Zebrafish embryos and larvae as a complementary model for behavioural research

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Chapter 5

Visually-mediated hyperactivity and habituation in larval zebrafish

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Abstract

Zebrafish larvae show hyperactivity, following the onset of sudden darkness which is known to wane over time during the same dark phase. It has been suggested in previous studies that the reduction in hyperactivity might be due to habituation but this has not been studied in detail. Here, we examined this issue by exposing wildtype zebrafish larvae at 4, 5 or 6 dpf to 1, 5 or 10 min intervals of alternating light and dark for 3 h. Movements of the larvae were tracked using video-recording and Zebratrack software. Zebrafish larvae at all stages tested with 10 min intervals showed hyperactivity during the dark phase which waned within the 10 min. However, during the next dark phase, zebrafish larvae again regained its previous peak value. Hence there was no habituation. Zebrafish larvae at 4 and 5 dpf showed hyperactivity with 5 min intervals which decreased in the first 3 dark phases and then it reached a constant level. Zebrafish larvae at 6 dpf also displayed hyperactivity but it did not wane over the subsequent 5 min phases. With a 1 min interval, larvae of all age groups tested showed hyperactivity which quickly waned and become stable within the first hour. To distinguish between habituation and fatigue, a sound stimulus, instead of a light-dark transition, was used after habituation with 1 min interval. A spike in locomotor activity after sound stimulus confirmed that the reduction in hyperactivity after the sudden onset of darkness was due to habituation. Light-to-dark transitions produced more spike in activity than in the dark-to-light transitions in all experiments. Together, these results show that zebrafish larvae are able to habituate to the sudden onset of darkness.

Introduction

The zebrafish has become a promising model in various fields of research in recent years. For example, both adult and larval zebrafish have been extensively used in behavioural studies which examine patterns of locomotion under various conditions [63,87,113,122,128,156,209,323,343,344]. A simple form of behaviour ('spontaneous coiling') is displayed as early as 17 hours post fertilization (hpf), and other more complex behaviours have been investigated, such as the photomotor response [46], the escape response [48,123,137], evoked swimming [134,138], the optomotor response [52,53,143,144], colour preference [345] turning behaviour [137,198], prey capture [137,147,148,198], anxiety-like-behaviours [219,297-299] and learning and memory [43,164,187,300] at various stages of larval development. Zebrafish have been used at different developmental stages for various assays involving locomotor activity [122,129,170,339] and information is available on larval zebrafish locomotor development at early stages [134], and

In many of the behaviours displayed by zebrafish larvae, (for example in prey capture and predator avoidance), vision, and a well-developed locomotor system play a pivotal role [171]. Zebrafish larvae can react to change in light intensities as early as 30 hours post fertilization (hpf), when they are still in the chorion, by exhibiting spontaneous movement [46]. After coming out of the chorion at around 3 days post fertilization (dpf), zebrafish embryos become free-swimming larvae but still rely on the yolk sac for food. However, as the yolk sac is rapidly becoming depleted, the larva will start feeding independently after 6 dpf when the yolk is completely utilized. At this time, rapid development of the visual and locomotor system takes place and the zebrafish larvae are able to take food in either by suction feeding while stationary or by engulfing the food after briefly swimming towards it [198]. Zebrafish initiate an escape response when threatened by a potential predator and by means of this innate behaviour, zebrafish visual performance can be assessed [172]. It is also known that dark acts a threatening stimulus for larval zebrafish, and that light environments are preferred over dark ones [122,173]. This stands in contrast to rodents, where the preference and aversion are reversed [84,346]. In the case of adult zebrafish, there are reports of dark preference [85] as well as light preference [225] and its seems that different experimental designs may have influenced the outcomes of these studies. There may also be an effect of ambient light levels used in one of the studies [314].

The visual motor response (VMR) test has been extensively used to observe the startle response in larval zebrafish in order to test the integrity of zebrafish visual and locomotor

systems [55,94,174]. In this simple test, larvae are placed in microtiter plates in a closed automated video tracking chamber. A light panel below the microtiter plate consists of white light from light emitting diodes, and an infrared light source, both of which can be controlled with the help of software. To initiate a startle response, light is suddenly switched off, and changes in larval activity are recorded with the help of a video camera and the infrared light which remains on throughout the test period.

In the VMR test, hyperactivity is induced by the light-dark transition [49]. However, this response soon wanes within each transition [122]. This waning of the response might be an indication of behavioural habituation, the simplest form of learning [347] also referred to as non-associative learning in a wide range of animals [348]. Habituation has been defined as "reduction in response by repeated exposure to novel stimulus which could be either positive (food) or negative (shock)" [191,192]. While adult zebrafish have been used to study habituation response to novelty [349-351], zebrafish larvae have also been examined for habituation in response to sound stimuli [352]. However, little information is available on whether zebrafish larvae are able to habituate to a dark challenge.

Here, the basal locomotor activity of zebrafish larvae is measured in the light phase (after 2 min acclimatization), by recording their activity with the help of an automated video-tracking device. Then, light is suddenly switched off (the 'dark challenge'), and any changes in locomotor activity recorded with the help of infrared light. It has been shown that zebrafish larval locomotor activity increases during the dark challenge and that it returns to basal levels after the light is switched on again. This test is helpful for checking the integrity of visual, nervous and locomotor system development, and has been used in various assays [55,94,174].

Here, we used the VMR to estimate the effect on locomotor activity of changes in lighting and whether zebrafish larvae are able to habituate to light and dark stimuli. We carried out this study to see if zebrafish larvae are able to habituate to repeated stimuli of alternating light and dark, and whether their response fulfils the criteria of habituation [192]. Decrease in the intensity of hyperactivity might be due to true habituation, or to fatigue caused by lack of energy. In principle, repeated stimuli could cause exhaustion and hence a reduction in the hyperactivity . If a reduction in hyperactivity occurs with stimulus A, then stimulus B is presented after the decrease in hyperactivity to test whether reduction in response is due to true habituation or to fatigue. In the case of true habituation, the hyperactivity is re-induced by the new stimulus (B), a phenomenon called dishabituation. If, however, the animal fails to respond to stimulus B, it is considered to be fatigued.

Although a decrease in locomotor activity has been observed after a brief period of hyperactivity in response to the onset of darkness [122], this phenomenon has not been investigated further to determine whether it is true habituation or fatigue. In the present study, we not only characterize the basal locomotor activity levels of the zebrafish larvae but also use simple VMR test consisting of alternating light-dark phases of varying periodicity to produce a hyperactivity . The alternating light-dark stimulus was repeated to see whether zebrafish larvae are able to display habituation at different developmental stages. To see whether reduction in the hyperactivity is due to true habituation, we also used a sound tone as stimulus B after habituation was observed.

Materials and Methods

Statement of ethics on animal use

All experimental procedures were conducted in accordance with The Netherlands Experiments on Animals Act that 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 (1145, Tecniplast, Germany) containing a plastic plant as tank enrichment, in a zebrafish recirculation system (Fleuren & Nooijen, Nederweert, The Netherlands) on a 14 h light: 10 h dark cycle (lights on at 07:00 AM: lights off at 09:00 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 (16 h) 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 onset of lights at 07.00 AM) and age was set as day post fertilization (dpf) 1. They were placed in 9.2 cm Petri dishes containing 100 ml egg water (0,21 g/l Instant Ocean Sea Salt and 0.0005% (v/v) methyl blue). Fifty to sixty eggs were place in one Petri dish in a climate room maintained at a temperature of 28 °C and 50% humidity and under a diurnal light-dark cycle of 14h:10h (lights on at 07:00 / lights off at 21:00). Note that in order to eliminate further sources of disturbance or stress, the medium was not refreshed except on 2 dpf where the medium was completely replaced by fresh egg water, and non-fertilized eggs were removed.

On 2 dpf, after removing unfertilized eggs and refreshing the egg water, each larva was gently taken up into a plastic Pasteur pipette (VWR International B.V., The Netherlands) and directly transferred to 48-well plate, one larva per well, each well containing 500 µl egg water. At the end of the behavioural testing, the larvae were rapidly euthanized with an overdose of tricaine mesylate (MS-222).

