Temperature-dependence of Early Development of Zebrafish and the Consequences for Laboratory Use and Animal Welfare

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Abstract

Zebrafish (*Danio rerio*) is widely used as a model organism in biological research. However, the impact of different incubation temperatures on specific developmental endpoints is still insufficiently studied.

This study quantifies developmental differences of zebrafish embryos incubated at two commonly used temperatures (26 °C and 28 °C), focusing on key developmental endpoints (onset of heartbeat, hatching time, eye size, yolk sac consumption, body length). For this purpose, we recorded a high-resolution time series comprising hourly observations of early developmental stages and key events and bi-hourly observations

of body length until 120 hours post fertilization. Additionally, we recorded a low-resolution time series at 72, 96, and 119 hours post fertilization for detailed measurements of eye size, yolk sac area, and swim bladder size.

Embryos incubated at 26 °C consistently exhibited delays in reaching developmental stages compared to those reared at 28 °C. Significant differences were apparent from the earliest stages of development, with the delays becoming more pronounced as the embryos progressed to more complex stages. Yolk sac consumption was delayed by approximately 10 hours at 26 °C by 120 hours post fertilization, indicating a delayed onset of independent feeding. These data suggest that exclusively time-based regulatory limits for rearing zebrafish eleutheroembryos, such as the 120 hours post fertilization threshold commonly interpreted from the German regulation TierSchVerV (Tierschutz-Versuchstierverordnung), do not adequately account for the temperature-dependency of zebrafish development.

The results highlight the need for guidelines that link incubation temperatures to developmental progress.

1 Introduction

The zebrafish, *Danio rerio*, is a widely used animal model in biological and biomedical research (Ferrandino, 2024; Simonetti et al., 2015), valued for the homology of many of its organs and their development with other vertebrates, including humans (He et al., 2014; Kinth et al., 2013). Therefore, experimental conditions are largely standardized either through legal requirements such as the EU Directive 2010/63/EU (European Union, 2010), individual countries, e.g. in the German regulation TierSchVerV (BMJV, 2019) or on the basis of widely cited analyses published in the scientific literature (Braunbeck et al., 2012; Kimmel et al., 1995; Reed & Jennings,

2011; Westerfield, 2007). These guidelines define good practice for zebrafish maintenance and laboratory use and form the basis for the standardized assessment of various zebrafish characteristics.

However, the optimal thermal conditions for the maintenance and laboratory use of zebrafish are still subject of considerable debate. While OECD and DIN EN ISO standards recommend temperatures of 26 ± 1 °C (DIN, 2009; OECD, 2013), the most frequently used maintenance temperature is 28.5 °C (Lawrence, 2007). Other scientific publications favor incubation temperatures ranging from 26 °C to 28 °C (Strähle et al., 2012).

Furthermore, the developing fish embryo is the most sensitive stage in the fish life cycle (Hallare et al., 2005). This stage is, therefore, particularly vulnerable to thermal stress, as embryos possess limited capacity to regulate membrane fluidity, adjust metabolic rates, or engage in behavioral thermoregulation (Brett, 1970; Rombough, 1997; Schaefer & Ryan, 2006).

The influence of temperature is particularly critical when analyzing endpoints that can only be recorded shortly before the zebrafish larvae enter their protected status according to EU Directive 2010/63 (European Union, 2010), such as locomotor behavior and the conclusions of the validation efforts for the fish embryo test under the OECD (Braunbeck et al., 2012). According to 2010/63 and as detailed in Strähle et al. 2012, early, non-"free-living" developmental stages of laboratory animals are not classified as vertebrates and are therefore not subject to special protection. Such developmental stages of fish are defined in the Directive as all stages that do not feed independently, even if already hatched, independent of the incubation temperature. Although Braunbeck and coworkers (2012) estimate that zebrafish eleutheroembryos do not feed independently prior to 120 hours post fertilization (hpf), they do not define

the underlying incubation temperature. In a follow-up paper by Strähle et al. (2012), the authors recommend 120 hpf as a safe limit for incubation at 28.5 °C and use an equation earlier developed by Kimmel et al. (1995) to extrapolate to an incubation time of 139.5 hpf at 26 °C before independent feeding starts. This equation was developed from staging data up to 72 hpf of three incubation temperatures (25 °C, 28.5 °C, 33 °C) and assumes a linear relationship between fish development rate and temperature. However, growth rates in biological systems typically follow a non-linear relationship that peaks at an optimum temperature (Arroyo et al., 2022), which was also demonstrated for fish growth processes (Jobling, 1997). We therefore, hypothesize that the current estimation of the maximum incubation duration before zebrafish eleutheroembryos commence independent feeding (and thereby enter into a life stage in which they enjoy special protection as vertebrates) is not sufficiently precise for extrapolation to other temperature conditions than 28 °C.

The aim of this study was to quantify the differences in zebrafish development that result from two incubation temperatures (26 °C and 28 °C) and to put them into the context of the considerations put forward by EU Directive 2010/63, Braunbeck et al (2012) and Strähle et al (2012). For this purpose, we analyzed the development characteristics in early embryonic stages, heartbeat onset and hatching time, as well as body length in detail using a twice repeated high-resolution time series, which was amended by a low-resolution time series (repeated thrice), in order to record details on eye and yolk sac size. This data can help responsible stakeholders in animal welfare make an informed decision on time limits for the use of zebrafish embryos dependent on experiment temperature.

2 Methodology

2.1 High-Resolution Time Series

All details of the set-up of the time series are presented in Miller et al. (2025). Briefly, we transferred 24 embryos per temperature group (26 °C and 28 °C) to 96-round-well-flat-bottom plates with 300 µL of embryo medium and photographed individually every hour using a digital microscope (Keyence VHX-970F, Neu-Isenburg, Germany). After all 24 embryos were hatched, we photographed them every two hours until 119 hpf.

We repeated the experiment once, resulting in data from 48 individuals per temperature group.

2.1.1 Classification of Early Developmental Stages

Based on the classification by Kimmel et al. (1995) we identified developmental stages between 1 hpf and approximately 40 hpf from the high-resolution time series (SI, Table 3). For accurate classification, we used the primary and easily recognizable developmental characteristics of each developmental stage. If an individual could not be clearly assigned to the next higher developmental stage, we classified it under the previous stage.

2.2 Low-Resolution Time Series

The low-resolution time series is presented in Miller et al. (2025). Briefly, after 72, 96 and 119 hours incubation and anesthesia, we individually adjusted 24 eleutheroembryos per temperature group (26 °C and 28 °C) laterally in a gelatinous fixative. This optimized the overlap of the two eyes, thereby facilitating the recording of an image that enables the quantitative comparison of the eleutheroembryo's

dimensions. In this experiment, we quantified the area of the top-facing eye, and the yolk sac size using the microscope software's internal scale.

We repeated the experiment twice, resulting in data from 72 individuals per temperature and time group (72 hpf, 96 hpf, 119 hpf).

