28.5 °C following methods used by Kimmel et al. [1]. For EED and EED/IED models the slopes of each temperature were averaged. The *y*-intercept for each model was derived by dividing the *y*-intercept of each temperature by the temperature at which the analysis was performed, resulting in a per-degree value. The per-degree values for the three temperatures tested were then averaged. This average per-degree value was added to the original *y*-intercept of 28.5 °C to obtain the final equation.

2.7. Image position analysis

Image position analysis was performed using theoretical modeling to compare the perceived distance to actual distance between the eye and inner ear, while taking into account the rotation of the zebrafish embryo. To determine the rotation in degrees required to obtain a specific amount of eye overlap, we calculated the tangent of a right triangle created by three lines: the midline of the back eye, the line between the eye edge of both eyes, and the line between the front eye edge and eye overlap point. To determine the impact of embryo rotation on the observed distance between the eye and ear as compared to the actual distance, we calculated the cosine of a right triangle created by three lines: the actual ear to eye distance, the perceived ear to eye distance, and the focus distance between the ear and front eye. The hypotenuse distance was arbitrarily set to 1.0.

2.8. Silver nanoparticle characterization and exposure

Silver nanoparticles were provided by nanoComposix (San Diego, CA, USA) and synthesized via methods explained by Park et al. [39]. Briefly, silver nanoparticles were synthesized using an aqueous reduction technique from silver salts to a final concentration of 2 mM in phosphate buffer. Nanoparticle size and shape was determined via transmission electron microscopy by examining 100 random particles. All nanoparticles were approximately spherical in shape and had an average diameter of 53.1 ± 4.1 nm (standard error). All zebrafish nanoparticle exposures occurred in 10 cm Petri dishes at a concentration of 0.5 mg/l and a volume of 10 ml in an environmental chamber (Sheldon Manufacturing, Model 2015, Cornelius, OR) set to 28.5 ± 0.1 °C. All nanoparticle solutions were probe sonicated immediately prior to use to ensure even dispersal of nanoparticles according to Bowman et al. [4].

3. Results

3.1. Effects of temperature

Comparison of developmental stages of zebrafish incubated at 24.5 °C, 26.5 °C, and 28.5 °C revealed a significant developmental delay ($F_{(1,35)} = 28.061$, $p < 0.001$) as temperatures decreased (Fig. 1). The extent of observed delay increased as development progressed with a delay of 2.9 h between 24.5 °C and 26.5 °C, 4.2 h between 26.5 °C and 28.5 °C, and 7.1 h between 24.5 °C and 28.5 °C by the final milestone, pigmentation.

The use of EED (Fig. 2) and EED/IED (Fig. 3) as markers of developmental delay also showed a significant delay in development as temperatures decreased. Statistical analysis showed that for each endpoint and each temperature a significant linear relationship existed between the measured EED or EED/IED distance and time ($p < 0.001$ for each endpoint at each temperature). ANCOVA analysis revealed that the slopes generated by EED and EED/IED data at 24.5 °C, 26.5 °C, and 28.5 °C were not statistically different ($F_{(1,199)} = 0.046$, $p = 0.831$ and $F_{(1,199)} = 0.902$, $p = 0.344$, respectively), but the *y*-intercepts were statistically different ($p < 0.001$ for both EED and EED/IED). Goodness of fit comparisons revealed that across all three temperatures, results from EED measurements were more precise (R^2 between 0.83 and 0.90) than EED/IED measurements (R^2 between 0.78 and 0.87).

3.2. Predictive modeling

Linear regression modeling for the influence of temperature (between temperatures of 24.5 and 28.5 °C) on developmental stage

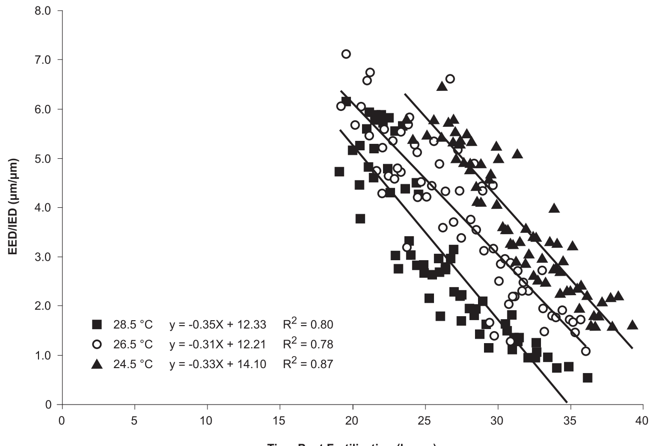

Fig. 3. Ratio of ear–eye distance to inner ear diameter in developing zebrafish. $n = 65$–69 for each temperature; data for each temperature derived from three independent image sequences.