Experimental procedures

Plating of zebrafish larvae

Fertilized eggs were washed and then placed in 9.2 cm Petri dishes at a density of 50-60 embryos per dish. The embryos remained in the Petri dishes until 24 hpf. After this, dead embryos were removed and live embryos transferred to 48-well plates, one embryo per well, each well containing 5 ml egg-water. The water was not refreshed for the duration of the experiment in order to avoid stress associated with egg water exchange.

Video-tracking device

To generate light-dark stimuli and track the movement of zebrafish larvae in response to that stimulus, the ZebraBox (View Point, Lyon, France) was used. This system monitors movements using automated video recording. Other publications using this software include [127,323]. The Zebrabox consist of microtiter plate holder, a video camera, a base to hold the microtiter plates, all enclosed in a box. The Zebrabox is run with a custom video processing software called Videotrack. A light-emitting diode (LED) illuminated the plate from below to track larvae during light and infrared panel used for tracking larvae during darkness. Both panels are a fixed component of the Zebrabox behavioural system.

Locomotor activity was assessed by a subtraction method used for detection of objects darker than background with a minimum object size. A threshold of 0.1 mm (minimum distance moved) was used for filtering all of the data to remove system noise. Locomotor endpoints were designed to express the changes in the general swimming activity in response to light-dark stimulus. The zebrafish larvae were acclimatized for 30 min before the start of light-dark stimulus to rule out effect of handling the plates which can affect locomotor activity.

Behavioural endpoints

The variables studied were speed of larval movement, number of active larvae and cumulative distance moved. The detailed methods are described below.

Experiment 1: Locomotor activity of larval zebrafish across developmental stages and time of day

First, we aimed to optimize the developmental stage of zebrafish larvae to be used in testing locomotor activity. To do this, zebrafish larvae of 4, 5 and 6 dpf were tested for their normal locomotor activity during light in the Zebrabox. The locomotor activity of the zebrafish larvae was recorded in the morning (09:00-12:00) and the afternoon (14:00-17:00 h) to look for a possible effect of time of the day [122].

Experiment 2: Habituation in larval zebrafish

We carried out the VMR test over a total of 3 hours, and with different inter-stimulus intervals (ISIs) to observe any change in the locomotor activity across time. We define the ISI's as follows:

10 min ISI: The zebrafish larvae were presented with alternating light and dark phases of 10 min each.

5 min ISI: This group was subjected to alternating light and dark phases of 5 min each.

1 min ISI: This group was subjected to alternating light and dark phases of 1 min each.

Experiment 3: recovery of hyperactivity and dishabituation

To further distinguish between habituation and fatigue, two experiments were performed as follows.

Recovery of hyperactivity

To differentiate between habituation and fatigue, two experiments were performed. In the first test, a basal locomotor activity level based on distance moved over time was established with 30 min in light. After this, larvae were exposed to alternating light and dark stimuli with 1 min ISI for one hour to see whether habituation took place. In the second phase, a light period of 30 min was given to allow the animals to recover and return to basal locomotor activity level. This was then followed by an hour of alternating light and dark with 1 min ISI to see whether they respond with the same magnitude of hyperactivity as in the first phase.

Dishabituation

In the second test, alternating light-dark stimuli with 1 min ISI were presented for three hours. However, at the start of the second and third hours (62 and 122 minutes, respectively), a sound tone was presented in place of dark stimuli to see whether decline seen in the first hour is due to exhaustion or habituation. The sound tone was 5 s in duration and was generated by a speaker placed 10 cm away from the plate inside the Zebrabox). The frequency of the tone was 220 Hz, 118 dB measured by Voltcraft SL-100 (Voltcraft, Hiraschau, Germany).

Statistical analysis

Statistical analysis was performed using SPSS© v. 19.0.0 for Windows (SPSS Inc.). In general, data from each study were compared using repeated-measures ANOVA. Activity throughout a test session was the dependent variable, whereas time of day and lighting condition (light vs. dark) were the independent variable. Significant interactions between time within a session and an independent variable were followed by step-down ANOVAs to assess lower-order effects. Total activity level was analysed using a chi-square (χ^2) test. P-values less than 0.05 were considered to be significant.

Results

Basal locomotor activity: effect of time of day

The basal locomotor activity of zebrafish larvae was assessed in the morning (09:00-12:00) and afternoon (14:00-17:00) for 4, 5 and 6 dpf and is shown in Figure 5.1. Two-way ANOVA analysis revealed significant interaction between developmental stage and time of day [$F_{(2,210)}$ =4.013, p=0.019]. The results further showed that the locomotor activity of the

4 dpf larvae was significantly lower than that of 5 and 6 dpf larvae ($p<0.0005$) while locomotor activity of the 5 dpf larvae was significantly higher than 4 and 6 dpf larvae ($p<0.0005$). There was no significant effect of time of day ($p=0.053$) as locomotor activity of zebrafish larvae in the afternoon did not differ significantly from that of morning.

Total activity level

The total activity level was assessed by counting the number of active larvae during the test period for a particular developmental stage (Figure 5.2). The 4 dpf zebrafish larvae were much less active 15/48 (31% of larvae showing movement) than the 5 dpf (39/48, 81%) and 6 dpf larvae (28/48, 58%) in the morning ($\chi^2 = 60.04$, $p < 0.0001$). There was no significant difference between morning and afternoon activities of the larvae showing 32, 84 and 65% activity at 4, 5 and 6 dpf, respectively ($\chi^2 = 0.096$, $p = 0.95$).

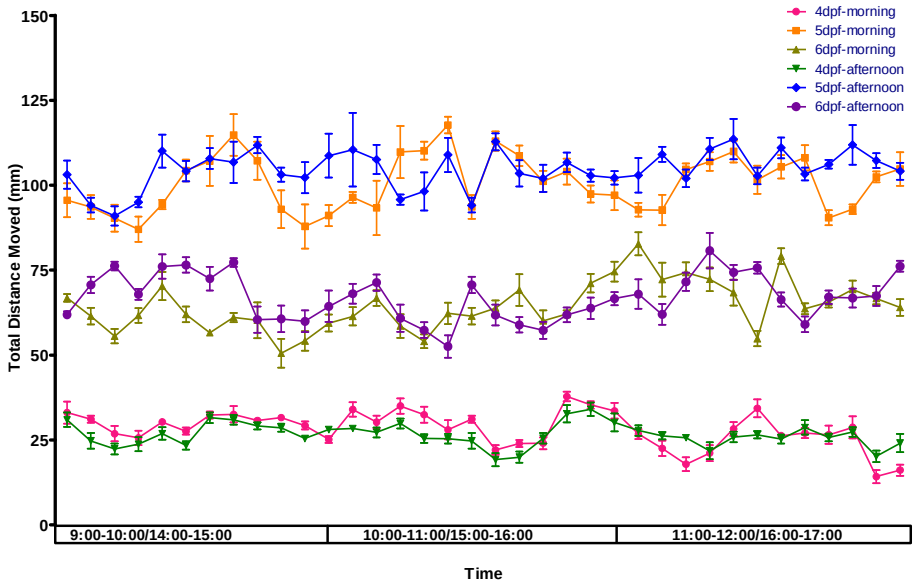

Figure 5.1 Basal locomotor activity. The locomotor activity of zebrafish larvae was recorded in the white light at 4, 5 and 6 dpf in the morning or afternoon. The 5 dpf zebrafish larvae were most active while 4 dpf were least active in terms of total distance moved. The activity of 6 dpf larvae was intermediate. However, no significant effect of time of day was observed. (n=48 in each case)