In general, the body length of eleutheroembryos from the low-resolution time series was smaller than those from the high-resolution time series after 119 hpf (by 0.48 mm when comparing the 26 °C data and by 0.43 mm when comparing the 28 °C data). This is likely caused by the more constant temperature profile of the eleutheroembryos from the low-resolution time series as those were only taken out of the incubators twice during the experiment.

2.3 Statistical Analysis

We performed all statistical analyses with R (R Core Team, 2024) in the RStudio IDE (RStudio Team, 2024) and used base R or the package *ggplot2* (Wickham, 2016) for plotting the data.

2.3.1 Early Developmental Stages

We assessed the significance of development speed by the Mann-Whitney test, with p-values being corrected for alpha inflation with the Bonferroni method. To quantify the delay, we calculated the median difference with bootstrapped 95% confidence intervals (CI).

We compared our observed data with the estimates provided by the equation of Kimmel et al. (1995), which relates development velocity to development at 28.5 °C as follows:

$$H_t = \frac{h}{0.055 * T - 0.57} \qquad (1)$$

With H_t = hours of development at temperature T, h = hours of development at 28.5 °C and T = temperature in °C. We also re-evaluated the equation, using Kimmel et al.'s own data series for 25 °C, 28.5 °C and 33 °C, retrieved from the paper with the help of the web plot digitizer (Rohatgi, 2024).

2.3.2-3 Onset of Heartbeat and Hatching

We tested the time to hatching and the onset of heartbeat for statistically significant difference of the medians with the Mann-Whitney test and a permutation test. To model the relationship between incubation duration and the fraction of embryos having a heartbeat we used a log logistic function (2 parameters) with package *drc* (Ritz et al., 2015). For analysis of the statistical difference, we compared the models for the two temperatures using the Wilks test with package *minpack.lm* (Elzhov et al., 2016).

2.1.4 Body Length

For the body length development over time, we considered two models, a linear and a logistic growth. If the linear model provided a better fit according to the Akaike Criterion, we selected it for further analysis. To quantify the delay in hpf between the temperatures at 119 hpf, we determined the time when eleutheroembryos incubated at 26 °C reached the same length as the ones incubated at 28 °C. Therefore, we defined the minimum delay as the distance between the lower CI of the 28 °C regression and the higher CI of the 26 °C regression. The maximum delay was defined inversely.

To estimate the impact of repeated handling of the embryos on growth, we compared the final lengths of the hourly to bihourly measured eleutheroembryos from the high-resolution time series with the ones measured in undisturbed individuals from the low-resolution time series.

As body length is one of the more commonly measured endpoints, we conducted a literature review of values at different temperatures. Most values were found for 120 hpf. We plotted them against the temperature at which they were measured and compared the fit of a linear model to that of a skewed Gaussian model, with the latter representing an optimum temperature growth curve. For comparison, we calculated the Akaike criterion and explained variation of each model:

$$1 - (variability \in model / variability \in null - model) \qquad (2)$$

2.1.5 Yolk Sac and Eye Size

Data for the areas of the yolk sac and eye are available at three time points: 72, 96, and 119 hpf, with n = 45 at 26 °C and n = 46 at 28 °C. To compare the delay that occurred at 119 hpf, we calculated the difference in medians with a bootstrapped confidence interval. For comparison of developmental rates for these endpoints, a linear regression over time was performed, and the slopes were tested against each other using a permutation test.

2.1.6. Data and Code

The underlying developmental data for both experiments have been previously published in a separate data publication (Miller et al., 2025). Additional data and analysis scripts are available in the supplemental material (SI, Table 5).

3 Results and Discussion

To determine the development delay between 26 °C and 28 °C we analyzed the differences seen in our endpoints over time.

3.1 High-Resolution Time Series

3.1.1 Early development stages

Figure 1 shows the time (hpf) zebrafish embryos need to reach a specific developmental stage at an incubation temperature of 26 °C or 28°C. The early cell stages (4-cell to 32-cell) of the first few hours in development show no difference between the two incubations. From the 75 %-epiboly stage on all stages are consistently reached significantly faster at 28 °C than at 26 °C with a CI of median difference not adjacent to zero. At around 1.5 days post fertilization (dpf) the embryos incubated at 26 °C have a median developmental delay of at least 2 hours (95% CI 2 – 4). Table 1 presents a detailed statistical analysis of the developmental stages, including the sample sizes (n), the differences in means, the bootstrapped CI of difference in medians and the p-values indicating the significance of the differences observed.

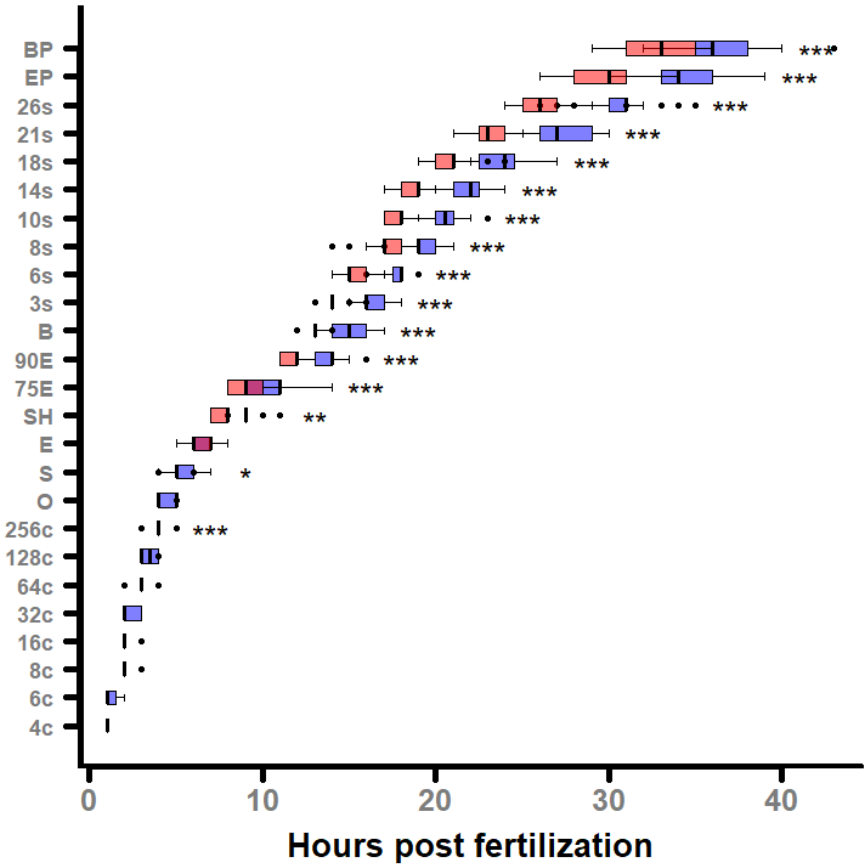

Figure 1: *Hours post fertilization (hpf) for various developmental stages at 26 °C (blue) and 28 °C (red). Data is shown as upper and lower quartiles (boxes), median (middle line), minimum and maximum values (bars), and outliers for each stage at both temperatures;* $n_{26\,°C} = 47$, $n_{28\,°C} = 48$. *Statistical significance according to Mann-Whitney test (α = 0.05) is given as * p < 0.05, ** p < 0.01 and *** p < 0.001. The explanations of the abbreviations are given in Table 1.*