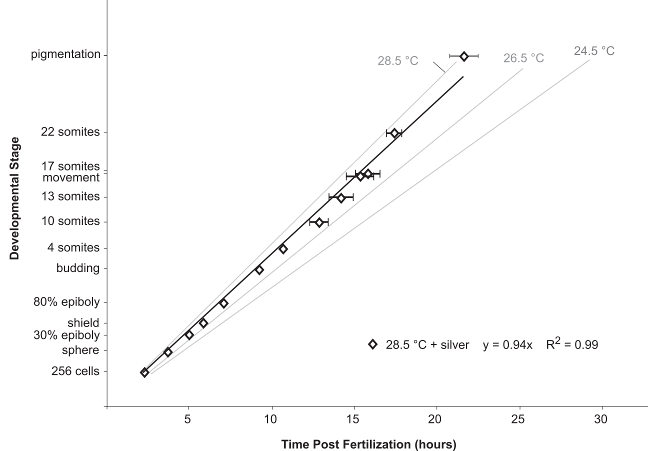

Fig. 4. Time to achievement of milestones after treatment with silver nanoparticles. Embryos were exposed to silver nanoparticles at 28.5 °C. $n = 3$ for each milestone. Data for each temperature derived from three independent image sequences. Data shown as mean ± SE.

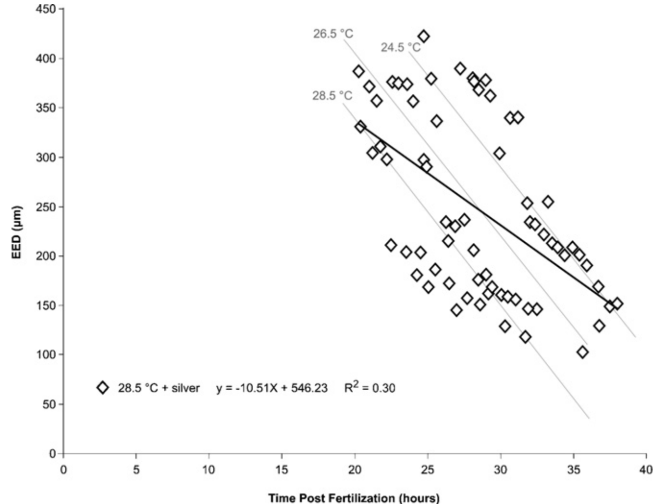

Fig. 5. Ear–eye distance in silver nanoparticle treated embryos. Embryos were exposed to silver nanoparticles at 28.5 °C. $n = 66$; data derived from three independent image sequences.

resulted in the following equation $H_T = h/(0.0675T - 0.92375)$; where H_T = hours of development at temperature T (°C), and h hours of development to reach the stage at 28.5 °C. The influence of temperature on EED resulted in the following predictive equation: EED distance = $-18.53x + (((28.5 - T) \times 32.23) + 714)$. Likewise, the influence of temperature on EED/IED resulted in the following equation: EED/IED = $-0.329x + (((28.5 - T) \times 0.43125) + 12.332)$. For both EED and EED/IED equations T = temperature (°C) and x = hours post fertilization.

3.3. Image position analysis

Theoretical image position analysis to determine the effect of body rotation on accuracy of EED and EED/IED measurements revealed that rotation of 14° produces a 75% eye overlap if the image is viewed from the side, resulting in a discrepancy of approximately 3% in observed ear–eye distance compared to actual distance (Fig. 7). Likewise, a rotation of 26.75° produces a 50% eye overlap if the image is viewed from the side, resulting in a discrepancy of approximately 11% in observed ear–eye distance compared to actual distance.