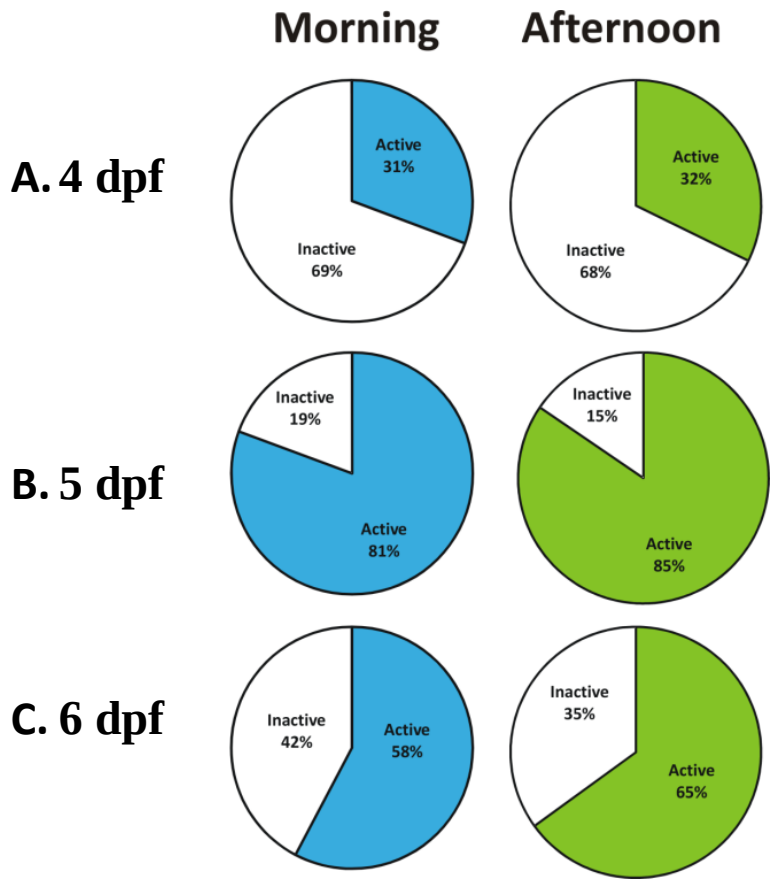

Figure 5.2: Activity of zebrafish larvae. The number of active and inactive zebrafish larvae in morning and afternoon are shown for (A) 4 dpf (B) 5 dpf (c) 6 dpf. The 4 dpf zebrafish larvae were least active while 5 dpf were most active while 6 dpf larvae showed itnermediate activity. No difference in the number of active larvae was found between morning and afternoon sessions. (n=48 in each case)

Habituation of zebrafish larvae to light-dark stimuli with different ISIs

To see whether zebrafish larvae are able to habituate to repeated stimuli of onset of darkness, we used three different ISIs of alternating light and dark cycles.

Locomotor activity in response to 10 min ISI

The locomotor activity of 4 dpf larvae is shown in Figure 5.3. Two-way ANOVA analysis revealed no significant interaction between lighting conditions and time [$F_{(8,162)}$ =0.258, p=0.978]. However, there was a significant effect of lighting conditions. The locomotor activity in the dark phases was significantly higher than in the light phases [$F_{(1,162)}$ =276.106, p<0.0001]. The locomotor activity across time was neither decreased nor increased and no significant effect of the number of stimuli was observed [$F_{(8,162)}$ =0.344, p=0.948]. We did not find any significant difference among light and dark phases over time (p>0.05)

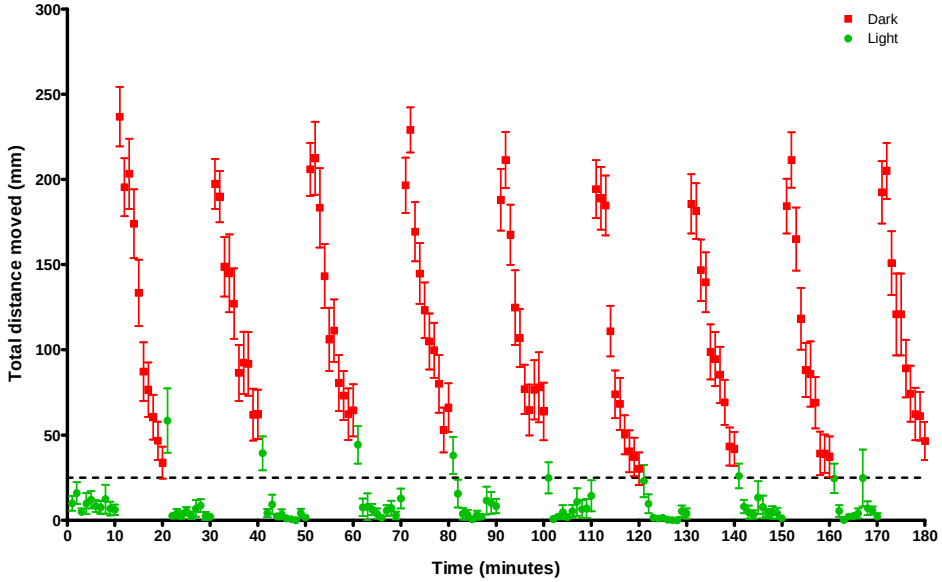

Figure 5.3: The locomotor activity of 4 dpf zebrafish larvae in response to a 10 min ISI of alternating light and dark. The locomotor activity in the dark phases after every light-to-dark transition was always higher than activity in the light phase. The activity of 4 dpf larvae in light was minimal. The larvae responded to sudden darkness and showed higher activity as expressed in total distance moved. However, this hyperactivity was progressively decreased in the 10 min dark phase. The same patterns were followed for the whole duration of the experiment. The dotted horizontal line represents the basal locomotor activity before start of experiment in light. The data are presented as ± SEM (n=48)

A similar pattern was observed when the locomotor activity of 5 dpf larvae was analysed (Figure 5.4). Two-way ANOVA analysis revealed no significant interaction between lighting conditions and time [$F_{(8,162)}$ =1.961, p=0.054]. However, there was a

significant effect of lighting conditions. The locomotor activity in every dark phases was significantly higher than in light phases [$F_{(1,162)}$ =1466.06, p<0.0001]. The effect of the number of stimuli was also significant [$F_{(8,162)}$ =14.1, p<0.0001]. There was a significant decrease in the average locomotor activity in successive dark phases after the initial peak in activity (p<0.05). In the case of light phases, the locomotor activity was also decreased after the initial peak of activity, but was not further decreased in subsequent light phases.

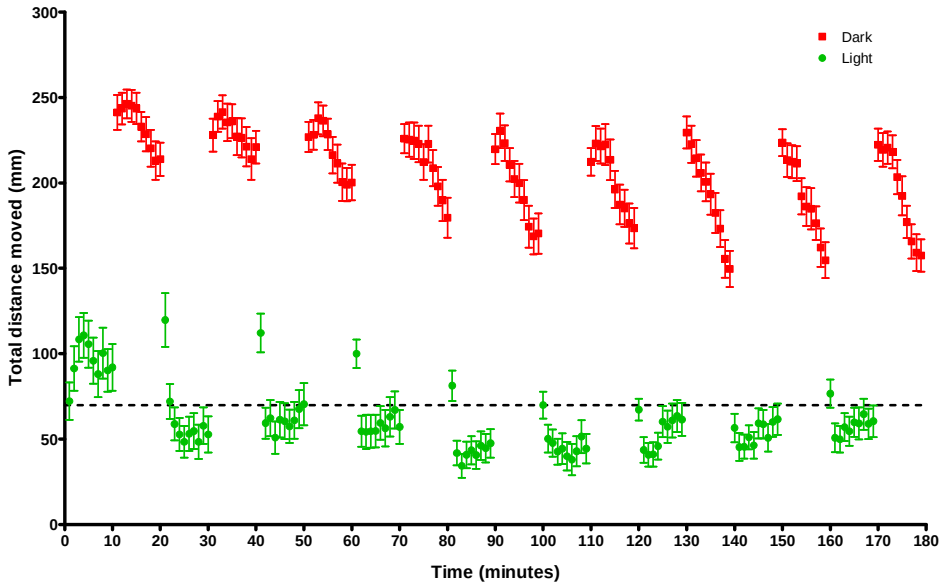

Figure 5.4: The locomotor activity of 5 dpf zebrafish larvae in response to 10 min ISI of alternating light and dark. The activity in the dark phases was always higher than activity in the light phase. The locomotor activity in first light phase was at higher in the first light phase and decreased in the second light phase. However, no further decrease was observed in the overall activity in the light phases. The zebrafish larvae showed higher activity in the dark phases than in the light phases. Within the first dark phase, there was no significant decrease of activity. However, more decay in the activity was shown in the subsequent dark phases. The dotted horizontal line represents the basal locomotor activity before start of experiment in light. The data are presented as ± SEM (n=48)

Locomotor activity of 6 dpf larvae in response to 10 min ISI is shown in Figure 5.5. Two-way ANOVA analysis revealed significant interaction between lighting conditions and time [$F_{(8,162)}$ =2.095, p=0.039]. There was a significant effect of lighting conditions on locomotor activity. The average locomotor activity in the dark phases was significantly higher than in light phases [$F_{(1,162)}$ =1257.76, p<0.0001]. The effect of number of stimuli

was also significant [$F_{(8,162)}$ =7.324, p<0.0001]. When dark and light cycles were tested separately, there was significant steady decrease in the average locomotor activity in each dark phase (p<0.05) while in the light phases, a significant decrease was observed after the first light phase but did not decrease in the subsequent light phases.