***Table 1**: Delay in hours post fertilization for zebrafish embryos to reach developmental stages at 26 °C and 28 °C. n = number of embryos this stage was observed in, CI = bootstrapped confidence interval of difference in medians, p-value: derived from the Mann-Whitney test and corrected for alpha inflation with the Bonferroni Method.*

Stage	Abbreviation	n (26 °C)	n (28 °C)	Difference in medians	CI	p-value
4-cell	4c	29	20	0.0	0.0 – 0.0	-
8-cell	8c	20	28	0.0	0.0 – 0.0	0.14
16-cell	16c	8	6	0.0	0.0 – 0.0	1
32-cell	32c	20	34	0.0	0.0 – 0.0	1
64-cell	64c	14	14	0.0	0.0 – 1.0	0.17
128-cell	128c	21	12	0.0	0.0 – 0.0	1
256-cell	256c	24	33	0.5	0.0 – 1.0	$9 \cdot 10^{-04}$
oblong	O	22	26	0.0	0.0 – 0.0	1
sphere	S	17	18	1.0	0.0 – 1.0	0.03
epiboly	E	28	33	0.0	0.0 – 1.0	0.48
shield	SH	40	41	1.0	0.0 – 1.0	$3 \cdot 10^{-03}$
75-epiboly	75E	29	26	1.0	1.0 – 2.0	$2 \cdot 10^{-07}$
90-epiboly	90E	41	44	2.0	1.0 – 2.0	$1 \cdot 10^{-06}$
bud	B	43	42	2.0	1.0 – 3.0	$6 \cdot 10^{-13}$
3-somite	3s	38	37	2.0	2.0 – 2.5	$4 \cdot 10^{-12}$
6-somite	6s	34	44	2.0	2.0 – 3.0	$4 \cdot 10^{-12}$
8-somite	8s	36	25	3.0	2.0 – 3.0	$6 \cdot 10^{-11}$
10-somite	10s	31	31	2.0	2.0 – 3.0	$1 \cdot 10^{-09}$
14-somite	14s	24	13	2.5	2.0 – 4.0	$2 \cdot 10^{-05}$
18-somite	18s	36	36	3.0	2.0 – 4.0	$7 \cdot 10^{-12}$
21-somite	21s	43	41	3.0	2.0 – 4.0	$1 \cdot 10^{-11}$
26-somite	26s	37	43	4.0	4.0 – 5.0	$8 \cdot 10^{-13}$
prim	P	43	39	5.0	4.0 – 6.0	$5 \cdot 10^{-10}$
pigmented eyes	EP	37	41	4.0	3.0 – 6.0	$1 \cdot 10^{-10}$
pigmented body	BP	44	46	3.0	2.0 – 4.0	$7 \cdot 10^{-08}$

When comparing our development data with the data and equation derived by Kimmel et al. (1995) it becomes clear that our data have an approximately 15 % lower developmental rate (compare SI, Figure 9). We attribute this to the different experimental set-ups: while we raised the embryos in 96-round-well-flat-bottom plates and removed them from the incubators for about 15 % of total time for the

measurements, Kimmel et al. (1995) incubated the embryos in 150 mL beakers from which they were only removed for approximately 8 % of the total incubation time.

The data we derived from the original plot indicate that the slopes predicted by Kimmel's formula deviate from the actual observed slopes by at least 2 % at 25 °C and at least 10 % at 33 °C (SI, Figure 8, Table 4). These deviations support our hypothesis that the relationship between temperature and growth rate is not linear but follows an optimal curve. The more significant downward deviation at 33 °C suggests that this temperature is further from the optimal range, which is likely around 28.5 °C. This is consistent with findings from Pype et al. (2015), who reported an increased rate of malformations at temperatures above 32.5 °C, further indicating that 33 °C is outside the ideal range for normal development.

3.1.2 Onset of Heartbeat

The median onset time of the heartbeat at 28 °C is 29 hpf, which is 4 hours (95% CI 4 – 5) earlier than the median onset time at 26 °C (Figure 2) (Mann-Whitney test $p < 0.0001$; permutation test $p < 0.0001$). The time of first heartbeat is approximately 4 hours earlier in the 28 °C incubation, and the slope of the model over time is about 21 % steeper, reaching 100 % of beating hearts approximately 7 hours earlier than at 26 °C incubation. The models are significantly different from each other (Wilks test $p < 0.0001$).

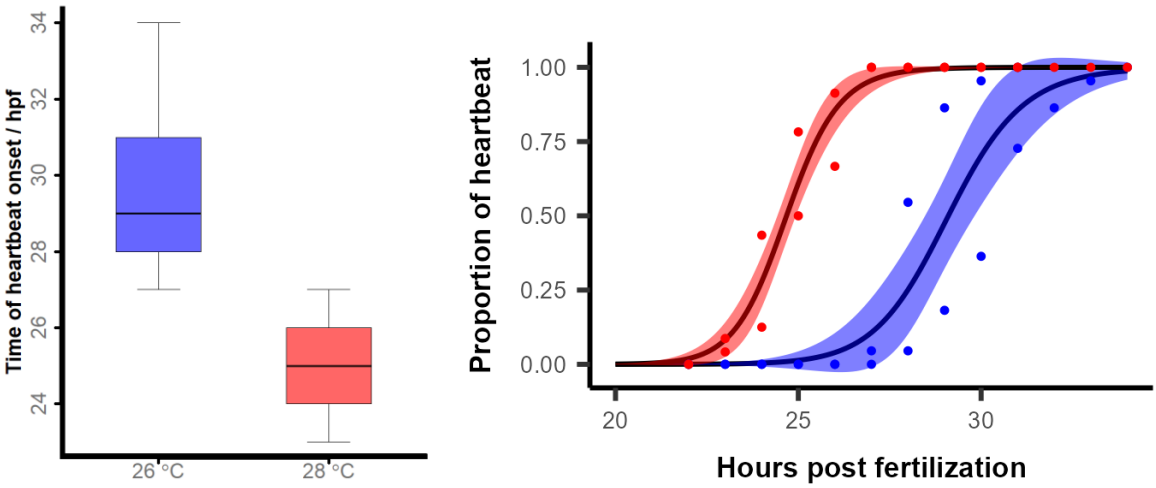

***Figure 2**: Time of heartbeat onset (hpf) at 26 °C and 28 °C (left). The proportion of embryos exhibiting heartbeat over development time modelled as log-logistic function (hpf) at 26 °C (blue) and 28 °C (red) (right). The shaded areas represent the 95 % confidence intervals ($n_{26\,°C} = 47$, $n_{28\,°C} = 48$).*

3.1.3 Hatching

The median hatching time was 68 hpf at 26 °C which is significantly longer (p=3.341e-11, Wilcoxon rank-sum test; p < 0.0001, permutation test) than the 60 hpf hatching time at 28 °C by 8 hours (95 % CI 4.5 – 9). The first hatch occur approximately 9 hours earlier at 28 °C, compared to 26 °C. Also, the slope of the model over time is 62 % steeper (Figure 3), reaching 100 % hatching success approximately 16 hours earlier than at 26 °C incubation. The model fits are statistically significantly different (Wilks test p < 0.0001).