3.4. Effects of silver nanoparticles

Significant linear relationships were observed for silver exposed zebrafish for developmental stage ($p < 0.001$; $R^2 = 0.99$) (Fig. 4), EED ($p < 0.001$; $R^2 = 0.30$) (Fig. 5), and EED/IED ($p < 0.001$; $R^2 = 0.34$) (Fig. 6). When comparing data for silver exposed embryos at 28.5 °C to non-silver exposed embryos at 28.5 °C using developmental stage no differences in slopes were observed ($F_{(1,22)} = 0.127$, $p = 0.911$). When this comparison was analyzed using EED and EED/IED significant differences in slopes were observed ($F_{(1,128)} = 12.840$, $p < 0.001$ and $F_{(1,128)} = 15.774$, $p < 0.001$, respectively).

4. Discussion

Zebrafish studies have been conducted at a wide variety of temperatures. Although recommended temperatures for zebrafish culture range from 26 to 28.5 °C [1,9,40], even a cursory review of literature reveals common experimental temperatures ranging from 24 °C to 29 °C [5–7,12,13,41]. This inconsistency clearly presents an issue in cross-temperature comparison of data unless normalized for temperature, since rate and efficacy of development is directly affected by environmental temperature. Previous studies investigating zebrafish development have shown that overall developmental rate slows or accelerates based solely on environmental temperature [1,2,30,31]; at temperatures just outside that range, abnormalities are more common [1].

Developmental staging endpoints are typically used as a straightforward means to measure and compare developing zebrafish. Schirone and Gross [2] identified 16 milestones for use in tracking developmental progress at different temperatures, while Kimmel et al. [1] identified 18. In the present study, thirteen morphological milestones were chosen to represent developmental stages in zebrafish embryogenesis (Table 1). Tracking time to achievement of each milestone showed that a temperature reduction of just 4 °C (24.5–28.5 °C) resulted in a delay of 7.1 h by the final milestone (Fig. 1). With increasing time, the divergence between times to achievement of each milestone appeared to increase as well. Comparison of developmental timing at 24.5 °C to 28.5 °C showed a 1.9-h delay in development by bud stage, which widened to a 7.1-h delay by pigmentation stage. Development appeared to occur correctly, regardless of the slower pace.

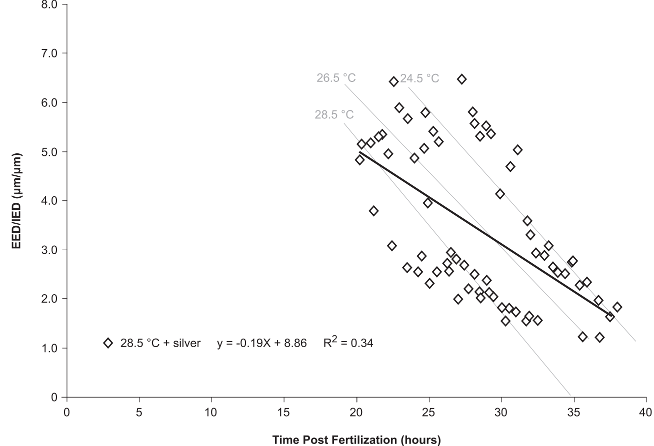

Fig. 6. Ratio of ear–eye distance to inner ear diameter in silver nanoparticle treated embryos. Embryos were exposed to silver nanoparticles at 28.5 °C. $n = 66$; data derived from three independent image sequences.

Table 2
Comparative zebrafish development time (h).

	Stage	[a,b]Kimmel et al. [1]	[c]Schirone and Gross [2]	[g]Present Study
[d]24.5 °C	Sphere	5.5	–	4.1 ± 0.3
	30% epiboly	–	6.8	5.5 ± 0.4
	Shield	7.7	7.3	6.7 ± 0.3
	80% epiboly	10.3	9.0	8.6 ± 0.4
	Bud	12.9	–	10.9 ± 0.2
	13 somites	20.6	–	17.7 ± 0.2
[e]26.5 °C	Sphere	4.8	–	4.1 ± 0.4
	30% epiboly	–	5.8	5.3 ± 0.3
	Shield	6.8	6.3	5.9 ± 0.8
	80% epiboly	9.0	8.3	8.0 ± 0.5
	Bud	11.3	10.0	9.8 ± 0.6
	13 somites	18.0	–	15.9 ± 0.7
[f]28.5 °C	Sphere	4.3	–	3.8 ± 0.4
	30% epiboly	–	5.5	4.7 ± 0.2
	Shield	6.0	6.0	5.4 ± 0.3
	80% epiboly	8.0	7.8	7.0 ± 0.4
	Bud	10.0	9.1	9.0 ± 0.5
	13 somites	16.0	–	13.3 ± 0.7

