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Incubation at 32.5°C and above causes malformations in the zebrafish embryo

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Abstract

Zebrafish embryos are increasingly used for developmental toxicity screening of candidate drugs and are occasionally co-incubated with a metabolic activation system at 32°C for 1, 2 or 4 h, depending on their developmental stage. As this temperature is higher than the optimal temperature for zebrafish embryonic development (26-28.5°C), we investigated whether

continuous incubation of zebrafish embryos from 2.5 until 96 hours post fertilization (hpf) at high temperatures (30.5-36.5°C) causes malformations. At 32.5°C tail malformations were observed as early as 24 hpf, and these became even more prominent at 34.5 and 36.5°C. Cardiovascular and head malformations, edema and blood accumulations throughout the body were present at 36.5°C. Finally, temperatures higher than 28.5°C accelerated embryonic development except for 36.5°C, at which a lower hatching rate and hatching enzyme activity were observed. In conclusion, incubation of zebrafish embryos at 32.5°C and above from 2.5 until 96 hpf causes malformations as early as 24 hpf.

Keywords: Zebrafish, Embryo, Development, Temperature, Morphology, Malformation, Hatching enzyme

1. Introduction

Zebrafish (*Danio rerio*) embryos are currently used for acute and/or developmental toxicity testing of pharmaceuticals, chemicals and cosmetics [1-3]. So far, only the (Zebra)Fish Embryotoxicity Test ((Z)FET, OECD TG 236) has been approved by the regulatory agencies, i.e. to test acute toxicity of chemicals [3,4]. In this test, zebrafish embryos are exposed to the test chemical for 96 h, covering the period of organogenesis, under standardized conditions. The endpoints in this test are limited to lethality, i.e. coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail bud from the yolk sac and lack of heartbeat [3,4]. However, several research groups and pharmaceutical companies are also using the ZFET study design to assess developmental toxicity of drugs and chemicals by adding several other endpoints such as edema, blood accumulations, malformations of the head and cardiovascular abnormalities [2,5-10]. Several acronyms are used in literature to denominate this assay, such as

Danio rerio Test (DarT) or zebrafish embryo developmental toxicity assay (ZETA) [2,5-8,10]. The latter term has been introduced in order to clearly differentiate this assay from other zebrafish embryo tests, as the DarT was originally introduced by Nagel in 2002 [11] to assess acute toxicity. In 2008, Busquet et al. introduced another term, i.e. mDarT [16]. In this assay, zebrafish embryos were only briefly exposed to the test substance, i.e. from 2 until 3 hours post fertilization (hpf), but in the presence of an exogenous metabolic activation system (MAS) at 32°C. They were then reared at 26°C and morphologically evaluated until 48 hpf (Figure 1). This MAS consists of induced rat liver microsomes, which contain high concentrations of cytochrome p450 enzymes (CYPs) that are important in phase 1 metabolism of xenobiotics [17,18]. Busquet et al. developed this DarT assay with metabolic activation (mDarT) to cover biotransformation of proteratogens [15,16]. Weigt et al. [15] initially followed this reasoning and slightly adapted this mDarT protocol by extending the rearing and evaluation period to 72 hpf (Figure 1). However, in later studies they showed that several proteratogens caused malformations in zebrafish embryos without addition of rat MAS [9,13], which could suggest that no metabolic activation system is required. The latter is still a matter of debate as some compounds, such as allyl alcohol [14], acetaminophen [15] and albendazole [10], appear not to be biotransformed by zebrafish embryos. Therefore, this has also been taken up as a potential limitation of the ZFET acute toxicity test [12]. This said, the published mDarT protocols also show limitations as induced rat MAS is toxic for the embryos and consequently only short exposure windows, not covering the entire period of organogenesis, are used in these assays [10,15,16]. Indeed, Mattsson et al. [10] showed that continuous incubation of 2 hpf zebrafish embryos with rat MAS alone caused a developmental delay after 5 h and embryonic death, which was noted after 2 days of exposure. A stepwise reduction of the MAS concentration to 1.4% of the original concentration (0.7 mg/mL microsomes; 10 mM NADPH) did not sufficiently reduce MAS toxicity to allow continuous exposure for days, starting at 2 hpf. After 24 h of exposure all embryos were still

developmentally delayed. Therefore, various incubation durations were tested, starting from 2, 12 and 24 hpf. The longest tested tolerable incubation times were 1 h from 2 hpf, 2 h from 12 hpf and 4h from 24 hpf and these exposure windows were applied in their final protocol (Figure 1).