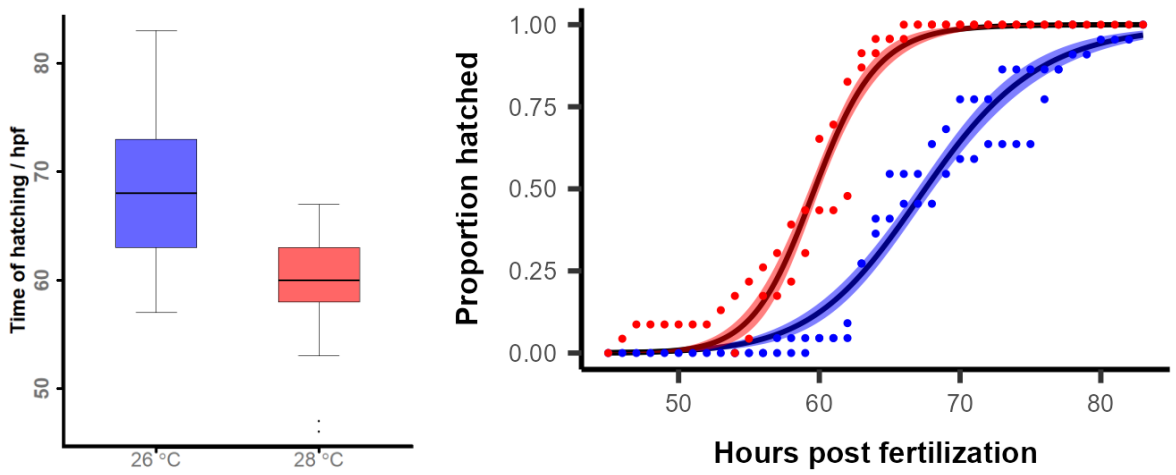

***Figure 3**: Time of hatching (hpf) at 26 °C and 28 °C (left). The proportion of embryos hatched over development time (hpf) at 26 °C (blue) and 28 °C (red) modelled with a log-logistic function (right). The shaded areas represent the 95 % confidence intervals (B); $n_{26\,°C}$= 45, $n_{28\,°C}$= 46.*

3.1.4 Body Length

Figure 4 illustrates growth (body length) over time at both incubation temperatures. We attribute the comparatively low R^2 values of the underlying linear regression (26 °C = 0.52, 28 °C = 0.67) to the variability introduced by the different depths to which the eleutheroembryos were submersed in the wells during measurement (which impacts the accurate measurement of the body length).

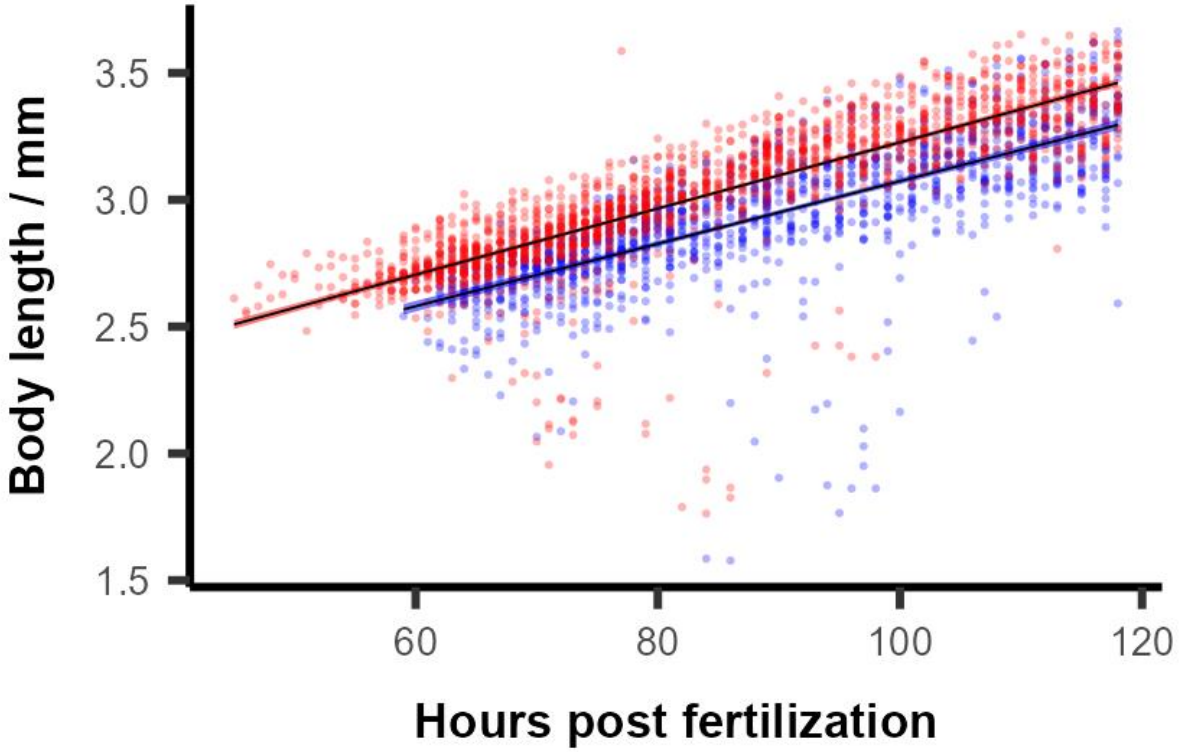

Figure 4: Body growth of zebrafish eleutheroembryos over development time in hpf at 26 °C (blue) and 28 °C (red). The shaded areas represent the 95 % CIs; $R^2_{26\,°C}$*= 0.52,* $R^2_{28\,°C}$*= 0.67;* $n_{26\,°C}$*= 45,* $n_{28\,°C}$*= 46.*

The difference in slopes is 6 % ($p < 0.05$), indicating only a slightly faster growth rate at 28 °C incubation temperature. We hypothesize that at this stage more energy is directed towards the development of physiological structures than to body size.

Eleutheroembryos incubated at 28 °C reached a median body length at 119 hpf of 3.46 mm (95% CI 3.44 - 3.47). In contrast, eleutheroembryos incubated at 26 °C have a median body length of 3.29 mm (95% CI 3.28 - 3.31). The average delay in body growth of the eleutheroembryos incubated at 26 °C is 12.5 hpf (95% CI 10 – 14.9) at approximately 117.5 hpf development.

Figure 5 presents the relationship between development rate (measured as body length at 120 hpf) and temperature based on data derived from literature (SI, Table 5). The data are best described by a skewed Gaussian model (Akaike value -3.55), which

explains 28.8 % of the variation relative to a null model. This is significantly better than the linear model (Akaike value -0.28), which accounted for only 2.5 % of the variation. The maximum of the curves lies at the 28.50 °C, which widely accepted as the optimal incubation temperature.