[a] Stage equivalents from Kimmel et al. (1995): sphere = dome; 80%; epiboly = 75% epiboly; 13 somites = 14 somites.
[b] Data presented for 24.5 and 26.5 °C were derived from the equation described in Kimmel et al.: $H_T = h/(0.055T - 0.57)$ where H_T = hours of development at temperature T, and h = hours of development to reach the stage at 28.5 °C.
[c] Stage equivalents used from Schirone: 30% epiboly = blastoderm 1/3 yolk sphere; Shield = blastoderm 1/2 yolk sphere; 80% epiboly = blastoderm 3/4 yolk sphere; Bud = closure of the blastopore.
[d] Data from Schirone and Gross collected at 24.0 °C.
[e] Data from Schirone and Gross collected at 26.0 °C.
[f] Data from Schirone and Gross collected at 28.0 °C.
[g] All values are presented as mean ± standard error. Dashed lines indicate no comparable data.

A comparison of rates of temperature-influenced development in zebrafish showed a considerable amount of variation between the present study, Kimmel et al. [1], and Schirone and Gross [2] (Table 2). The variation between these data sets is difficult to interpret since no amount of error surrounding the data collected from Kimmel et al. and Schirone and Gross was determined. However, the same general pattern exists within each dataset and shows that developmental rate varies as a linear function of incubation temperature. To date, only one study has provided a normalization equation to account for temperature variability in developing zebrafish [1]. This equation was developed to estimate when embryos developing at temperatures between 25 °C and 33 °C will reach a specified developmental stage. Kimmel et al. provided the equation $H_T = h/(0.055T - 0.57)$; where H_T = hours of development at temperature T, and h = hours of development to reach the stage at 28.5 °C. When data from the present study was used to develop a normalization equation in the same manner as Kimmel et al. a similar, yet different equation was calculated ($H_T = h/(0.0675T - 0.92375)$. The differences between these two predictive normalization equations are difficult to interpret since no amount of variability in the data used by Kimmel et al. was determined. It is important to note that these two equations were developed using data that spanned different temperature ranges (present study = 24.5–28.5 °C; Kimmel et al. = 25–33 °C), which could, in part, explain the differences observed. For both equations, the linear nature of the data would suggest possible extrapolation to temperatures outside of the range used to determine the equation; however, further study is recommended to verify accuracy at extended temperatures.

Another approach to quantitatively assess developmental timing is the measurement of EED and EED/IED [1,2,4]. In a previous study by Walpita et al. [5], EED/IED was used to show that increased thyroid hormone (T_3) availability in developing zebrafish embryos accelerated development. In the present study investigating the effects of temperature, measurements across the 24.5–28.5 °C range yielded similar results to those using morphological milestones; although development appears normal, significant delays were observed at lower temperatures. In contrast to morphological data, the rate of development at 24.5 °C, 26.5 °C, and 28.5 °C as measured by EED (Fig. 2) and EED/IED (Fig. 3) appeared to be constant. Slopes at all three temperatures were not significantly different, while the starting point of each line shifted as temperature was lowered. EED appeared to be a more reliable endpoint than EED/IED, based on the goodness of fit across the three temperatures tested. To date, this is the first study to investigate the effects of temperature on the quantifiable endpoints EED and EED/IED.

Previous research has shown that silver nanoparticles cause deleterious effects in zebrafish [35–37,42]. Bowman et al. [4] observed delays in hatching, slower heart rates, and differences in EED measurement in zebrafish exposed to silver nanoparticles when compared to controls, which indicated a potential delay in overall rate of development. We therefore used silver nanoparticles similar to those used by Bowman et al. in shape, diameter, and concentration to demonstrate the relative efficacy of the endpoints measured in this study.