Figure 1. Overview of published mDarT protocols. The exposure of the zebrafish embryos to the test substance (TS) with induced rat MAS was performed at 32°C in all protocols. Further rearing of the embryos until 48 hpf, 72 hpf or 144 hpf was performed at 26°C in all protocols.

As a high temperature (32°C) is used when co-incubating zebrafish embryos with induced rat MAS, we wondered whether this could also be a contributing factor to the embryotoxicity that Mattsson et al. observed under continuous incubation of 2 hpf embryos, even with very low

MAS concentrations [10]. Although adult zebrafish tolerate extreme water temperatures in the wild, ranging from 6°C in winter up to 38°C in summer, zebrafish have a much lower threshold for temperature fluctuations during their embryonic and larval development [19]. The optimal temperature for zebrafish embryonic development is considered to be 26 - 28.5°C [15,20-22]. A higher temperature (32°C) is applied when co-incubating zebrafish embryos with rat MAS, as mammalian microsomes are considered to function optimally at 37°C [16]. Busquet et al. chose 32°C as a compromise between optimal metabolic activation and optimal embryonic development, because Kimmel et al. [21] mentioned that temperatures above 33°C may impact embryonic development after prolonged incubation [16]. However, so far no morphological characterization of this temperature has been performed under continuous incubation conditions until the end of organogenesis. Therefore, the aim of this study was to investigate whether high incubation temperatures from 2.5 until 96 hpf cause malformations in zebrafish embryos.

2. Materials and Methods

2.1 Methods

2.1.1 Adult zebrafish housing and egg production

Adult wild type zebrafish (*Danio rerio*, in house wild type AB zebrafish line), designated for breeding, were kept in a recirculating Zebtec standalone housing system (Tecniplast, Hohenpeißenberg, Germany). The water temperature was set to 28°C ± 0.2°C and the conductivity and pH of the water were 500 ± 15 µS/cm and 7.5 ± 0.3 respectively. The renewal of

water was sustained at 25% of its total volume/day in order to keep ammonia, nitrite and nitrate levels below detection limits. An automated light cycle of 14/10 hours light/dark was applied.

The fish were fed thawed food (*Artemia nauplii*, *Daphnia*, Chironomidae larvae or *Chaoborus* larvae) twice a day, as well as granulated food (Biogran medium; Prodac International, Cittadella, Italy) at a rate of 1.5% of their mean wet weight per feeding, also twice a day.

The day before mating, one male and two female fish were transferred from the stock to separate breeding tanks. The next morning, the fish were allowed to breed and spawn during 45 minutes, after which the embryos were collected. To remove faeces and coagulated eggs, multiple washing steps in embryo solution (pH 7.5), i.e. Instant Ocean® Sea Salt (Blacksburg, VA, USA) and sodium bicarbonate (VWR, Leuven, Belgium) dissolved in reverse osmosis water (conductivity <10 $\mu\text{S}/\text{cm}$), were performed. The conductivity of the solution was set to 500 $\mu\text{S}/\text{cm}$. Within 2 hpf, all of the embryos were transferred to a 48 well plate (Greiner Bio-One, Cellstar, Wemmel, Belgium) filled with approximately 1 ml of embryo solution and checked under a Leica S8 APO microscope with Leica L2 lighting (Leica, Diegem, Belgium) for normal (i.e. no asymmetric) cell division of the first cell cycles (4- to 64-cell blastomeric stage). Unfertilized or asymmetric eggs were discarded from the experiment and replaced by other eggs.

2.1.2 Test conditions

Each experiment consisted of a control group (n= 96 embryos) and a test group (n=96). The controls were incubated at $28.5^{\circ}\text{C} \pm 0.3^{\circ}\text{C}$, which is the standard temperature used for rearing in our zebrafish facility. Test embryos were kept at temperatures ranging from 30.5°C up to 36.5°C with a 2°C interval ($t = 30.5^{\circ}\text{C}, 32.5^{\circ}\text{C}, 34.5^{\circ}\text{C}$ and $36.5^{\circ}\text{C} \pm 0.3^{\circ}\text{C}$). The selected temperature range was based on the physiological temperature of zebrafish embryos (28.5°C) and an extreme of 36.5°C , which was previously shown to cause approximately 100% embryonic death [23-25].