We conclude that the temperature dependency of zebrafish development does not follow a linear trend in this temperature spectrum, as previously suggested by Kimmel et al. (1995) and adopted for extrapolation by Strähle et al. (2012). However, data availability and variability are somewhat limiting, and a more systematic data generation for the temperature range between roughly 22 °C and 32 °C might be called for.

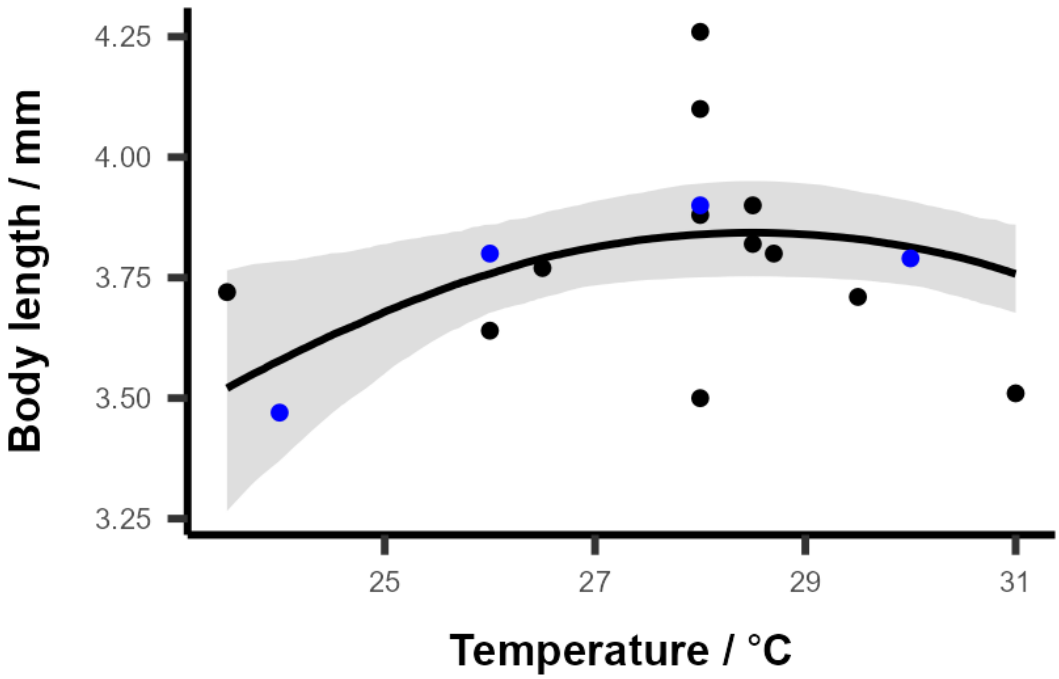

Figure 5*: Relationship between body length and incubation temperature of 120 hpf zebrafish eleutheroembryos modelled using a Gaussian model. The shaded grey area represents the bootstrapped lower and upper 95% CIs. Blue dots correspond to data from our study, while black dots represent data extracted from the literature (SI, Table 5) The maximum of the curve lies at 28.5 °C.*

3.2 Low-Resolution Time Series

3.2.1 Yolk Sac Consumption

The measurements of 72, 96 and 119 hour old eleutheroembryos show a continuous consumption of the yolk sac with progressive development. Yolk sac degradation is 1.2 times faster at 28 °C (Figure 6, highly significant difference, $p < 0.0001$). As a result, the eleutheroembryos incubated at 26 °C are delayed in their development by 10.2 hpf at the end of the incubation (95% CI 9.0 - 11.8). We used a linear model, as according to Schwartz et al. (2021), the depletion of yolk is still moving in a linear scope at the observed time points.

Both temperature groups of eleutheroembryos had not fully consumed their yolk sacs after 119 hours, indicating ongoing reliance on yolk as an endogenous energy source. This aligns with previous findings by Kalasekar et al., (2015), who found that the eleutheroembryos' functional gut develops at approximately 120 hpf, initiating external food ingestion while still utilizing yolk reserves. This is corroborated by Wilson (2012), who argue that at 120 hpf at 28.5 °C, the intestinal tract is still not fully developed, thus limiting the eleutheroembryos' ability to absorb nutrients from exogenous sources efficiently. And finally, Jardine & Litvak (2003) report that the yolk sac consumption of zebrafish larvae that were incubated at 28.5 °C is only fully consumed only after 165 ± 12 hpf. Taken together, these findings indicate that the yolk sac is the primary energy source of the developing eleutheroembryo until 120 hpf at an exposure of 28 °C, and that its role begins to diminish thereafter, as the eleutheroembryo gradually transitions to external feeding. We therefore conclude that eleutheroembryos that are incubated at 26 °C should not be classified as "independent feeders" at 120 hpf.