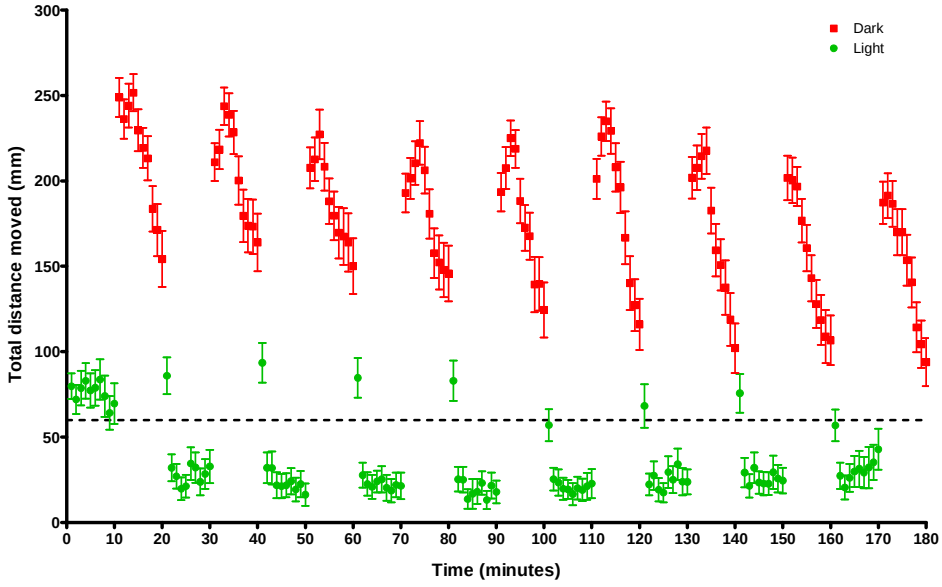

Figure 5.5: The locomotor activity of 6 dpf zebrafish larvae in response to 10 min ISI of alternating light and dark. The activity in the dark phases was always higher than activity in the light phases. The locomotor activity in light was at the basal level in the first light period and decreased in the subsequent light phases. The activity within the 10 min dark phase also decreased over time. However, no significant decrease in activity in dark periods over time was observed. The dotted horizontal line represents the basal locomotor activity before start of experiment in light. The data are presented as ± SEM (n=48)

Locomotor activity in response to 5 min ISI

The locomotor activity of 4 dpf larvae is shown in Figure 5.6. Two-way ANOVA analysis revealed significant interaction between lighting conditions and time [$F_{(17,144)}$ =6.846, p<0.0001]. There was also a significant effect of lighting conditions. The transition from light to dark and vice versa produced hyperactivity. However, the transition from light to dark produced a more pronounced response which did not decrease to the basal level (as it did in the dark-to-light transition). The hyperactivity observed after the transition from dark to light was of low magnitude and decreased to the basal level in the subsequent light

phases. The locomotor activity in the dark phases was always significantly higher than the baseline activity in light phases [$F_{(1,144)}$ =682.038, p<0.0001]. It was observed that the average total distance moved in the dark phases declined across the duration of the experiment (180 min) [$F_{(17,144)}$ =16.343, p<0.0001]. To compare the light and dark phases, a Dunnet post hoc test was performed for multiple comparisons. Significant differences were found in total distance moved between light and dark phases (p<0.05). The locomotor activity in the first dark phase decreased very rapidly. However, with subsequent dark phases, the decay in activity during the dark phase was less pronounced. By the 4th dark phase, the total distance moved had stabilised and remained constant per dark phase until the end of the experiment (180 min). The locomotor activity in the first light phase was higher than that in subsequent light phases.

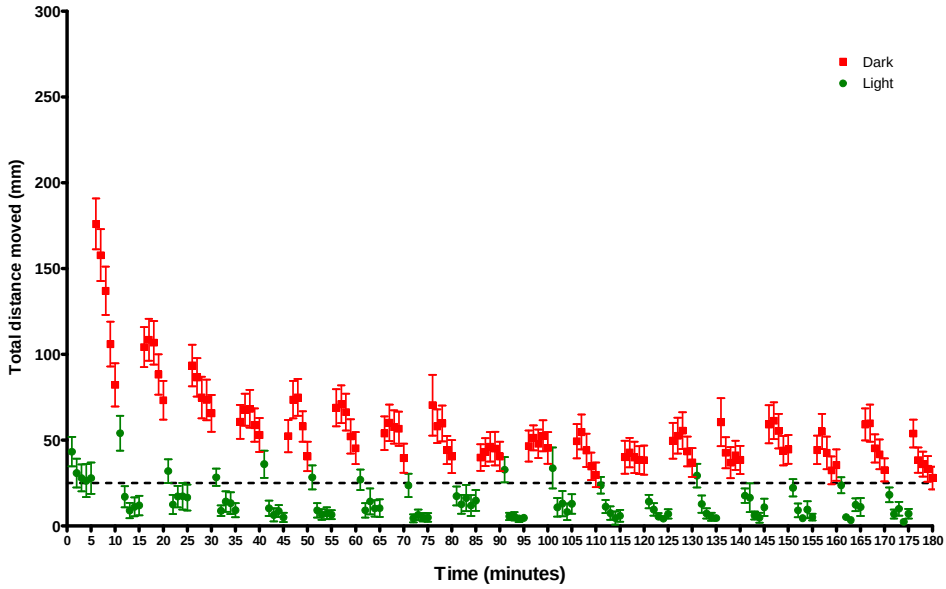

Figure 5.6: The locomotor activity of 4 dpf zebrafish larvae in response to 5 min ISI of alternating light and dark. The activity in the dark phases was always higher than activity in the light phase. The activity of 4 dpf larvae in light was minimal. They responded to sudden darkness and showed a hyperactivity. However, this hyperactivity continued to decrease in the subsequent 5 min dark phases and became stable after four dark phases. The dotted horizontal line represents the basal locomotor activity before start of experiment in light. The data are presented as ± SEM (n=48).

The locomotor activity of 5 dpf zebrafish larvae with a 5 min ISI of light and dark is depicted in Figure 5.7. Two-way ANOVA analysis showed a significant effect of light

conditions [$F_{(1,144)}$ =1416.09, p<0.0001] and time [$F_{(17,144)}$ =4.141, p<0.0001]. However, there was no significant interaction of lighting conditions and time [$F_{(1,144)}$ =0.530, p=0.934]. A post hoc Bonferroni test was used for further multiple comparisons. The initial hyperactivity due to light-to-dark transition was decreased in the 2nd and 3rd dark phases then remained stable for the rest of the experiment. The zebrafish larvae also showed a surge in activity at the dark-to-light transition in the first minute; however, it was of low magnitude and did not decrease over time. The locomotor activity of zebrafish larvae for the rest of the four minutes in the light phases was well below the basal locomotor activity and this pattern was observed for the rest of the test period.

