Zebrafish embryos and larvae as a complementary model for behavioural research

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Chapter 4

Effect of lighting conditions on development and locomotor activity in embryonic and larval zebrafish

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Abstract

Light plays an essential role in the development of visual and motor systems, and hence in the development of the behavioural repertoires of organisms. This study examines the role of abnormal lighting conditions during development on the hatching process, development and locomotor activity of zebrafish. Embryos were raised from fertilization to 6 days post fertilization in one of three lighting regimes: (i) normal daily light-dark cycle, (ii) constant darkness; (iii) constant light. Morphology, hatching and locomotor behaviour were studied. The results showed that rearing in constant light accelerated the hatching process while constant dark delayed it, as compared to rearing in a normal light-dark cycle. Constant dark also produced an excess of morphological abnormalities and mortality. By contrast, the morphology and mortality did not differ between larvae raised in constant light or in a light-dark cycle. The locomotor activity of the larvae was tested using the visual motor response test. The results showed that the larvae raised in constant darkness were abnormal in that they did not demonstrate a startle response in the test. The larvae raised in constant light conditions showed a normal startle response, but also higher basal activity than those raised in a normal light-dark cycle. The zebrafish larvae which were given a recovery time of two hours in light, after rearing in abnormal lighting conditions, showed normal locomotor activity patterns. Together, these results suggest that abnormal lighting conditions during rearing have a transient and reversible effect on behaviour. It also confirms previous reports that a normal light-dark cycle is essential for normal morphogenesis.

Introduction

In the last few years, the developing zebrafish (both embryonic and larval) has become a useful model in the study of behaviour [45,49,60,173]. It displays a wide variety of complex behaviours such as addiction [295,296], anxiety [219,297-299], learning and memory [43,164,187,300]. One of the readily assayed behaviours displayed by zebrafish is visually-mediated behaviour [240]. In these behaviours, light plays an important role in the response to a visual, external stimulus and the latter can be controlled in quality and intensity. Zebrafish display a number of well-defined behavioural responses of varying complexity to visual stimulation [60].

Light is also important for other physiological responses. Disturbance of the normal light-dark cycle (and therefore the circadian rhythm) is of considerable interest in clinical medicine because it may be involved in common disorders such as insomnia and depression [301]. To gain more insight into these phenomena in humans, it is important to develop assays in other organisms, as has been done with rodents [80]. However, the use of warm-blooded animals is sometimes the subject of ethical discussion.

Assays developed for rodents have been widely used; similar assays in zebrafish have only recently been developed [80]. Nonetheless, the use of zebrafish in developmental research has gained considerable popularity due to certain advantages that the zebrafish model offers. Zebrafish are quick breeders, can lay many eggs which develop rapidly and have transparent tissues at early stages [302], making the progress of development relatively easy to follow [33]. Behavioural data from zebrafish larvae could give us valuable insight into the possible consequences of disturbance of the natural circadian rhythm [80], and this knowledge could complement what has been learned from experiments with rodents [303,304].

The locomotor activity of zebrafish larvae plays an important role in their feeding and survival, and has been studied in detail [135,156]. The zebrafish embryo display a startle response to an external stimulus as early as 30 hpf [32]. Free-swimming episodes occur after larvae hatch from the chorion around 48-72 hpf [134]. After hatching, zebrafish larvae display a wide range of behaviours such as optomotor response [52,53,144], optokinetic response [50,139,140,179,259], visual startle response [46,305], C-starts [145], slow scoots, burst swimming and routine turns [198,204,306], J-turns [148], O-bends [163] and capture swimming [203]. All of these visual behaviours are dependent on the integrity of the musculoskeletal system, nervous system and visual pathways [174].

Adult zebrafish have, like many teleost fishes, a natural preference for dark environments [85], called scototaxis. This is thought to be mediated by anxiety or fear [298,307]. Gerlai *et al.* [225] concluded that early zebrafish larvae do not show the preference for a dark environment shown by adults. In an open field test, larvae initially avoid the dark chamber, but after habituation they spent equal amounts of time in the illuminated and dark chambers. These findings were also confirmed by another recent study [67].

Why zebrafish larvae prefer light environments over dark ones, and whether they can learn to prefer darkness, is still debated. Nonetheless, there is reason to believe that higher locomotor activity in light environments might increase the chance of evading predators [163]. Another reason that could explain why larvae prefer light, whereas adult zebrafish do not, is that the early development of the zebrafish might be influenced by, or dependent on, light conditions.

Studies in several species have demonstrated that the developing visual system is influenced by environmental conditions [65,308-312]. Light is also one of the factors which has been studied for its effect more generally on development and growth of zebrafish [313]. Previous studies have shown that exposure to either constant dark or constant light during embryonic development of zebrafish larvae leads to deficits in visually-mediated behaviour [174] and also causes reduced spectral sensitivity [65]. Little is known about the effects of light conditions, during embryonic development, on the preferences for, or adaptation to, dark or light. It is clear from the published literature summarised above that the prior rearing conditions of larvae, in terms of light and temperature [132], should be known before they are used for behavioural testing. This is likely to be particularly important in assays that examine the effects of light and dark, or of visual cues, including the light/dark preference test [116,314], visual motor response test [49,315], optomotor response test [316] and optokinetic response test [50,51,139,140].