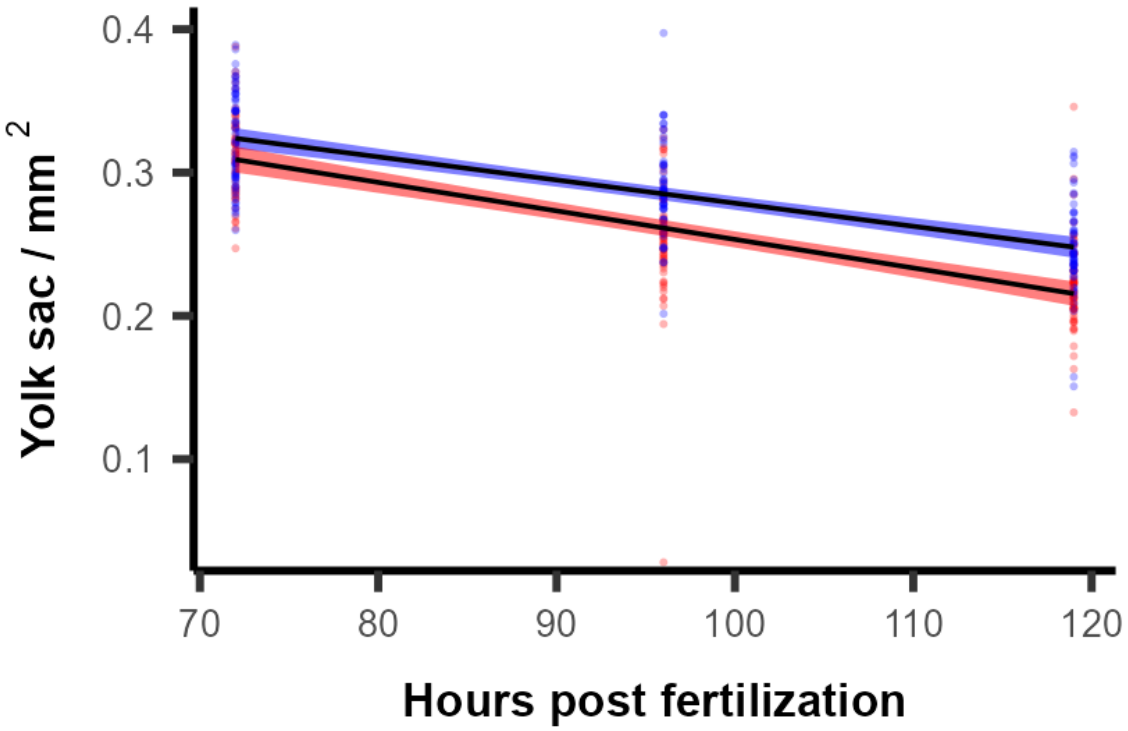

Figure 6: *Yolk sac size of zebrafish eleutheroembryos over development time in hpf at 26 °C (blue) and 28 °C (red). The shaded areas represent the 95 % CIs;* $p_{26\,°C}$ *= 2.2e-16;* $R^2_{26\,°C}$ *= 0.86,* $p_{28\,°C}$ *= 2.2e-16,* $R^2_{28\,°C}$ *= 0.89.* $n_{26\,°C}$ *= 45,* $n_{28\,°C}$ *= 46.*

3.2.2 Eye Size

The initiation of eye development occurs at the 6-somite stage at both incubation temperatures, which is consistent with previous findings (Schmitt & Dowling, 1994). However, the rate of eye growth in eleutheroembryos incubated at 28 °C is 1.16 times faster compared to those incubated at 26 °C (Figure 7, $p < 0.0001$). The developmental delay in eleutheroembryos incubated at 26 °C is 13.2 hours at 119 hpf (95 % CI 5.2 - 20.1).

As observed in many teleost species, eye growth and visual system development continue throughout a zebrafish's life (Fadool et al., 1997). Therefore, the influence of temperature on eye development likely persists beyond early embryonic stages, potentially affecting long-term visual function and growth dynamics. This would be in

line with Scott & Johnston (2012) observation that thermal acclimation capacity of adult zebrafish can be modified by temperature also after early development.

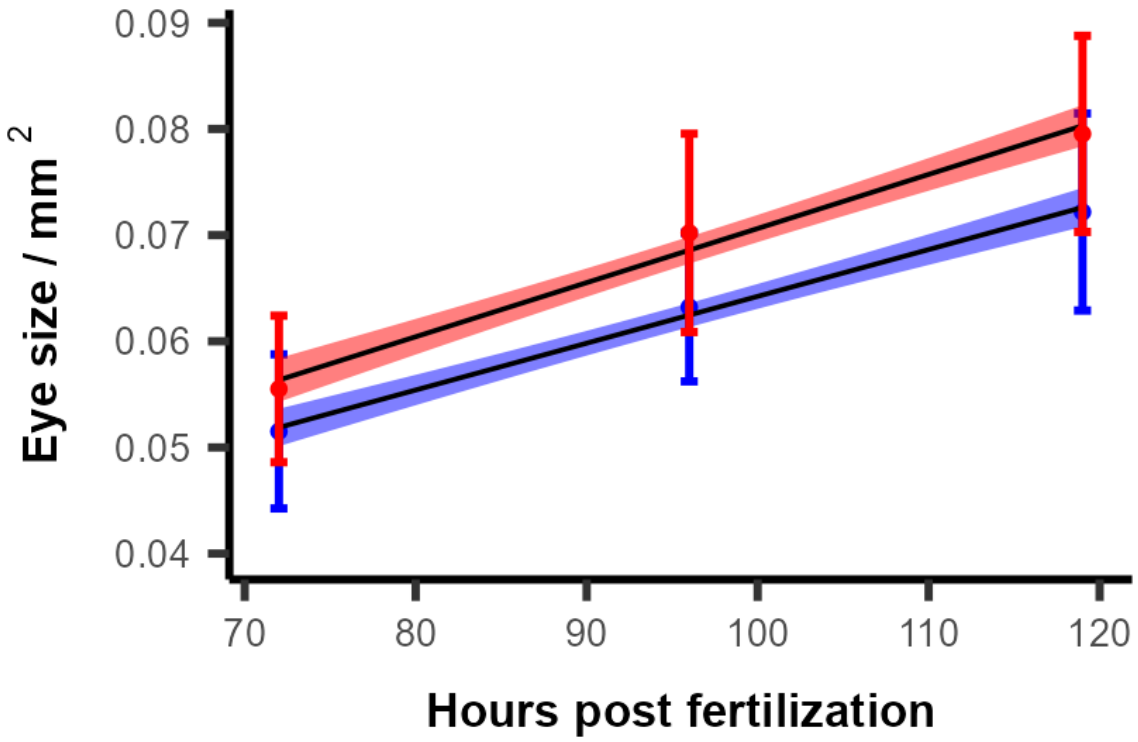

***Figure 7**: Eye size of zebrafish eleutheroembryos over development time in hpf at 26 °C (blue) and 28 °C (red). The shaded areas represent the 95 % CIs; $p < 0.0001$ (F-test); $R^2_{26\,°C} = 0.49$, $R^2_{28\,°C} = 0.49$. $n_{26\,°C} = 45$, $n_{28\,°C} = 46$.*

3.2.3 Summary

A summary of the developmental delays of zebrafish eleutheroembryos incubated at 26 °C, compared to 28 °C, is shown in Table 2. We observed a progressing delay between successive endpoints. At the final development stage (119 hpf) a minimum delay of 5 hours and a maximum delay of 21 hours was found.

***Table 2**: Summary of the developmental delays of the respective examined endpoints of the zebrafish eleutheroembryos incubated at 26 °C when compared to 28 °C. Minimum and maximum delay are given as bootstrapped 95 % confidence intervals.*

		Hours of delay		
Endpoint	dpf	min	average	max
Embryonic Stages	1	2	3	4
Onset of Heartbeat	2	4	4	5
Hatching	3	4.5	8	9
Body Length	5	10	12.5	15
Eye Size	5	5	13	20
Yolk Sac Consumption	5	8	15	21

4 Conclusions

Our study reveals that the sensitivity of zebrafish eleutheroembryos to incubation temperature is more complex than previously recognized. The analyzed endpoints respond differently to the temperature difference. The regulatorily accepted age limit of 120 hpf appears to be based on standard incubation temperatures of 26 °C - 28.5 °C, but our findings suggest that this limit should be closely linked to a narrow temperature range.

4.1 Temperature Sensitivity and Developmental Delays

The criteria for independent feeding assembled by Strähle et al. (2012) are based on studies using a maximum incubation temperature of 28.5 °C. Our study demonstrates a minimum delay of 8 hours at the end of the incubation time in yolk sac consumption when comparing embryos incubated at 28 °C versus 26 °C. This estimate is conservative, as the developmental trajectories increasingly diverge over time. This delay is further corroborated by other endpoints, such as body length and eye size, indicating significant sensitivity to temperature variations from early developmental stages.