Control and test embryos were kept in a 14/10h light/dark cycle in a separate incubator. To avoid acidification and oxygen deprivation of the medium and to make it easier to handle the embryos during examination, 48-well instead of 96-well plates were used. At the latest at 2.5 hpf, the control and test plates with embryos were placed in their respective incubators. After 48 hpf, the medium was renewed in order to maintain high oxygen levels, reduce acidification of the medium and prevent accumulation of molecules excreted by the embryo.

In order for a batch of eggs to be valid, at least 80% of all eggs had to be fertilized and mortality of the controls had to be lower than 10% throughout the experiment. Since larvae are able to swim shortly after hatching (normally before 72 hpf and especially when manipulating them), scoring of the larvae at 96 hpf was performed under anesthesia with 0.1 g/l MS-222, pH 7.4 (ethyl 3-aminobenzoate methanesulfonate, tricaine; Sigma-Aldrich, Diegem, Belgium). At the end of the experiment, the larvae were euthanized by placing them in a 1 g/l solution of MS-222, pH 7.4. Each experiment was performed in duplicate to assess reproducibility of our results.

2.1.3 Morphological evaluation of the embryos

The embryos were evaluated at several time points (5.25, 10, 24, 48, 72 and 96 hpf; see Table 1), which represent the transitions of one developmental stage to another according to Kimmel *et al.* [21]. Apart from coagulation, a series of morphological parameters, adapted from Nagel [11], were examined depending on the time point. An overview of all parameters is given in Table 1.

Table 1. Coagulation and morphological parameters scored at the different time points.

Stage (hpf)

	5.25	10	24	48	72	96
Coagulation	X	X	X	X	X	X
Hatching				X	X	X
Tail deviation			X	X	X	X
Oedema			X	X	X	X
Blood accumulation			X	X	X	X
Malformation of the cardiovascular system			X	X	X	X
Malformation of the head			X	X	X	X
Malformation of the pectoral fins					X	X

hpf = hours post fertilization

Coagulation or embryonic death was distinguished from living embryos by the turbid content of the chorion and the absence of a heartbeat. Hatching, normally occurring between 48 and 72 hpf, was scored as successful when the head of the embryo was outside the chorion. As a consequence, a non-hatched score was given when only the tail was outside this structure. Deviations of the tail included slight curves or complete bends. Edemas and blood accumulations were registered in the entire embryo, including the pericard and the head. Malformations of the cardiovascular system included absent or disturbed blood circulation in the tail and abnormal looping of the heart. Malformation of the head included malformations of the ear (e.g. number of otoliths and position of the ear), eye and mouth, whereas malformations of the pectoral fin comprised missing or deformed fins. All parameters were scored 0 if normal and 1 if a malformation was present. This binary scale was used due to the high number of analyzed

parameters. As such, only a distinction had to be made between normal and abnormal development and no gradation of the severity of a malformation had to be made.

2.1.4 Cathepsin L assay

In order to hatch properly, embryos need to have reached the stage at which the hatching gland releases enzymes that break down the chorion [26]. As hatching was impaired at 36.5°C (see section 3.2), we evaluated whether this could be related to a loss of hatching enzyme release. Therefore, we assessed cathepsin L activity, which is an enzyme that is highly expressed in the hatching gland [27]. Cathepsin L cleaves the substrate z-Phe-Arg-7-amido-4-methylcoumarin into the fluorescent product 7-amido-4-methylcoumarin (AMC). The protocol was adapted from Trikić *et al.* [27] and 2 biological replicates were performed. Single embryos were placed into a 96-well plate in 110 µl of embryo solution at 28.5°C (n = 16) and at 36.5°C (n = 24). We included more embryos in the test group (36.5°C) than in the controls (28.5°C) to compensate for the possible loss of embryos (coagulation) at 36.5°C. At 48 and 72 hpf, 100 µl of the medium was collected and replaced with new medium. The collected medium was transferred to dark 96-well plates. To each well, 50 µl of assay buffer (340 mM sodium acetate, 60 mM acetic acid, 4 mM EDTA P.H 5.5, 8 mM DTT) and 50 µl of 20 µM substrate (*i.e.* 5 µM as final concentration) was added and immediately placed in a Tecan Infinite M200 Pro (Tecan, Männedorf, Switzerland) after which the signal (excitation of 370 nm, emission of 460 nm) was measured every 5 minutes during half an hour.