In teleost fish, light influences not only embryo development [317] but also sexual maturation in adults [318]. During larval development, vision, pineal gland and deep brain photoreceptors play important roles [319]. Thus, light is required for vision, foraging and feeding [319,320], for synchronising hatching [278] and for optimal growth [320,321]. The development of larval structures, including fins, teeth and swim bladder is also dependent on light [319]. For these reasons, we wish to study the effect of light on zebrafish larval development and behaviour.

Three different developmental stages of zebrafish larvae for locomotor activity measurement were chosen (4, 5 and 6 dpf). The effects of lighting conditions during rearing on the hatching, mortality and morphology were recorded. The zebrafish embryos were reared under a normal light-dark cycle (LD), constant light (LL) or constant dark (DD) conditions. Finally, the effects of these three lighting conditions on locomotor activity were studied using the visual motor response test (VMR) to see whether the zebrafish larval response to darkness is innate or learned.

Materials and methods

Ethics statement

All experimental procedures were conducted in accordance with local and international regulations. The local regulation *Wet of de dierproeven* (Article 9) of Dutch Law was followed as well as the same law administered by the Bureau of Animal Experiment Licensing, Leiden University. This local regulation serves as the implementation of "*Guidelines on the protection of experimental animals*" by the Council of Europe (1986), Directive 86/609/EC and were performed only after a positive recommendation of the Animal Experiments Committee had been issued to the license holder.

Animal husbandry

Male and female adult zebrafish (*Danio rerio)* of AB wild type were purchased from Selecta Aquarium Speciaalzaak (Leiden, The Netherlands) who obtains stock from Europet Bernina International BV (Gemert-Bakel, The Netherlands). Fish were kept at a maximum density of 12 individuals in plastic 7.5 L tanks (Overall Dimensions (L x W x H): 14.53 x 6.142 x 5.197 inch; Floor area: 67.43 $inch^2$, Tecniplast, Germany) containing a plastic plant as tank enrichment, in a zebrafish recirculation system (Fleuren & Nooijen, Nederweert, The Netherlands) on a 14h light : 10h dark cycle (lights on at 7h AM: lights off at 21h PM). Water and air temperature were maintained at 24 ^{o}C and 23 ^{o}C, respectively. Fish were purchased at the juvenile stage and were allowed to adapt to our facility for at least 2 months before being used as adult breeders. The fish were fed daily with dry food (DuplaRin M, Gelsdorf, Germany) and frozen artemias (Dutch Select Food, Aquadistri BV, The Netherlands).

Zebrafish eggs were obtained by random mating between sexually mature individuals. Briefly, on the day before eggs were required, a meshed net allowing eggs to pass through but preventing adult fish from accessing/eating them, was introduced in the

home tank of a group of 12 adult fish. Each breeding tank was only used once per month to avoid handling stress and ensure optimal eggs quantity and quality.

The eggs were harvested the next day (30 min after the switching on of lights at 07:00 h). They were placed in 9.2 cm Petri dish containing 100 ml egg water (deionized water with 0.2 g/l Instant Ocean, 0.075 g/l NaHCO3, 0.008 g/l CaSO4, and 50 ppb methylene blue to inhibit fungal growth). All eggs were distributed randomly into Petri dishes. Petri dishes were housed in a climate room maintained at a temperature of 28°C and 50% humidity.

Experiment 1: Effect of lighting conditions on hatching and mortality of zebrafish embryos

Zebrafish eggs, after collecting and cleaning for any debris and removing unfertilized eggs, were transferred (one egg per well) to 48-well plates by using plastic Pasteur pipette (VWR International B.V., The Netherlands). After this, these plates were exposed to the appropriate light conditions. A climatic room with a temperature of 28°C was used to maintain a normal light-dark cycle (LD) of 14:10h (lights on at 7:00/lights off at 21:00) [322]. Constant dark (DD) condition was obtained by wrapping the 48-well plates in aluminium foil to prevent any light penetrating into the plate. Constant light (LL) was achieved by placing zebrafish embryos in 48-well plates in a separate climatic room where the white light remained ON all the time (400 lux, measured by digital lux meter MASTECH®, MS 6612). Hatching and mortality was monitored daily until all larvae were hatched. All experiments were done in triplicate. Note that in order to eliminate further sources of disturbance/stress, the egg water was not refreshed.