Developmental delays become apparent very early in the life cycle of zebrafish. Markers of early development, such as heartbeat initiation and hatching, show a smaller delay. In contrast, later endpoints, such as yolk sac consumption, show increased delays, which supports the hypothesis that temperature-related differences in development will continue to increase over time. This aligns with the findings of Scott et al. (2012), who demonstrated temperature-dependent variations in gene expression in adult zebrafish, when developing temperatures variated, particularly in genes involved in energy metabolism, muscle contraction and remodeling, and cellular stress.

4.2 Implications for Experimental Protocols

Incubating zebrafish embryos at 26 °C presents several advantages. The slower development at 26 °C allows researchers more time to observe specific developmental stages or endpoints, which is especially valuable for studies focusing on organogenesis or detailed gene expression patterns. On the other hand, the slower pace of development at 26 °C might hamper the analysis of late endpoints, and therefore the study of more complex ones like behaviors. This holds especially true, if decision-makers in animal welfare adhere to the 120-hour limit proposed by Strähle et al. without taking temperature into consideration. For instance, there are currently efforts to standardize a behavioral biotest with zebrafish eleutheroembryos, which is increasingly used in ecotoxicological research (Light-Dark Transition Test (LDTT)) (Haigis et al., 2022). The standard operating procedure under revision recommends a measurement at around 123 hpf for reproducibility. Based on our findings we therefore recommend extending the current German time limit for zebrafish embryo tests from 120 hpf to 125 hpf for embryos incubated at 26 °C (as opposed to the 139.5 hpf estimate made by Strähle et al. (2012) with the formula derived by Kimmel et al. (1995)). This adjustment accounts for the observed developmental delays and

incorporates a conservative safety margin. This seemingly minute extension from 120 to 125 hpf is critically important for the implementation of the LDTT. Our findings also have implications for another ecotoxicological test, the Fish Embryo Toxicity Test (FET). Here the guideline allows a range from 25 °C to 27 °C. In light of our results and previous publications of the influence of temperature on toxicity (Heugens et al., 2003; Patra et al., 2015) we consider this margin to be too large.

Incubating at 28 °C accelerates development, making this temperature well suited for high-throughput and large-scale studies. Higher temperatures compromise embryo survival (Pype et al., 2015) and should therefore be avoided.

The results of the presented study advocate for better acknowledging the links between incubation temperature and the development of zebrafish embryos. We found a minimum 5-hour developmental delay for later endpoints at 26 °C compared to 28 °C, indicating significant developmental plasticity. This also complicates data interpretation and comparability across studies.

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Competing interests

The authors declare no competing interests.

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8 Supplemental Information

8.1 Embryonic Stages Categorization

***Table 3**: Descriptions of the embryonic stages determined during the high-resolution time series. Stages were categorized according to the developmental criteria of Kimmel et al. (1995). The table shows the selection of criteria from Kimmel et al. (1995) used to determine the respective stage.*

Stage	Abbreviation	Description
4 cell	4c	2 x 2 array of blastomeres
8 cell	8c	2 x 4 array of blastomeres
16 cell	16c	4 x 4 array of blastomeres
32 cell	32c	2 regular tier (horizontal rows) of blastomeres, 4 x 8 array
64 cell	64	3 regular tier of blastomeres
128 cell	128	5 blastomeres tier, cleavage planes irregular
256 cell	256c	7 - 9 irregular blastomeres tiers, yolk syncytial layer develops
oblong	O	> = 11 irregular blastomeres tiers, beginning of blastodisc cell asynchrony and flattening. Flattening produces an elliptical shape. Animal-vegetal axis of the blastula shortens, with the blastodisc compressing down upon the yolk cell
sphere	S	Continued shortening along tha animla-vegetal axis generates a late blastula of smooth and approximately spherical shape, flat border between blastodisc and yolk, start of yolk cell bulging (doming) toward animal pole as epiboly begins
epiboly	E	Epiboly is the thinning and spreading of the yolk syncytical layer and the blastodisc over the yolk cell. 30%-50% epiboly.
shield	SH	germ ring/ shield visible from animal pole. After reaching a 50% epiboly a thickened marginal region (germ ring), nearly simultaneously all around the blastoderm rim. Convergence movements then, nearly as rapidly, produce a local accumulation of cells at one position along the germ ring, the embryonic shield
75 epiboly	75E	Dorsal side distinctly thicker, thin evacuation zone on ventral side
90 epiboly	90E	Brain rudiment thickened, notochord rudiment distinct from segmental plate
bud	B	tail bud prominent, early polster, along dorsal side the neural plate is thickened along the embryonic axis. The thickening is most prominent near the animal pole in the prospective head region, where the head will form
3 somite	3s	first three somites furrow
6 somite	6s	brain primordium has distinctively thickened, eye primordium
8 somite	8s	8 - 10 somites without otic placode
10 somite	10s	otic placode developed
14 somite	14s	yolk cell begins to invert and look like kidney-bean. Somites begin to take on chevron shape
18 somite	18s	yolk cell extension is clearly delimited from the yolk ball as the tail straightens out
21 somite	21s	lens primordium
26 somite	26s	Straightening of posterior trunk is nearly completed, but elongating tail still curves ventrally. Cerebellar primordium is prominent
prim	P	Cerebellum is evident at the hindbrain/midbrain boundary region
Eye pigmentation	EP	eye pigmentation begins
Body pigmentation	BP	body pigmentation begins