2.2 Calculated data and statistical analysis

The number of coagulated embryos in the different temperature groups was used to calculate the survival rates of all six developmental stages. To determine the general developmental speed of the embryos at different incubation temperatures, hatching was reported from 48 hpf onwards and only living embryos were included in the analysis. From 24 hpf, all malformations (except malformations of the pectoral fins, which can only be evaluated from 72 hpf onwards) within a single embryo were pooled to calculate the percentage of malformed embryos per group. Additionally, the percentage of embryos showing no, one or several malformations or death was calculated for the different test groups as well. Moreover, the incidence of each scored malformation at 96 hpf was reported for the test groups and their respective controls to present an overview of the temperature induced malformations at the end of organogenesis.

To assess the reproducibility of our results, the two replicates were analyzed with a chi²-test (IBM SPSS Statistics v.22, Brussels, Belgium). SPSS was also used to analyze the percentage of embryos showing no, one or several malformations in each test group compared with their controls by means of a Mann-Whitney U test. For our binary data (i.e. 0 or 1), using binary logistic regression analysis was not possible due to so-called “zero cell count” of several parameters (i.e. very rare malformations leading to zero affected controls, or when all embryos of a certain group are affected). Therefore, odds ratios (OR) were calculated (MedCalc v. 13.1., Ostend, Belgium) with a Haldane correction, where needed. The latter means that a value of 0.5 is added to each cell with a zero-cell count (28). With a z-test, the p-value indicates whether the odds are significantly different between controls and the test group. For the cathepsin L data, we compared the group of embryos reared at 28.5°C with those reared at 36.5°C at 48 and 72 hpf using the Independent Samples T-Test (IBM, Belgium). Outcomes were considered significant when the p-value was ≤ 0.05 .

3.Results

3.1 Validity and reproducibility of the experiments

All batches of eggs were valid as the mortality in all control groups never exceeded 10% and the overall percentage of control embryos showing malformations was less than 5%. The results of the experiments were reproducible as no statistical difference for any of the parameters was present between the two replicates. Only for hatching at 48 hpf a difference between the two replicates was noted ($p < 0.05$). However, hatching is known to be a very variable parameter, normally occurring between 48 hpf and 72 hpf [21]. Therefore, all values reported in this paper were taken arbitrarily from the first replicate.

3.2 Coagulation, hatching and total number of malformed embryos

Coagulation, or embryonic death, occurred only in the highest temperature groups (34.5°C and 36.5°C) and late during development (Figure 2). At 34.5°C and 96 hpf, coagulation was significantly higher ($p = 0.0014$) than the controls. Embryos incubated at 36.5°C had a 12.2 ($p < 0.0001$) and 55 ($p < 0.0001$) times higher chance to coagulate at 72 and 96 hpf, respectively. At 36.5°C, this resulted in a 74% chance that the embryos/larvae would coagulate whilst incubated at this temperature.

Figure 2. Survival curve for the pooled controls and all tested temperatures. FOR THE WEB VERSION IN COLOUR, FOR THE PRINTED VERSION IN BLACK AND WHITE, PLEASE (SEE ALSO IN ATTACHMENT).

Embryos incubated at 30.5°C, 32.5°C and 34.5°C showed a significantly higher chance of hatching compared to the controls (OR = 0.08, 0.005 and 0.23, respectively, $p \leq 0.0001$). At 36.5°C, the opposite effect was noted (OR = 19.72, $p = 0.0411$). In Figure 3, this is visually represented as the percentage of embryos that hatched and did not hatch at 48 hpf in the different test groups.

Figure 3. Proportions of hatched embryos at 48 hpf for the pooled controls and the different test groups. All test groups were significantly different from their respective controls, as indicated by the white asterisks (*). P-values for 30.5°C – 34.5°C ≤ 0.0001 and 0.0411 for 36.5°C. From 30.5 - 34.5°C, there was an increase of hatched embryos, whereas there was a decrease of already hatched embryos at 36.5°C.

At 36.5°C, most embryos also failed to hatch at 72 hpf (OR = 150.27; $p < 0.0001$) and at 96 hpf (OR = 285.92; $p = 0.0002$). Often embryos remained with their heads and upper bodies in the chorion, whereas the tails were partially out of the chorion (see Figure 6D). Additionally, embryos that were not hatched at 72 hpf had a 4.35 fold ($p = 0.0477$) higher chance of being coagulated at 96 hpf. This could not be explained or predicted by morphological effects at earlier time points as non-hatched embryos at 72 hpf showed a similar incidence and type of malformations at 48 hpf and 72 hpf compared with the embryos that did hatch at 72 hpf.