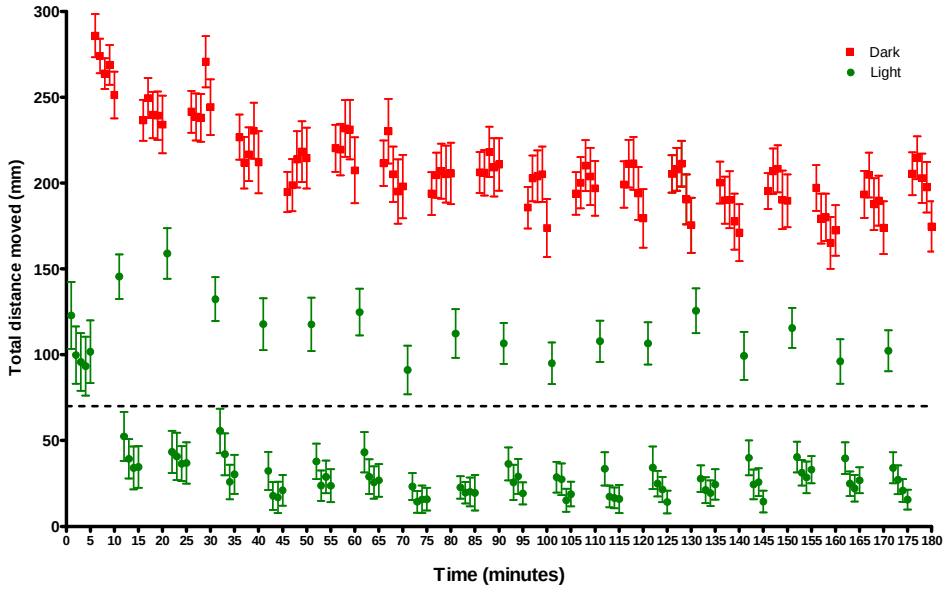

Figure 5.7: The locomotor activity of 5 dpf zebrafish larvae in response to 5 min ISIs of alternating light and dark. The average locomotor activity in the dark phases was always higher than activity in the light. The locomotor activity in light was at basal levels and decreased in the second light phase. However, no further decrease was observed for the duration of the experiment. The zebrafish larvae showed hyperactivity of higher magnitude in the dark phases. The activity marginally decreased in the second and third dark phases but remained stable in the subsequent dark phases for the duration of experiment. The dotted horizontal line represents the basal locomotor activity before start of experiment in light. The data are presented as ± SEM, (n=48).

The 6 dpf zebrafish larval activity is displayed in Figure 5.8. Two-way ANOVA analysis revealed a significant interaction between lighting conditions and time [$F_{(17,144)}$

=2.125, p<0.009]. A post hoc Bonferroni test was used for further multiple comparisons. A significant effect of lighting conditions [$F_{(1,144)}$ =2017.21, p<0.0001] and time [$F_{(1,144)}$ =7.895, p<0.0001] was also observed. The transition from light to dark always produced a hyperactivity of higher magnitude as compared to hyperactivity by dark-to-light transition. However there was a different pattern observed in both cases. The hyperactivity from light to dark did not wane either across time or within a given dark phase. The activity in the light phases initially increased but then decreased in the subsequent light phases.

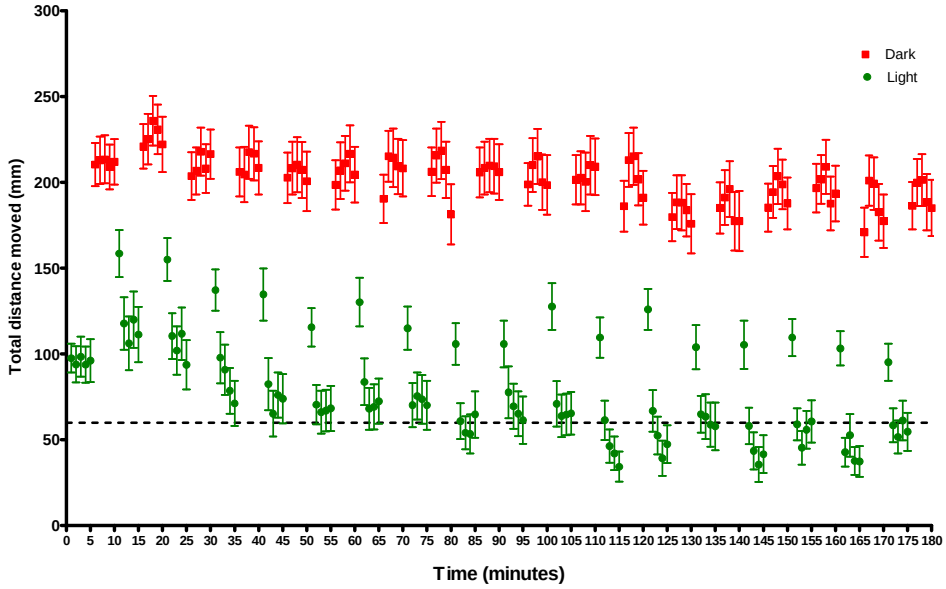

Figure 5.8: The locomotor activity of 6 dpf zebrafish larvae in response to 5 min ISI of alternating light and dark. The activity in the dark phases was always higher than activity in the light phase. The locomotor activity in light was at basal level in the first light phase and did not decrease for the duration of the experiment. The activity within the 5 min dark phase did not decrease over time. There was also no significant decrease in activity subsequent dark phases. The dotted horizontal line represents the basal locomotor activity before start of experiment in light. The data are presented as ± SEM, (n=48).

Locomotor activity in response to 1 min ISI

The locomotor activity of 4 dpf zebrafish larvae in response to 1 min ISIs is displayed in Figure 5.9. The zebrafish larvae showed a similar hyperactivity of high magnitude in dark phase. However, this hyperactivity quickly waned across successive dark phases. Two-way ANOVA analysis showed no interaction between light conditions and time [$F_{(2,174)}$ =1.475, p=0.232]. However, significant effect of time (p<0.0001) and lighting conditions

($p<0.0001$) was observed. The activity in the light was also increased from the basal level and then declined with subsequent light stimuli.

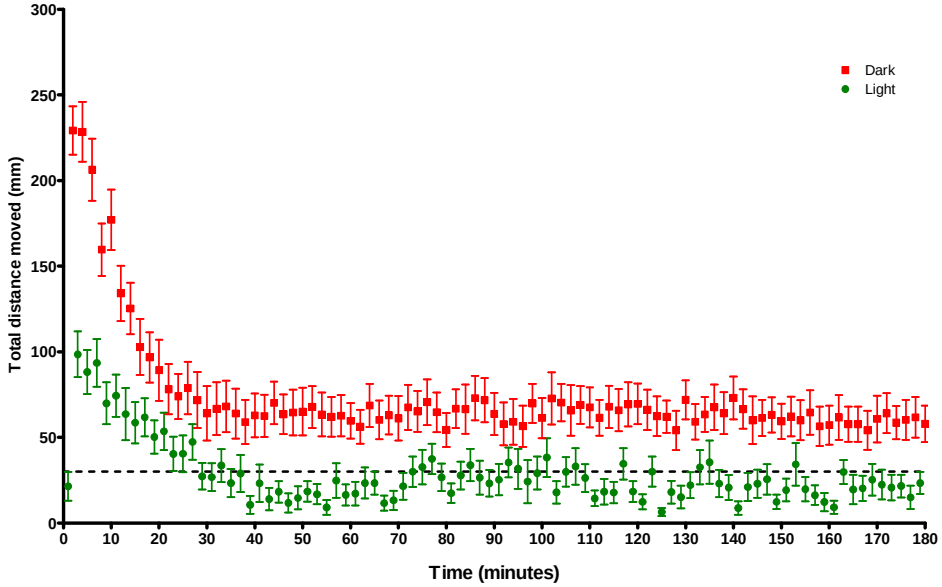

Figure 5.9: The locomotor activity of 4 dpf zebrafish larvae in response to 1 min ISI of alternating light and dark. The activity of 4 dpf larvae in the first light phase was minimal, but increased sharply at the subsequent light phases. It then declined to basal level after 30 minutes. The zebrafish larvae responded to sudden darkness by showing a burst of activity. This hyperactivity progressively decreased in the subsequent 30 minutes, but became stable after 30 minutes. The dotted horizontal line represents the basal locomotor activity before start of experiment in light. The data are presented as ± SEM (n=48).

The locomotor activity of 5 dpf zebrafish larvae also displayed a strong surge in the first dark phase (Figure 5.10). The distance moved with subsequent dark phases, alternated with light phases of 1 min ISI, declined steadily but become stable after 40 minutes and did not decrease further across subsequent dark phases. The transition from dark to light also resulted in a surge in activity, but this was of lower magnitude and quickly waned and returned to basal locomotor activity level after 10 cycles. Two-way ANOVA analysis revealed a significant effect of lighting conditions [$F_{(1,174)}$ =416.16, $p<0.001$] and time [$F_{(2,174)}$ =27.498, $p<0.0001$]. However, no significant interaction between lighting conditions and time was observed [$F_{(2,174)}$ =1.438, $p=0.24$].