8.2 Reanalysis of the Data derived by Kimmel et al. (1995)

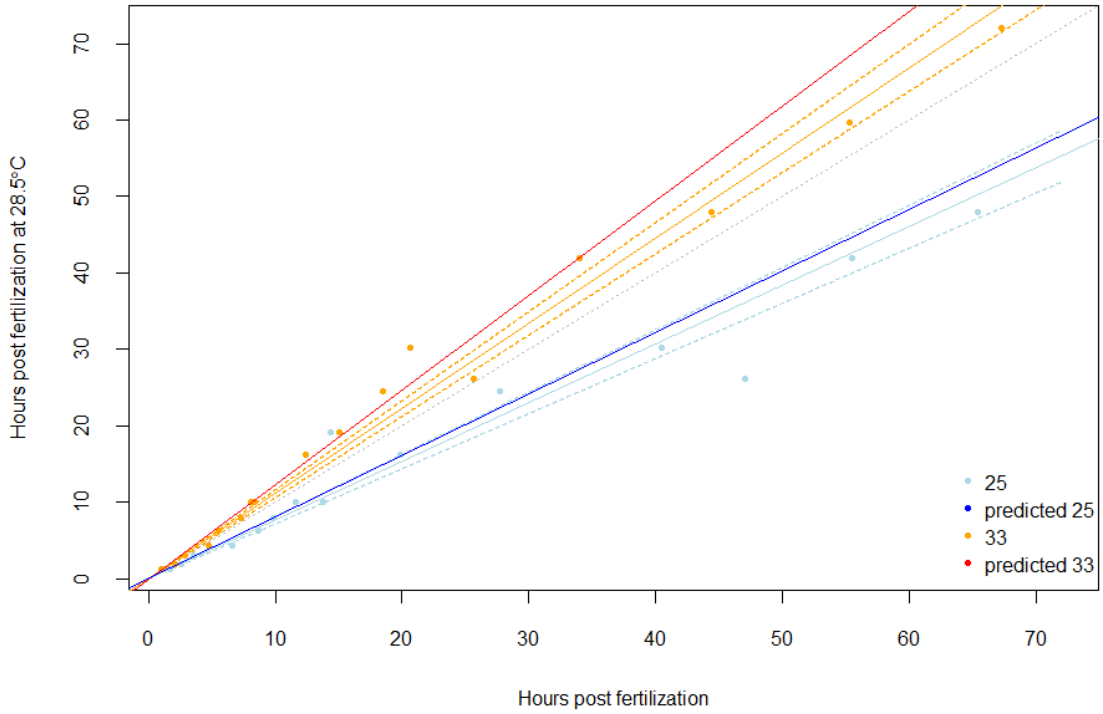

Figure 8: Relationship between the hours post fertilization (hpf) at different temperatures and the corresponding normalized hpf at 28.5 °C, comparing observed data points with predicted values based on fitted regression lines (adapted from Kimmel et al. (1995)). Raw data is listed in Table 4.

Based on our analysis of the development of zebrafish embryos in hpf derived by Kimmel et al. (1995) at SI25 °C, the predicted development rate is 0.805, calculated using the formula:

$$0.055 * 25 - 0.57 = 0.80 \qquad (3)$$

The slope of the fitted regression line for this temperature is 0.76783 ± 0.02208. The observed data points (Figure 8, light blue) and the predicted values (Figure 8, dark blue line) show that the actual development rate is slightly slower than predicted.

At 33 °C, the predicted development rate is 1.245, calculated as:

$$0.055 * 33 - 0.57 = 1.245 \qquad (4)$$

The slope of the fitted line here is 1.11440 ± 0.02414. The observed data points (Figure 8, orange) and the predicted values (Figure 8, red line) indicate that the development rate at 33 °C is also slightly lower than the model's prediction.

Overall, the data show that while the development rate increases with temperature, as reflected in the steeper slopes of the lines at higher temperatures, the observed rates are consistently lower than predicted. This suggests that the model used for prediction may overestimate the development rates, particularly at the more extreme temperatures of 25 °C and 33 °C. The variation in the slopes and their uncertainties indicates that the relationship between temperature and development rate might require further refinement for more accurate predictions.

Table 4: *Raw data of developmental rates derived from Kimmel et al. (1995) by using Web Plot Digitizer (Raharzi, 2024). Shown are the hpf at 28.5 °C, which is the standard incubation temperature, and the development of the eleutheroembryos in hpf when incubated at higher or lower temperatures.*

hpf at 25 °C	hpf at 28.5 °C	hpf at 33 ° C
1.7	1.3	1
2.6	2	2.1
3.6	3	2.9
6.6	4.3	4.7
8.7	6.3	5.5
9.8	8	7.3
11.6	10	8.1
13.8	10.1	8.4
14.4	16.3	12.4
19.9	19.2	15.0
27.7	24.5	18.5
40.5	26.1	20.6
47.0	30.2	25.6
55.5	41.9	34.0
65.4	47.9	44.4
76.7	59.7	55.3
87.9	72.1	67.3

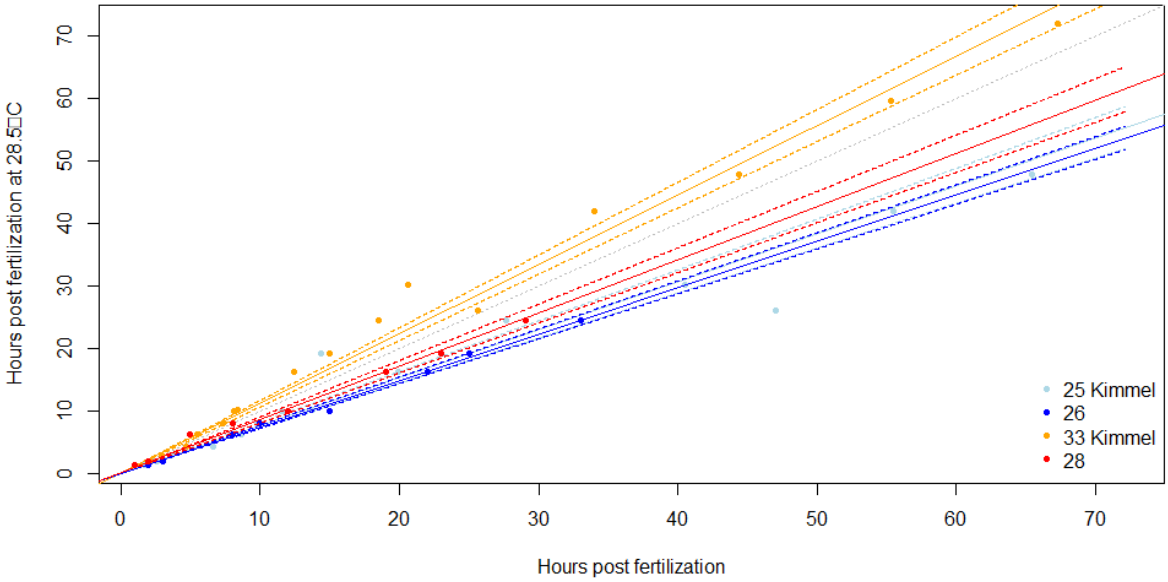

Figure 9: Relationship between the hours post fertilization (hpf) at different temperatures and the corresponding normalized hpf at 28.5 °C, comparing data generated on zebrafish embryo stages by Kimmel et al. 1995 to our data on staging, where comparable.

8.3 Body Length Data derived from Literature

***Table 5**: Larval body length measurements after 120 hours of incubation at various temperatures: Data obtained directly or derived using Web Plot Digitizer (Rohatgi, 2024)*

Length in mm	Temperature	Reference
3.72 ± 0.12	23.50	*van de Pol et al., 2021*
3.47 ± 0.13	24.00	*Miller et al., 2025*
3.80 ± 0.12	26.00	*Miller et al., 2025*
3.64 ± 0.12	26.00	*Chen et al., 2017*
3.77±0.11	26.50	*van de Pol et al., 2021*
3.90 ±0.12	28.00	*Miller et al., 2025*
3.50 ±0.08	28.00	*Pan & Hunt von Herbing, 2017*
4.10 ± 0.10	28.00	*Zizioli et al., 2023*
3.88 ± 0.08	28.00	*Dang et al., 2018*
4.26 ± 0.08	28.00	*Liu et al., 2022*
3.82 ± 0.21	28.50	*Schwartz et al., 2021*
3.90±0.45	28.50	*Hughes et al., 2019*
3.80 ± 0.10	28.70	*Mentor et al., 2020*
3.71 ± 0.13	29.50	*van de Pol et al., 2021*
3.79 ± 0.14	30.00	*Miller et al., 2025*
3.51 ± 0.10	31.00	*Pan & Hunt von Herbing, 2017*