Regarding the total number of affected embryos, no significant increase was found at 30.5°C compared to the controls. However, at 32.5°C these numbers significantly increased as early as 24 hpf, up until the end of the experiment (OR ≥ 5.68 ; $p \leq 0.0024$) (Figure 4B). In the highest

temperature groups (34.5°C and 36.5°C) this observation was even more distinct, with ORs for the different time points (48 - 96 hpf) of $OR_{34.5^{\circ}C} \geq 11.55$, $p \leq 0.0001$ and $OR_{36.5^{\circ}C} \geq 56.62$, $p \leq 0.0001$, respectively (Figure 4C and 4D). At 34.5°C, the amount of embryos having at least one malformation appeared to decrease over time. This could be linked to one parameter, i.e. edema. At 24 hpf, the number of recorded edemas (head, yolk and pericard) was 73, whereas the numbers of edema that were observed at 48, 72 and 96 hpf were 35, 27 and 7, respectively. At 36.5°C, the amount of embryos that was impacted was already high at 24 hpf and remained high at the later time points (Figure 4D).

Figure 4. Representation of the percentages of embryos having at least one malformation at the different time points. A. 30.5°C, B. 32.5°C, C. 34.5°C and D. 36.5°C. Statistical differences compared to their respective controls (28.5°C) are indicated with asterisks (*).

Regarding the average number of malformations per embryo, the analysis yielded results that were in line with the above-mentioned results. At 30.5°C, no difference was evident at any of the time points ($p \geq 0.173$). For temperatures of 32.5°C and above, there was a statistically significant higher amount of malformations per embryo from 24 hpf onwards ($p \leq 0.012$). For 34.5 and 36.5°C, a proportional increase in the amount of malformations with increasing temperature was seen ($p \leq 0.004$). Results at 96 hpf are shown in Table 4.

Table 4. Representation of the percentages of embryos for each test group having one or more malformations at 96 hpf.

Temperature	Number of malformations per embryo								p-value
	0	1-2	3-4	5-6	7-8	9-10	11-12	Unscored*	
30.5°C	88.5	4.2	0.0	0.0	0.0	0.0	0.0	7.3	0.577
32.5°C	69.8	19.8	4.2	1.0	2.1	0.0	1.0	2.1	0.002
34.5°C	28.1	35.4	2.1	3.1	0.0	1.0	0.0	30.2	< 0.001
36.5°C	0.0	2.1	1.0	2.1	8.3	1.0	2.1	83.3	< 0.001

*Unscored embryos are either coagulated or their body parts were too malformed to score the embryo.

3.3 Detailed evaluation of the surviving embryos at 96 hpf

Figure 5 gives an overview of the percentage of malformations for all scored parameters at 96 hpf in all temperature groups. Embryos incubated at 30.5°C barely showed malformations. At 32.5°C, one parameter was affected, i.e. 21.51 % of the embryos showed a tail malformation (Figure 6B). The incidence of this malformation increased with higher temperatures. At 34.5°C, tail malformation was still the only parameter that was significantly more abundant in the test group. It should however be noted that some of the other parameters showed a tendency towards statistical significance (e.g. edema pericard (6.85 %), malformation of the cardiovascular system (5.48 %), malformation of the head (6.85 %) and blood accumulation in the heart (6.85 %)). At the highest temperature (36.5°C) almost all parameters were affected (Figure 5). So, not only did the embryos die (Figure 2), all surviving embryos suffered from a malformation and the amount of malformations per embryo was also higher than in the lower temperature groups (Figures 5 and 6).

Figure 5. Percentage of embryos having a specific malformation for all temperature groups. Significant differences of the test groups compared to the controls are indicated with white asterisks (*).

Figure 6. Overview of several observed malformations. A. Normally developed embryo (96 hpf). B. Typical example of an embryo reared at 32.5°C showing a tail malformation (72 hpf). C & D. Embryos reared at the highest temperature showing several malformations (96 hpf). Abbreviations: B: Blood accumulation, M: Malformation mouth (underdeveloped), N: Non-hatching, O: Edema, T: Tail malformation (bended tail), Y: Malformation yolk.

3.4 Cathepsin L assay

Cathepsin L activity (Figure 7) was higher at 48 hpf for the embryos incubated at 28.5°C (controls) compared to 36.5°C in both replicates ($p = 0.0005$ and 0.006 , respectively). At 72 hpf, no difference in cathepsin L activity was noted between controls and 36.5°C in the first replicate ($p = 0.397$). However, the cathepsin L activity was still higher in the controls than at 36.5°C in the second replicate ($p = 0.025$).