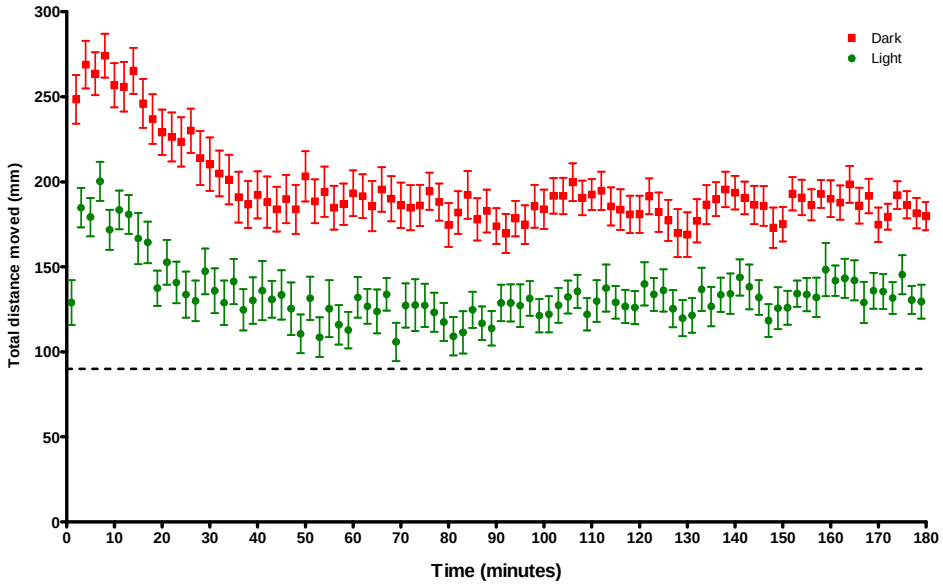

Figure 5.10: The locomotor activity of 5 dpf zebrafish larvae in response to 1 min ISI of alternating light and dark. The activity of 5 dpf larvae in light was at basal level and it increased in the second light phase. The locomotor activity in light then gradually decreased and become stable in 20 minutes without further decrease for duration of experiment. The zebrafish larvae showed hyperactivity of higher magnitude in the dark phases. However, the activity marginally decreased in subsequent dark phases but did not decrease after 30 minutes and become stable in the subsequent dark phases for the duration of experiment. The dotted horizontal line represents the basal locomotor activity before start of experiment in light. The data are presented as ± SEM, (n=48).

The locomotor activity of 6 dpf zebrafish larvae also displayed a robust response in the first dark phase Figure 5.11. Two-way ANOVA analysis revealed a significant effect of lighting conditions [$F_{(1,174)}$ =416.16, p<0.001] and time [$F_{(2,174)}$ =27.498, p<0.0001] but no significant interaction between lighting conditions and time was observed [$F_{(2,174)}$ =1.438, p=0.24].The distance moved with subsequent dark phases, alternated with light phases of 1 min ISI, declined steadily; after 40 minutes, however, the response become stable and did not decrease further across dark phases. The transition from dark to light also produced a surge in activity, but of lower magnitude and quickly waned and returned to basal locomotor activity level.

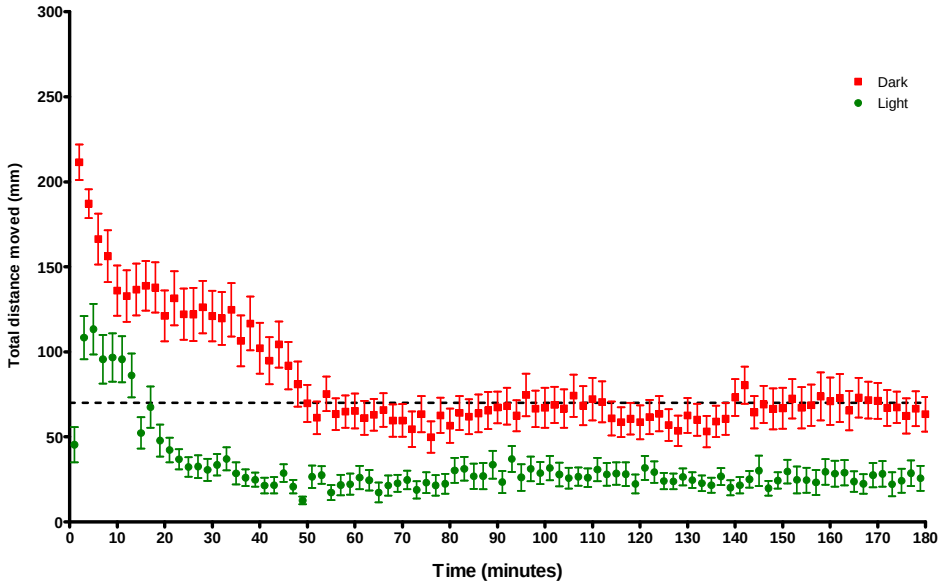

Figure 5.11: The locomotor activity of zebrafish larvae in response to 1 min ISI of alternating light and dark. The locomtor activity of 6 dpf larvae in light was at basal level in the first light phase and increased in the next light phases. However, the locomotor activity in the light phases decreased in the first 30 minutes and become stable for rest of the experiment. The response to first onset of dark produced hyperactivity of higher magnitude . Howeve, it decreased with time and became stable after 40 minutes. The dotted horizontal line represents the basal locomotor activity before start of experiment in light. The data are presented as ± SEM (n=48).

Recovery from hyperactivity

To determine whether zebrafish larvae are able to recover the iterative reduction in hyperactivity, two series of light-dark stimuli with 1 min ISI separated by 30 min intervals were carried out. There was an effect of dark stimulus on the distance moved which significantly increased as compared to baseline activity ($p<0.0001$). The distance moved decreased over time with subsequent dark stimuli. However, the distance moved in response to dark stimulus was always higher than distance moved in light (Figure 5.12A)