Experiment 2: Morphological assessment of zebrafish larvae exposed to abnormal lighting conditions

After exposure to different lighting conditions as stated above, all living embryos at 6 dpf were cleared, stained, and stored as described [94]. In short, embryos were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline at pH 7.2 at 4^0C overnight. They were then rinsed 5 times in distilled water and dehydrated in a graded series of ethanol (25, 50, and 70%) for 5 min each. Embryos were rinsed in acid alcohol (1% concentrated hydrochloric acid in 70% ethanol) for 10 min. They were then placed in filtered Alcian blue solution (0.03% Alcian blue in acid alcohol) overnight. Embryos were subsequently differentiated in acid alcohol for 1 h and washed 2 x 30 min in distilled water. Finally, they

were cleared and stored in glycerol. All embryos remained in their original multi-well plates, so that each individual could be tracked throughout the entire experimental and analysis procedure.

Morphological evaluation of the surviving embryos was performed at the end of experiment (144 hpf). Analysis of embryo morphology was carried out using a dissecting stereo microscope for normal development and morphological defects. The phenotypes were scored according to the criteria listed in Table 4.1.

Table 4.1: Phenotypes scored at the end of experiment in living zebrafish larvae having active locomotion, heart beat and transparent tissues. The zebrafish larvae with no locomotor activity, no heartbeat and opaque tissues were considered dead and not included in the analysis.

Phenotype	**Normal**	**Abnormal**
Melanocytes appearance	The melanocytes appears quite distinct and separated from each other.	The melanocyte congregate and disperse forming a cluster.
Yolk sac oedema	Yolk sac is almost depleted at 5 dpf.	yolk sac enlarged by fluid accumulation
Pericardial oedema	No enlargement of pericardial sac	pericardial sac enlarged by fluid accumulation
Bent tail	Straight tail	The tail was bent either dorsoventrally or laterally
Bent body	Straight trunk	The trunk was bent on the dorso-ventral axis
Meckel cartilage	It is the bilaterally paired, rod-like, cartilaginous ventral component of the lower jaw, or ventral mandibular arch	Absence or reduction (both in size and rostral extent) or displacement of the Meckel cartilage
Hatching time altered	Embryo emerges from chorion at 48 – 72 hpf	Embryo remain in the chorion

Experiment 3: Effect of lighting conditions on locomotor activity

This was done in two steps. In the first step, the 48-well plates with one embryo per well were prepared and were moved to respective lighting conditions as stated above. On the day of testing, plates from each lighting regimes were tested immediately for locomotor activity of 4, 5 and 6 dpf zebrafish larvae. In the second step, before behavioural recording, the zebrafish larvae in the 48-well plates were given 2h recovery period in light after taking out from respective light regime to see the differences in the locomotor activity. Note that plates from any lighting regime were not used again.

The ZebraLab was used to automatically record the locomotor activity of larvae in the 48-well plates with the help of VideoTrack software (View Point, S.A., Lyon, France). A light-emitting diode panel (LED) illuminated the 48-well plate from below. Recording was done under infrared light which, like the LED panel, is a fixed component of the ZebraLab system.

A short test, comprising of 14 minutes, and called the 'visual motor response test', was performed to examine the effect on locomotor activity of 4, 5 and 6 dpf zebrafish larvae as described elsewhere [94]. We did not replace or refresh the buffer prior to the experiment. All experiments were done at a temperature of 28 ± 0.5 °C. The visual motor response test has been previously used as frequently alternating periods of light and dark for a very short duration (not more than 10 minutes). This test is used to check abrupt change of locomotor activity (also called as visual startle response) after sudden shift from light to dark [54,55,94,117]. The experimental recording protocol consisted of three phases. First 2 minutes were given in the ZebraLab to acclimatize to the new environment. This phase was necessary to make sure that basal locomotor activity of zebrafish larvae is without any bias resulting in handling of the plate or change of location and hence was not used in the further analysis. After this acclimatization, basal phase started consisting of 4 minutes to measure the basal locomotor activity while light in the ZebraLab remained ON. Immediately after basal phase, the lights were suddenly turned off for 4 min to record sudden change of locomotor activity which is called as 'challenge phase'. Behavioural activity in the dark was also automatically recorded during this period with the help of infrared light. A third phase called 'recovery phase' was started immediately for 4 min in light after challenge phase to give zebrafish larvae time to recover from shock of darkness. All three phases consisted of 4 min to prevent habituation, and also to obtain more robust

responses. At the end of the behavioural testing, the larvae were euthanized with an overdose of tricaine mesylate (MS-222).

Behavioural end-points

Video images were analysed in ZebraTrack software (Viewpoint, Lyons, France). Other publications using this software include [127,323]. The parameter measured was total distance moved (mm) over time.

Statistical analysis

Statistical analysis and graphs were performed using GraphPad Prism version 5.04 for Windows, GraphPad Software, San Diego California USA (www.graphpad.com). One-way ANOVA test was performed to analyse mortality among larvae exposed to different abnormal lighting conditions (Figure 4.1B). A Dunnett's post hoc test was used to analyse multiple comparisons. Two-way ANOVA analyses were performed to determine effect on hatching (Figure 4.1A), malformations (Figure 4.2) and locomotor activity of the zebrafish larvae (Figure 4.3A-C, Figure 4.4A-C). Significant main effects were further decomposed using pair-wise comparisons with a Bonferroni's correction for multiple comparisons. Data are presented as mean ± SEM, and a probability level of 5% was used as the minimal criterion of significance.