Silver nanoparticles did not cause a significant developmental delay in zebrafish when using developmental staging as an endpoint (ANCOVA, $p < 0.001$) (Fig. 4). However, when using EED (Fig. 5) and EED/IED (Fig. 6) to measure developmental timing, significant differences were observed. Consider developmental staging; exposure of zebrafish to silver nanoparticles occurred at 28.5 °C. Therefore, had silver exposure caused a significant delay, a shift away from the 28.5 °C line would have been observed. When EED data for embryos exposed to silver was layered over EED data for embryos exposed to lower temperatures (Fig. 5) variability was apparent. This same pattern was observed for EED/IED data (Fig. 6). Comparison of results among these endpoints (developmental stage, EED, and EED/IED) revealed an inconsistency. If silver nanoparticles' effect on developmental rate had been assessed only by examination of developmental staging then no effects would have been detected. This interpretation is vastly different than an assessment utilizing EED and/or EED/IED as the measurement tool for developmental rate. It is possible that the effect of silver nanoparticles on developing zebrafish may not occur until late

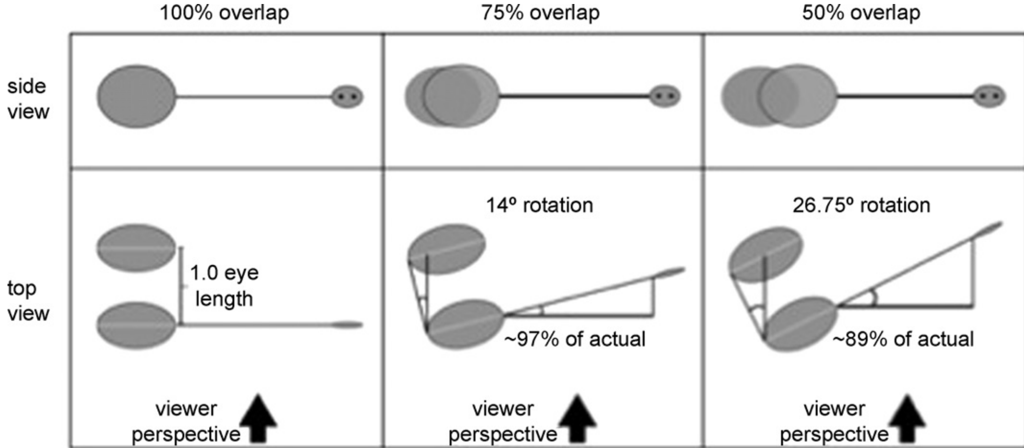

Fig. 7. Influence of image position on measurement of EED and EED/IED.

development, past such time as can be accurately measured using developmental staging. It is also possible that silver nanoparticles directly affect specific periods of development that overlap measurement endpoints of EED and EED/IED. Results from the present study should be used with caution when describing effects of silver nanoparticles on zebrafish development, since use in this study was focused solely on demonstrating the variability that exists between endpoints. However, further investigations into the effects of silver nanoparticles on zebrafish development are warranted and should include testing multiple nanoparticle sizes while measuring both development endpoints and biomarkers of exposures and/or effects.

The use of a quantitative measure of developmental delay, such as EED or EED/IED, can mitigate the subjectivity inherent in methods that rely on observation, such as developmental staging. However, the use of such a measurement must be reliable, and given that this is a 2-dimensional measurement on a 3-dimensional image, certain limitations exist that must be addressed. Image position of a developing zebrafish is vital to achieve consistent measurements. The transparency of the zebrafish embryo allows visualization of both eyes, which makes precise measurement possible from a side view. Results from the present study, using theoretical modeling, showed that for images being analyzed for EED with more than 75% eye overlap, measurement error associated with image rotation would be less than 5% (Fig. 7). This would be considered an appropriate amount of eye overlap to allow, while remaining within the 5% error commonly accepted in biological studies.

In summary, all endpoints investigated in this study (developmental staging, EED, and EED/IED) consistently revealed significant delays in development as temperature decreased. However, developmental staging showed an increasing delay in development as time progressed, while EED and EED/IED delays remained constant over time. Developmental milestones were useful to determine changes in rate of development during early stages (2–24 hpf), while EED and EED/IED were appropriate for later stages of development (20–40 hpf). EED proved a more reliable endpoint than EED/IED. Predictive models for the influence of temperature on zebrafish development were constructed that allow for cross-temperature data analysis. In addition, determination of the influence of image position on EED and EED/IED measurements showed that more than 75% eye overlap was necessary to ensure actual measurement with less than 5% error. The effectiveness of these endpoints in zebrafish was demonstrated, underscoring the existing variability in cross-temperature comparisons and the risks of selecting any single endpoint as a measure of such a complex process as development.

Conflict of interest

None declared.

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