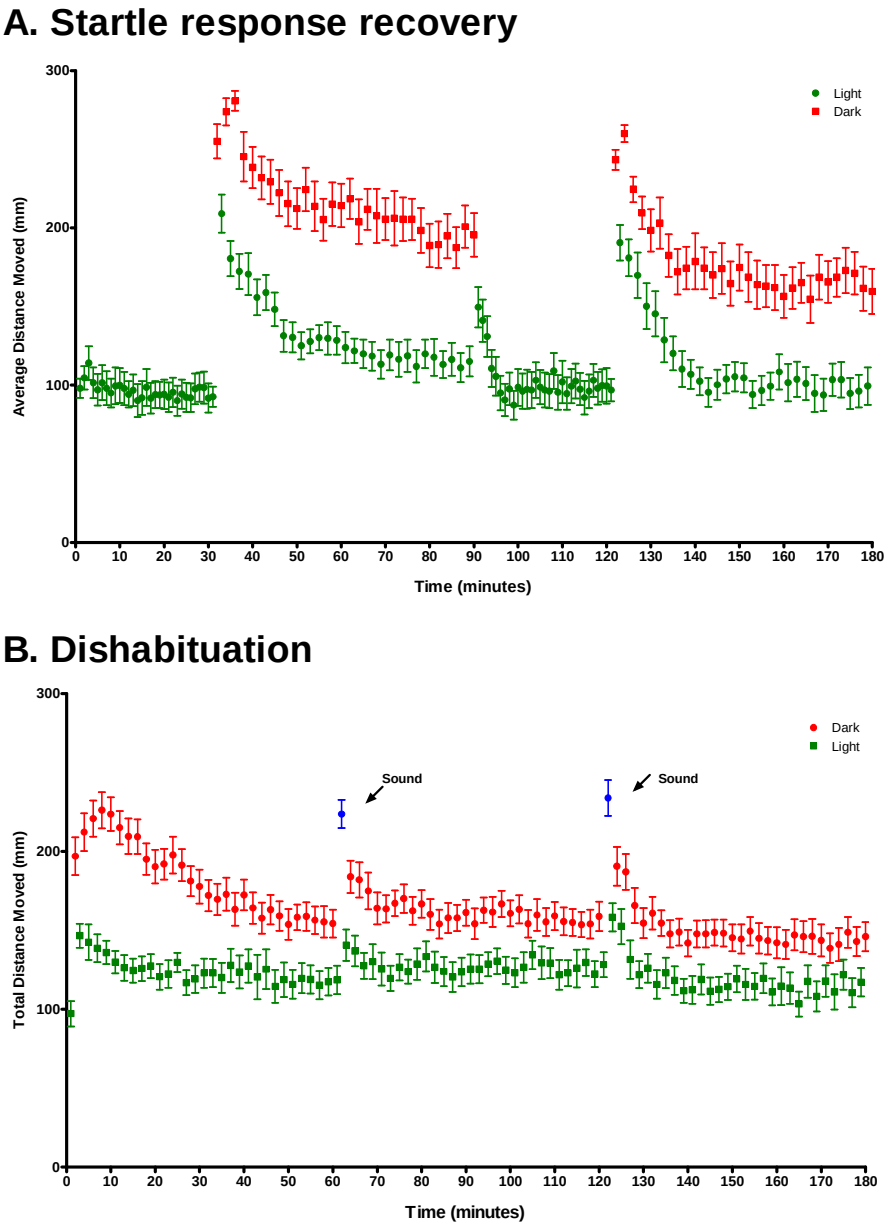

Figure 5.12: (A) The recovery from hyperactivity. The basal locomotor activity of larval zebrafish was recorded for 30 minutes and then 1 min ISIs of alternating light-dark phases was continued for one hour during which a decline in the response to dark and light was observed. After this, a recovery time was given in light to allow the larvae to return to their

basal locomotor activity level. The zebrafish larvae were then again presented with 1 min ISIs of alternating light-dark cycles for one hour. The activity in light and dark decreased steeply during this second phase and became stable more rapidly than in the first phase. (B) This experiment consisted of three phases. In the first phase, alternating light-dark stimuli were presented for one hour, during which a decline in activity was seen in response to dark stimuli. In the second phase of one hour, alternating light-dark stimuli of 1 min ISIs were presented again. This time, a sound pulse was presented during the 62nd minute in place of a dark stimulus. This was to determine whether the decline in the activity in dark phase is due to habituation or to exhaustion. The activity of the larvae increased again to a higher level but then showed rapid decrease in activity with subsequent dark stimuli. The third phase was exactly the same as the second phase, and a sound pulse was presented in 122nd minute instead of a dark stimulus. The zebrafish larvae again showed hyperactivity of higher magnitude, but then decreased with subsequent dark stimuli more rapidly than in the first phase. The data are presented as ± SEM (n=48)The zebrafish larvae were given a 30 min recovery phase in light in which larval activity returned to basal levels. Then, light-dark stimuli with a 1 min ISI were repeated again for one hour in which zebrafish larvae moved significantly more in response to dark stimulus ($p<0.0001$). However, the distance moved in dark decreased more rapidly than in the first set ($p<0.0001$).

Dishabituation

To see whether reduction in the hyperactivity represents habituation or whether it represents fatigue, another stimulus (sound) was presented instead of the one with which reduction took place (transition from light to darkness). We used sound as stimulus B in this study which enabled the zebrafish larvae to re-induce the hyperactivity. The hyperactivity in response to dark stimulus increased and then gradually decreased to a stable level both in light and dark (Figure 5.12B). However, when sound was presented, the distance moved significantly increased ($p<0.001$). The distance moved in response to a sound tone was equivalent to the distance moved in response to the dark stimulus in the first phase ($p>0.05$).

Total activity level

The total activity level was assessed by counting the number of active larvae for each developmental stage and ISI. The total activity level of zebrafish larvae in response to different ISI of light-dark is shown in Figure 5.13. The number of active 4 dpf larvae increased with decreasing ISI ($\chi^2 = 10.66$, $p < 0.005$). However the number of active larvae was not significantly different in the case of 5 dpf ($\chi^2 = 3.47$, $p = 0.207$) and 6 dpf zebrafish larvae ($\chi^2 = 2.782$, $p = 0.2489$).

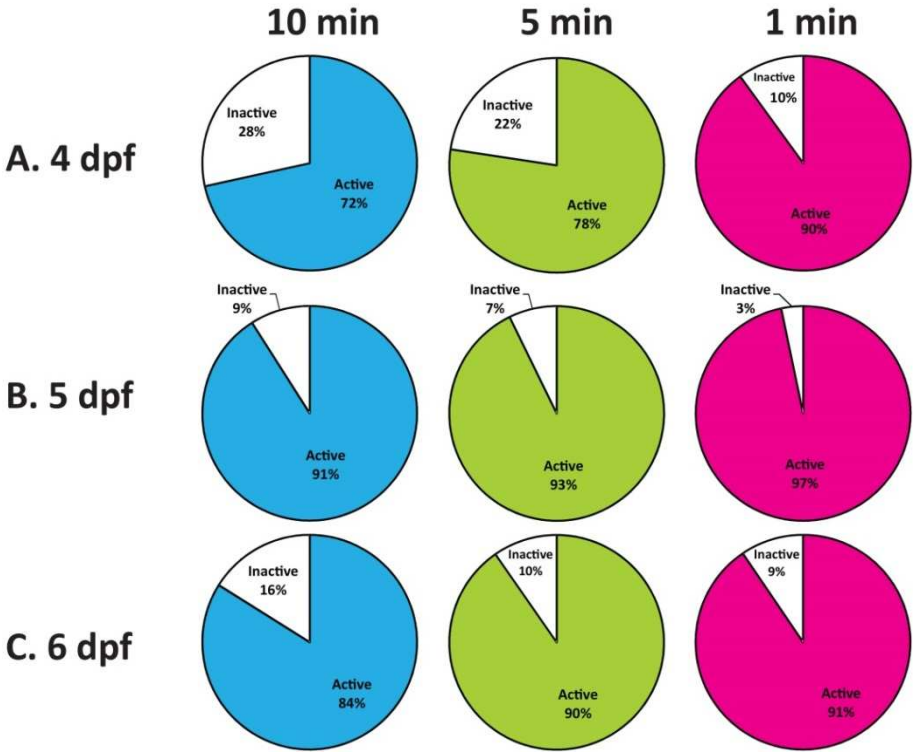

Figure 5.13: Total activity level of zebrafish larvae during light-dark stimulus of different ISI. The % of active 4 dpf larvae significantly increased with decreasing ISI ($p<0.005$). The number of active 5- and 6 dpf zebrafish larvae did not increase significantly ($p>0.05$)

Discussion

Basal activity in light at different developmental stages

The locomotor activity of 4, 5 and 6 dpf zebrafish larvae was monitored to look for differences in the locomotor patterns across different developmental ages. The basal locomotor activity of the larvae was monitored in the presence of light because zebrafish larvae have different responses to light and dark [49]. In the light, zebrafish larvae show low activity, while in darkness a relatively high activity is observed [32,129,163,339]. However, this higher activity is transient, consisting of a peak or spike of activity following the light-dark transition [122]. We used microtitre plates, and these certainly restrict the

spatial movement of the larvae. However they do allow large numbers of larvae to be monitored simultaneously.

Although, zebrafish larvae moved spontaneously at all developmental stages tested, they were far less active at 4 dpf than at 5 or 6 dpf. Their locomotor activity was highest at 5 dpf, a finding consistent with other studies [353-355]. The very low locomotor activity exhibited by 4 dpf larvae suggests that a key developmental transition takes place between 4 and 5 dpf perhaps in the development of the musculoskeletal system or nervous systems. These findings are in agreement with previous studies [32,129]. The higher activity observed in the 5 and 6 dpf larvae is presumably related to the fact that, at these stages, the yolksac is almost completely resorbed and larvae must start seeking food [148,198,203,356,357]. The locomotor activity of 4 dpf larvae was less as compared to 5 and 6 dpf larvae and which is consistent with other studies [129].