Results

Effect of abnormal lighting conditions on hatching and mortality

The hatching of zebrafish embryos was monitored until they all hatched in all light regimes and the results are shown in Figure 4.1A. Two-way ANOVA analysis reveals a significant interaction between developmental stage and lighting regimes [$F_{(18,56)}$=188.4, $p<0.0001$]. A post hoc Bonferroni test was used for multiple comparisons. The results show a significant difference in terms of hatching percentage between LL and DD larvae as compared to LD larvae. Specifically, the larvae raised in LL (regardless of time of exposure to LL except LL 1-24) hatched early ($p<0.0001$) as compared to LD larvae. The LL 1-24 larvae at 48 and 72 hpf did show significant delay in hatching as compared to LD larvae ($p<0.001$).

The larvae raised in DD took a significantly longer time to hatch as compared to LD and LL larvae ($p<0.0001$). The stage at which the embryos were exposed to the constant dark

period had little or no effect on the hatching rate. Thus, embryos in DD from 1-24h, 25-48h and 1-48h all had a similar, extremely low level of hatching (Figure 4.1A). By contrast, the acceleration of hatching in LL embryos appeared to be influenced by the length of exposure to light. Thus hatching was more accelerated in embryos exposed to continuous light than in embryos with periodic exposure of light.

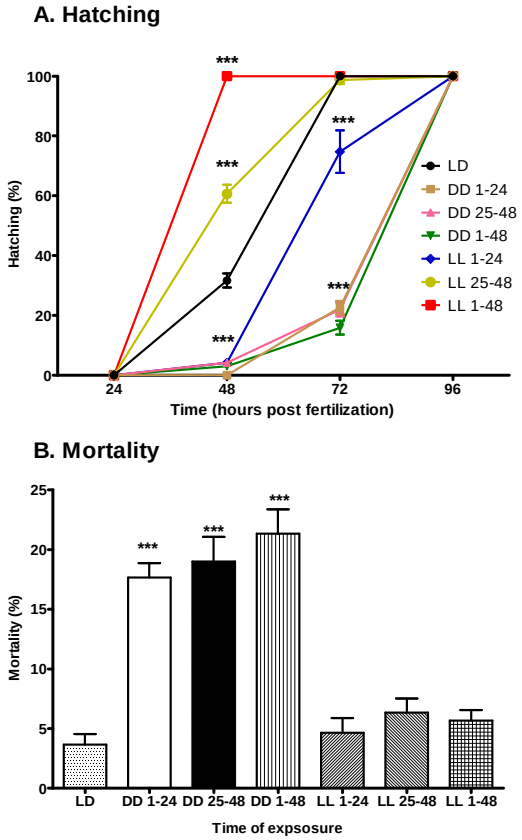

Figure 4.1Hatching and mortality of zebrafish embryos exposed to abnormal lighting conditions at different time points. (A) Hatching; zebrafish larvae showed delayed hatching when exposed to dark regardless of the exposure time. Constant light led to precocious hatching. (B) Mortality; the zebrafish larvae exposed to constant dark showed higher mortality as compared to larvae exposed to constant light or normal alternating light/dark cycle. DD=constant dark, LL=constant light, Control=Normal light/dark cycle while numbers after these abbreviations corresponds to the time period in which they were exposed to these conditions in hours. ($p<0.05$)

Figure 4.1B shows the mortality of the embryos exposed to abnormal lighting conditions. A one-way ANOVA analysis for repeated measures shows that DD larvae (regardless of their time of exposure to DD) had higher mortality as compared to LL and LD larvae [$F_{(6,20)}$=29.23, p<0.0001]. The mortality in LL larvae didn't differ from that of LD larvae regardless of their time of exposure to LL (p>0.05).

Effect of abnormal lighting conditions on the morphology of zebrafish larvae

The results of malformations observed are shown in Figure 4.2. Two-way ANOVA analysis showed a significant interaction between light regimes and types of malformation observed [$F_{(8,20)}$=36.92, p<0.0001]. A post hoc Bonferroni test was used to evaluate multiple comparisons. The results show a significant difference between the number of malformations observed in DD larvae as compared to LL and LD larvae (p<0.0001). Specifically, DD larvae showed higher susceptibility for yolk sac oedema, pericardial oedema, dispersed pigmentation, bent tail or bent body axis and malformed Meckel cartilage. However, there was no significant difference between LL and LD larvae for any malformation observed (p>0.05).

Effect of abnormal lighting conditions on visual motor response

4 dpf

Figure 4.3A shows the effect of abnormal lighting conditions on the locomotor activity of 4 dpf larvae immediately after taking out from their respective light regime. A two-way ANOVA analysis reveals a significant interaction between light conditions and activity phases [$F_{(4,99)}$=16.71, p<0.0001]. A post hoc Bonferroni test was used to further decompose the interactions and correct for multiple comparisons. The results show a significant difference between the basal locomotor activity level of LL and DD zebrafish larvae compared to LD larvae. The basal locomotor activity level was significantly high in case of LL than either DD or LD larvae (p<0.001).