Figure 7. Cathepsin L activity (expressed as μM of AMC formed after 30 minutes) for the control (28.5°C) and test (36.5°C) groups at 48 and 72 hpf, respectively. A. First replicate. B. Second replicate. Statistical differences between control groups and test groups are indicated by bars with asterisks (*) above them.

4. Discussion

Heat is a well-known teratogen in a wide variety of vertebrates, and biota in general [29-31]. The observed effects range from embryonic death to malformations and are heavily dependent on the degree, duration and timing of the exposure. This has also been shown for the zebrafish as several studies already showed embryonic death from 34°C onwards, with reported survival rates of only 43 to 45% [23,24]. In our study, in which we focused on malformations, the temperature threshold appeared to be even lower, i.e. near 32°C . This was evidenced by the high percentage of embryos (ca. 30%) that showed tail malformations, which appear to be a sensitive marker for teratogenicity in the zebrafish as this has also been reported for several other toxicants [32-37]. This seems to be in contrast with data from Kimmel *et al.* and Schirone and Gross, who described that embryos survive normally between 25 and 33°C [21,25].

However, their conclusion was based on studying embryos that were only evaluated until 9 hpf [25]. As mentioned before, duration and timing are also very important factors for teratogenicity. At 9 hpf, only a very limited part of organogenesis (5 ¼ to 96 hpf) is covered and the evaluation of these embryos is restricted to developmental progression and survival. Malformations of the tail, cardiovascular system, head and pectoral fins and edema, and blood accumulation can only be assessed from 24 hpf onwards. So, as Schirone and Gross did not expose or evaluate embryos during organogenesis, possible effects may have been missed. This is further substantiated by a study on muscle function in the zebrafish. In this study, zebrafish embryos were incubated at 32°C until 120 hpf and then reared at 27°C until the age of 8-9 months. Interestingly, these embryos had a lower body weight and a smaller size when reaching adulthood compared to embryos that were kept at 27°C until 120 hpf. Persistent effects on metabolic enzymes of the muscle of these zebrafish were still present when reaching adulthood [23]. In a similar study, performed earlier, shifts in the proportions of the muscle fibers, and thus the muscle phenotype, had been examined and the effects also remained present in the adult fish [24]. Although a morphological scoring of the embryos was not performed in these studies, they substantiate our findings that embryos do not tolerate 32-32.5°C and that the threshold of a teratogenic effect by increased temperature is even lower than previously suggested [25].

Increased temperatures also accelerate zebrafish embryonic development [25]. Previous studies reported a faster development at 30°C than at 26-28°C and the fastest development was observed at 32°C and 34°C [23-25]. These findings are in full accordance with our results. However, there was a sharp cut-off at 36.5°C as most embryos did not hatch at this time point. In addition, our study showed that non-hatching at 72 hpf was a strong indicator for mortality at 96 hpf in this temperature group. This is in line with the general principles of physiology, in which altered temperatures can push organisms from a homeostatic condition to compensatory mechanisms at first (such as a higher metabolic rate), which may breakdown at a certain

threshold and further lead to failure, inducing permanent effects or even death. We already showed this previously in adult zebrafish [38]. However, in our current study non-hatching of embryos at 36.5°C could not be explained by the presence of specific or more malformations in these embryos at earlier time points, when compared to the minority of embryos that did hatch at 36.5°C. Therefore, we investigated the possible role of the hatching gland and noted a lower hatching enzyme activity at 36.5°C, which may explain the low hatching rate in this temperature group compared to controls (28.5°C). Variability between the two replicates was noted but at 48 hpf both replicates showed a significantly lower cathepsin L activity at 36.5°C compared to controls. Variability in timing of release of the hatching enzymes and consequently of hatching itself under control conditions has been reported previously [27]. Therefore, it is not surprising to see this variability in our replicates as well.

Based on the above, it appears that zebrafish embryos, in contrast to adult zebrafish, have a low upper temperature threshold, which is situated at less than 4°C above the upper limit of optimal developmental temperature (28.5°C).

5. Conclusion

Incubation of zebrafish embryos at 32.5°C and above from 2.5 until 96 hpf causes malformations as early as 24 hpf. From 4 degrees centigrade above the normal developmental temperature, the total amount of affected embryos is significantly higher than at 28.5°C and these embryos mainly show tail malformations.

6. Acknowledgements

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7. Conflict of interest

The authors declare no conflict of interest.

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