The LL and LD larvae responded to sudden offset of light and their activity was significantly higher in challenge phase but DD larvae responded poorly (p<0.0001). In the recovery phase, LL larvae again showed higher activity than DD or LD larvae (p<0.001).

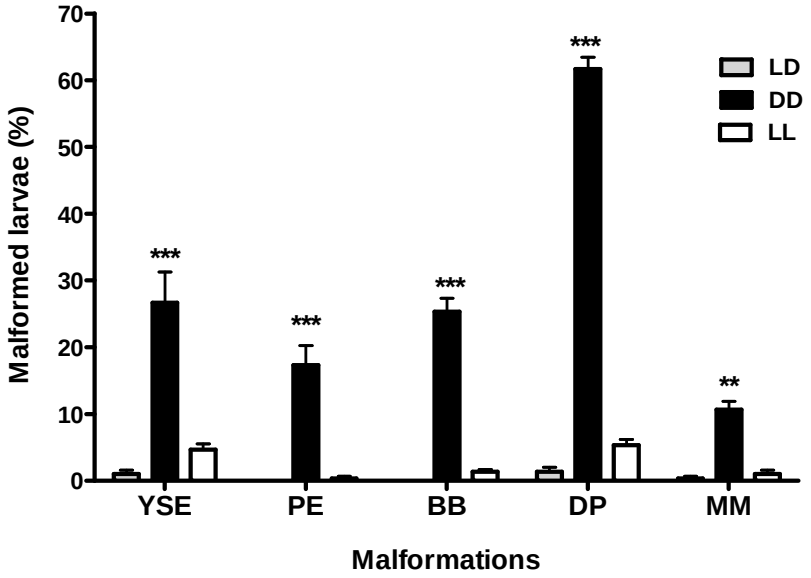

Figure 4.2. Morphological assessment of zebrafish larvae. The zebrafish larvae exposed to constant dark (black bars) showed a higher number of malformations. The zebrafish larvae raised in constant light (white bars) didn't differ from the larvae raised in normal light/dark cycle. YSE = Yolk sac oedema, PE = pericardial oedema, BB = bent body axis or tail; DP = dispersed pigmentation; MM = malformed Meckel's cartilage. (p=0.0004)

5 dpf

The locomotor activity levels of 5 dpf zebrafish larvae in all three phases of VMR test are presented in Figure 4.3B. A two-way ANOVA analysis reveals a significant interaction between light conditions and activity phases [$F_{(4,99)}$=10.88, p<0.0001]. A post hoc Bonferroni test was used to further decompose the interactions and correct for multiple comparisons. The results show that DD larvae moved significantly less in the basal, challenge and recovery phases when compared to LL and LD larvae.

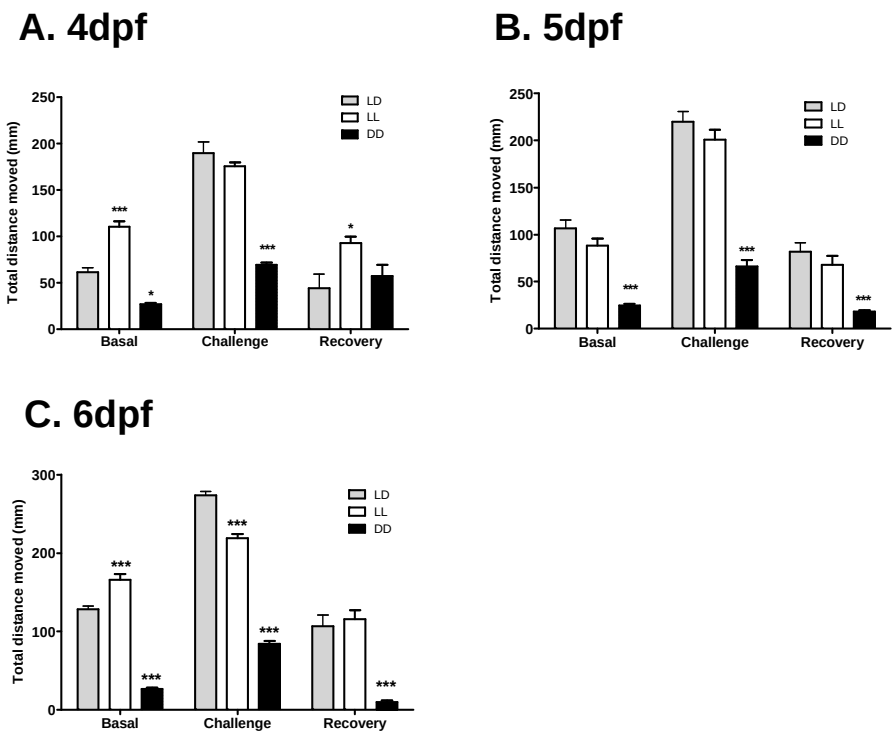

Figure 4.3. Locomotor activity of the zebrafish larvae, tested immediately after taking out from their respective light regimes. (A) The LL and DD zebrafish larvae showed significantly higher locomotor activity in the basal base. However, their response to sudden darkness in challenge phase was not significantly different from the control larvae raised in normal light-dark cycle. (B) The response of 5 dpf larvae to the sudden onset of dark in the challenge phase was not different in any light conditions. However, basal locomotor activity in dark reared larvae was significantly less than LL and LD larvae. (C) The LL and DD zebrafish larvae also showed a higher basal locomotor activity level as compared to LD larvae. The response to sudden onset of dark in challenge phase was not significantly different. Data are presented as ±SEM, $p<0.05$

6 dpf

Effect of abnormal lighting conditions on 6 dpf larvae has been shown in Figure 4.3C. A two-way ANOVA analysis reveals a significant interaction between light conditions and activity phases [$F_{(4,99)}=16.14$, $p<0.0001$]. A post hoc Bonferroni test was used to further decompose the interactions and correct for multiple comparisons. The DD larvae showed a significantly less movement in the basal phase compared with LD and LL larvae

($p<0.0001$). The DD larvae also failed to respond to challenge test during light off and moved significantly less as compared to LL and LD larvae.

Zebrafish larvae recovery from abnormal lighting conditions

4 dpf

The zebrafish larvae locomotor activity in response to VMR after giving recovery time of two hours in constant light is shown in Figure 4.4A. A two-way ANOVA analysis reveals a significant interaction between light conditions and activity phases [$F_{(4,123)}=9.313$, $p<0.0001$]. A post hoc Bonferroni test was used to further decompose the interactions and correct for multiple comparisons. In the basal phase, the activity of DD and LL zebrafish larvae after recovery was significantly higher than LD larvae ($p<0.0001$). However, in the challenge phase, the zebrafish larvae responded equally and no significant differences were found in the response to sudden darkness ($p>0.05$). In the recovery phase of the VMR test, the locomotor activity of LL larvae was significantly higher than either DD or LD larvae ($p<0.01$).

5 dpf

A two-way ANOVA analysis followed by post hoc Bonferroni test was used to analyse activity of zebrafish larvae in response to VMR of 5 dpf zebrafish larvae after 2h recovery (Figure 4.4B). The analysis revealed a significant interaction between lighting regimes and locomotor activity phases [$F_{(4,135)}=9.811$, $p<0.0001$].The DD zebrafish larvae moved significantly less in the basal phase as compared to LL or LD larvae ($p<0.0001$) while in the challenge phase there was no significant difference among zebrafish larvae in any lighting regime ($p<0.05$). In the recovery phase, LD zebrafish larvae moved significantly more than LL ($p<0.01$) and DD ($p<0.0001$).

6 dpf

The results of locomotor activity in the VMR test, of 6 dpf zebrafish larvae raised in abnormal lighting regimes then given 2h recovery, are shown in Figure 4.4C. A two-way ANOVA analysis was used, followed by post hoc Bonferroni test for multiple analysis which showed significant interaction between lighting conditions and locomotor activity phases [$F_{(4.99)}=18.20$, $p<0.0001$]. The DD and LL zebrafish larvae moved significantly more in the basal phase as compared to LD larvae ($p<0.0001$). In the challenge phase, the zebrafish larvae in all lighting regimes responded equally and no significant differences

were found among them (p>0.05). In the recovery phase, the LL zebrafish larvae moved significantly more as compared to DD or LD larvae (p0.01).

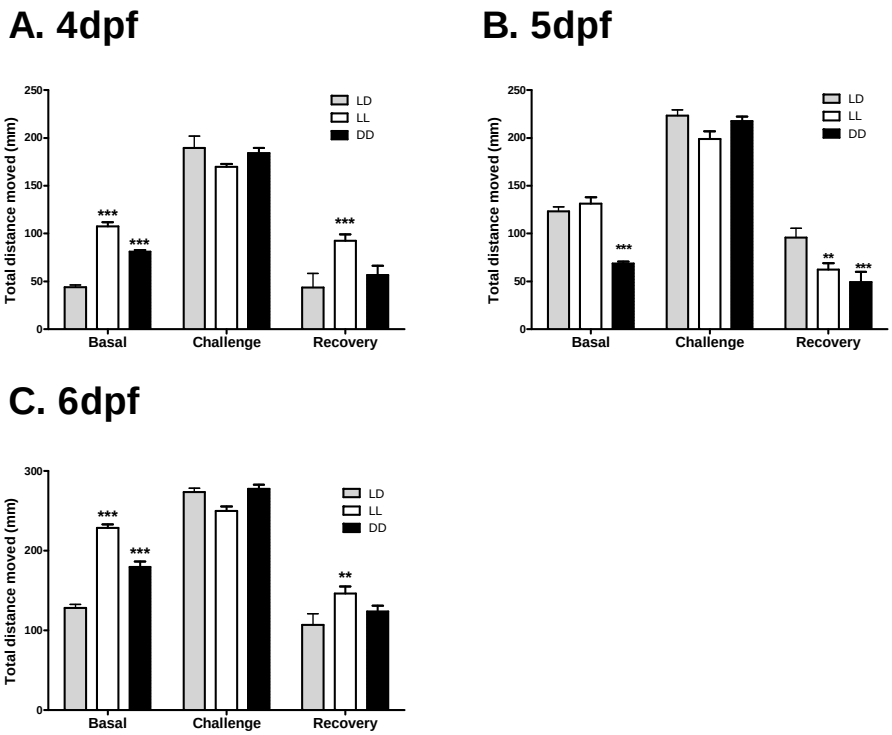

Figure 4.4. Locomotor activity of the zebrafish larvae, tested after taking out from their respective light regimes and giving them 2 hours of recovery time in constant light. (A) The LL and DD zebrafish larvae showed significantly higher locomotor activity in the basal base. However, their response to sudden darkness in challenge phase was not significantly different from the control larvae raised in normal light-dark cycle. (B) The 5 dpf larvae response to the sudden onset of dark in the challenge phase was not different in any light conditions. However, basal locomotor activity in dark reared larvae was significantly less than LL and LD larvae. (C) The LL and DD zebrafish larvae also showed a higher basal locomotor activity level as compared to LD larvae. The response to sudden onset of dark in challenge phase was not significantly different. Data are presented as ±SEM, p<0.05

Discussion

Hatching and mortality

In the present study, constant darkness delayed the hatching process irrespective of the length of darkness which is known to delay hatching in *Artemia* [324]. Zebrafish embryos normally hatch between 48-72 hpf [33,43,322]. The chorion of zebrafish embryo undergoes a thinning process called ‘‘chorion softening’’ before the larva emerges [325]. Proteolytic enzymes from the hatching gland cells facilitate this process [326]. This process is influenced by many factors such corticosteroids [327,328], epinephrine [329], the anaesthetic MS222 [329] and electric current [330]. The timing of the teleostean hatching process with mechanical release of hatching enzyme has also been studied in detail [331] while temperature and oxygen content of the water are known to influence the time at which fish embryos are hatched [329]. Taken together, all these factors suggest that not only environmental factors but also nervous and/or endocrine system may also be involved in determining the timing of hatching. Delayed hatching in DD larvae suggest that light is another factor which is essential for hatching.

The delayed hatching in LL (1-24 hpf) larvae and precocious hatching in LL(25-48 hpf) larvae suggests that light plays important role during 2 dpf. The precocious hatching of larvae in continuous light in the present study suggests that light is another environmental factor for regulating the time of hatching. Early hatching in embryos reared under LL conditions could indicate that the general development of the embryos had been accelerated. However, this was not the case, as all embryos at 48 hpf were at the same stage (long-pec) as the DD- and LD-reared embryos. The other possible explanation of early hatching could be that some select aspect of hatching, such as secretion of proteolytic enzymes into the perivitelline space, has been accelerated relative to the rest of the embryo’s development.

Continuous exposure to light might also act as a stressor. It has been found that zebrafish embryo as old as 30 hpf respond to changes in illumination by means of a robust motor excitation phase characterized by vigorous shaking (a behaviour known as the photomotor response [46]). Continuous movement in response to light may help to break the chorion mechanically. However, these phenomena were not studied in detail here and remain to be investigated.

The adult zebrafish spawns every 2-3 days and produces several hundred eggs per clutch. However, not all the eggs survive, and a natural mortality of 5-40% may occur, depending on density, handling and temperature [332]. Some laboratories have reported a natural spontaneous mortality of less than 10% in their zebrafish early embryos [98,242]. The influence of individual embryo variability was addressed here by randomization of treatment. In the present study, the mortality of the LD and LL embryo was 3.7% and 5.7% respectively at the end of experiment. The mortality in DD larvae, however, was significantly higher (19%) at the same time point. These results are in accordance with another study where zebrafish larvae raised in the dark had a lower survival rate than embryos raised in diurnal light-dark cycle [174]. It will be important to identify mechanisms by which abnormal light conditions during rearing lead to higher mortality.

Morphological abnormalities

Light plays an important role in the normal development of fish and can affect many processes depending on the photoperiod and the intensity of light and its duration [311]. The LL zebrafish larvae did not show an excess of malformations during development compared to LD larvae. These results are in accordance with another study [174] in which zebrafish larvae exposed to constant light were not phenotypically different from controls. They hatched on time, had a similar survival rate and began swimming at the same time [174]. By contrast, the DD zebrafish larvae in present study had a number of malformations including bent tail or body axis, malformed Meckel cartilage and pericardial oedema. Constant dark had a significant effect on the pigmentation. The DD larvae had a dispersed pigmentation phenotype as compared to LD or LL larvae. The number of larvae with yolk sac oedema was also higher as compared to LD and LL larvae.

In the zebrafish, pineal photoreceptors embody an endogenous circadian oscillator which is synchronized to a 24 h daily cycle for rhythm of melatonin secretion [333]. In the absence of light, light-dark rhythm of zebrafish larvae might have been disturbed. Furthermore, light has a great influence on pigmentation and serious developmental abnormalities appear when light is insufficient [334]. Dispersed pigmentation has also been seen as a sign of stress [335]. These results together correlate well with our findings in the locomotor activity analysis, where DD larvae showed a low level of basal locomotor activity along with low response to sudden onset of darkness during VMR.

Locomotor activity

The basal locomotor activity of LD zebrafish larvae at 4 dpf in response to the VMR test is lower than that of 5 and 6 dpf larvae. However, the response to sudden darkness is the same. The lower activity in the basal phase has be attributed to that fact that the larva is in a transitional developmental stage when the swim bladder is not yet inflated [32]. Thus, inflation of the swim bladder is an essential step in the transformation from a larva that has intact, touch-evoked swimming responses but negligible spontaneous swimming, into a free-swimming and self-feeding larva with a beat-and-glide motor system. Presence of the swim bladder also enables larvae to manoeuvre in all three dimensions [135,170,336,337]. It has been shown by Thirumalai and Cline [170] that zebrafish larvae at 3 dpf are capable of generating fictive swimming episodes at a high frequency but that the initiation of episodes is suppressed by endogenously released dopamine. These findings are in accordance with other studies which showed that the locomotor activity of 3-4 dpf zebrafish larvae is minimal [32,128].

The results of this study indicate that lighting conditions during initial development affect zebrafish locomotor activity. The zebrafish larvae raised in constant dark showed relatively little response to the sudden onset of darkness, as compared to LD larvae, at all developmental stages tested. This is not very surprising considering that they were raised in constant dark and so one might expect the light to be more unfamiliar, and therefore stressful. However, the DD zebrafish larvae also showed a very low level of basal locomotor activity level in light as compared to LD and LL larvae. This might be an indication that the musculoskeletal, nervous or visual systems of the zebrafish larvae did not properly develop in the absence of light. Although these aspects were not studied, previous work suggest that zebrafish larvae raised in constant dark had no motor defects and responded like control larvae when their visual behaviour was tested using the optomotor response [174].

The response to the sudden onset of darkness during the VMR test in both LL and LD larvae was similar. This suggests that light plays a more important role than dark in normal development including the development of the normal behavioural repertoire. Zebrafish larvae raised in constant light showed a higher basal locomotor activity than DD and LD larvae, but their response to sudden darkness was not different from that of LD larvae at any developmental stage tested. In a previous study [174], a decline in visual acuity was noticed when zebrafish larvae were exposed to constant light. However, the present results

show that the zebrafish is at least able to discriminate between light and dark and to respond in the same manner as LD larvae.

It appears that the cyclic nature of the lighting conditions is a very important factor in visual development and can have drastic effects on various systems such as visual, nervous and skeletal system [310,311,338]. The 4 dpf larvae showed a lower locomotor activity and response to sudden darkness. This might be due to the fact that during early stages, wiring of the visual and nervous systems is taking place, while by contrast, the 5 dpf larvae are well-developed with most of their organs differentiated, and they can spontaneously swim aided by their possession of an inflated swim bladder [106,165]. No difference was found in the locomotor activity between 5 and 6 dpf larvae in terms of basal phase and challenge phase. Thus, we suggest that larvae at 5 dpf or older should be used when studying swimming behaviour. Further, it has also been shown in another study [174] that timing of exposure to abnormal lighting condition is an important determinant of the severity of the effects. That study reported that one missing dark cycle can produce drastic changes in visual acuity. Together, these finding suggest that zebrafish larvae have an intrinsic response to the sudden onset of darkness, rather than acquiring this response from the environment.

Surprisingly, DD zebrafish larvae showed a restored response to the sudden onset of darkness after receiving a 2h recovery time. This suggests that the startle response to the sudden onset of darkness is intrinsic and not learned. However, prolonged exposure to either constant light or constant dark may lead to adverse effects on development. Thus, it is evident from the results in this study and others [174,313] that light plays an essential role in normal development . It has been shown in previous studies that zebrafish locomotor activity is regulated by a circadian clock [339,340]. However, this aspect is sometimes ignored or at least not mentioned in the studies [170,198,341,342]. Our future work will focus on circadian mechanisms and on the physiological changes that take place due to the absence of either light or dark exposure.

Conclusions

We have shown that the zebrafish larvae at 4 dpf is in a transitional stage between a less active larva that shows no spontaneous swimming, and the 5 dpf a larva with a more robust, spontaneous swimming activity. This transition might be associated with inflation of the swim bladder. Thus, 5 dpf larvae are well suited for behavioural assays. A diurnal light/dark cycle is needed for the development of a normal locomotor repertoire. Abnormal

lighting conditions can have drastic effects on hatching and behaviour. Furthermore, constant darkness can result in various malformations and high mortality.