The increasing use of zebrafish larvae in drug discovery and developmental toxicology highlights the need for a detailed understanding of their behaviour repertoire. This challenge is difficult because many factors can contribute to activity patterns and behaviour. These factors include microtiter plates, developmental malformations [129] and lighting conditions [49,122]. Previous studies used different developmental stages for the tests involving locomotor activity [122,129,170,339]. Our results presented highlight the importance of selecting specific developmental stages for tests involving locomotor activity.

Effect of time of day

Zebrafish larvae are reported to have a peak locomotor activity in the morning and to be less active in the afternoon [122]. However, in our study, we did not find any differences in locomotor activity of zebrafish larvae in the morning and afternoon sessions. These contrasting results might be explained by the different light conditions used here. We monitored the locomotor activity of zebrafish larvae in the light in contrast to the study by MacPhail and colleagues [122] where activity was monitored in the dark. Thus, gradual decrease in the activity in afternoon session might not be due the timing of the day but due to the light condition used.

The zebrafish larva has been shown to respond to sudden onset of dark with hyperactivity [49,55]. However, this response soon waned and locomotor activity decreased with time [122]. Thus, lower activity in the evening in the study of Macphail and colleagues

[122] study might be due to the habituation for dark stimulus. In our study, no decrease in activity make sense because the activity is recorded in the day time when light should have been ON according to the normal light-dark cycle. Thus, it is important to use basal locomotor activity in the light because in the dark the zebrafish larvae are hyperactive and then activity is gradually decreased [353]. To set the optimal timing of our further experiments, we recorded the locomotor activity of zebrafish larvae in the morning.

Habituation and the ISI in larvae of different ages

We found that, for larvae of all ages (4, 5 and 6 dpf) the shorter the ISI, the greater the habituation which is a simplest form of learning [352]. Previous studies demonstrated that zebrafish larvae are able to respond to a light-dark transition with hyperactivity [49,55] which was expressed as a transient spike [122]. It has been suggested [122] that decrease in hyperactivity may be due to habituation. Various criteria have already been proposed for true habituation [192,358] and we tested zebrafish larvae to see whether their behavioural patterns meet these criteria.

Initially, we compared the response of zebrafish larvae to different light levels (0 or 100% light) to determine if there was any difference in the response to light level. An optimum measureable response was achieved during darkness, when the white light was completely off (but the infrared remained on so that the video recording could continue druing darkness). We examined total distance moved by 4, 5 and 6 dpf larvae in response to different ISIs (10 min, 5 min and 1 min).

The 4 dpf old larvae showed no habituation in response to 10 min ISIs. The hyperactivity did not decrease in any of the subsequent light-to-dark transitions for the duration of the experiment. The activity of these larvae in the light phase was also stable across the experiment. In the case of 5 min ISI, the activity of 4 dpf larvae did decrease from the second light-dark transition onwards. The response of 4 dpf larvae to light-dark transition further decreased during subsequent dark phases but become stable after one hour. The locomotor activity of 4 dpf old larvae with 1 min ISI also decreased significantly with the passage of time and became stable after 30 minutes. These findings suggest that 4 dpf zebrafish larvae are unable to habituate with longer ISIs, but showed, with shorter ISIs (5 min and 1 min) a decrease in activity which could be due to habituation.

For 5 dpf old larvae exposed to alternating light-dark phases with 10 min ISIs there was no sharp decrease in the light-dark hyperactivity in subsequent transitions. However,

habituation within the same dark phase over 10 minutes was observed. With a 5 min ISI, zebrafish larvae did show a slight decrease in the hyperactivity in subsequent dark phases. A decrease in the locomotor activity in the light phases was also observed after the first light phase. With a 1 min ISI, the 5 dpf zebrafish larvae also showed decrease in the hyperactivity in dark phases and then a stable response was observed in subsequent dark phases. The average activity in the light phases also decreased and was stabilized at the same time as in the dark phase.

The 6 dpf old larvae with 10 min ISIs did not show a sharp decrease in the hyperactivity in the subsequent dark phase. However, decrease in the activity within the same dark phase was observed as with 5 dpf old larvae. With a 5 min ISI, the zebrafish larvae did not show a decrease in hyperactivity in dark phases, but did show a decrease in locomotor activity in the light phase.

The longer ISIs resulted in a slower habituation than did shorter ISIs. This conforms with one of the criteria of habituation, namely that the stimuli of rapid frequency result in more rapid habituation than do stimuli of lower frequency [192]. Interestingly, the 5 and 6 dpf zebrafish larvae showed an increase in activity during the first minute of the dark phase, and an even higher activity in the subsequent minutes suggesting a potentiating effect. It is possible that, after the first peak of hyperactivity, zebrafish larvae could become more alert, thereby anticipating the following stimulus more acutely [358-360]. More work is required to fully understand this phenomenon.

In the present study, the percentage of active larvae at 4 dpf was significantly less (48%) than at 5 (81%) and 6 dpf (58%). However, when the light-dark stimulus was applied with different ISIs, the percentage of larvae showing movement increased to 71% with 10 min ISIs, 77% with 5 min ISIs and 75% with 1 min ISIs. The number of active larvae in the case of 5 dpf larvae increased from 81% in the normal light-dark experiment to 91, 93 and 97% when ISIs of 10, 5 and 1 min, respectively, were used. The percentage of active 6 dpf larvae increased from 58% in the normal light-dark experiment, to 84, 90 and 91%, when ISIs of 10, 5 and 1 min, respectively, were used. This might be explained on the basis of the innate response of zebrafish larvae to a dark stimuli [49,55]. Thus, zebrafish larvae respond to dark stimulus with hyperactivity, and this could explain the increase in the percentage of active larvae.

The decrease in activity after early hyperactivity in the current study might be an ancestral form of non-associative learning or it might be related to the conservation of

energy. One possible explanation is that zebrafish larvae at 5 and 6 dpf need to conserve energy because they have depleted yolksac and are just beginning to self-feed. Furthermore, the zebrafish larvae generally reduce their locomotor activity at night [49,127,260]. Thus, early hyperactivity in the darkness may be attributed to shock when zebrafish larvae are not expecting night (darkness) and further decline might be associated with a need to conserve energy.

Light-to-dark and dark-to-light transitions

In all age groups tested, we have shown that light-to-dark transition produced hyperactivity of higher magnitude than dark-to-light transitions. Other studies have also reported that onset of sudden darkness induces hyperactivity [49,122]. One possible explanation for this hyperactivity might be the fact that they are diurnal and seek shelter at night [361]. Another explanation may be that the hyperactivity caused by sudden darkness may be a form of predator avoidance or escape, although Burgess and Granato [163] have suggested that this is unlikely because larvae might not interpret sudden onset of dark as a potential threat. The spikes in motor activity during darkness, stable basal levels during light and decrease in activity with increasing time, may all reflect habituation.

The fact that the response to dark-to-light transition is smaller than the resonse to a light-to-dark transition might be explained by the circadian rhythm of zebrafish larvae. The zebrafish larvae were raised in light-dark cycle of 14:10 h and were tested during the day when light would normally remain switched ON, thus turning lights back ON might have had a calming effect. It would be interesting to examine the light-to-dark and dark-to-lighting transitions during the night, when the light should remain switched off.

Dishabituation and a control for exhaustion

After observing a decline in the hyperactivity displayed in the dark phases in all age groups tested, we then determined whether this reduction was a true habituation, or was simply caused by lack of physical energy (exhaustion). To examine these issues we examined 5 dpf zebrafish larvae with 1 min ISIs because we had shown that this produced the most significant habituation to light-dark transitions. It has been suggested that, if habituation is occurring, then, if response to a certain repeated stimulus wanes with repeated exposure, a second series of the same stimulus (after allowing a period of recovery) will result in a similar magnitude of response as in the first series [192]. Our results showed that this indeed was the case, suggesting that the effect is due to habituation.

The next criterion of habituation is that presentation of a different stimulus results in the recovery of the habituated response [191,192]. Recovery of the hyperactivity did indeed occur after the exposure to an audible stimulus rather than a visual one. Our results showed that zebrafish achieved a basal locomotor level in the first 30 min in light. Then the spike in motor activity was noticed in response to the first onset of dark with 1 min ISI. However, this response was decreased with increasing time and become stable in the first hour of light-dark transitions. In the next phase of 1h light-dark transitions, the zebrafish larvae response waned more rapidly. Thus, the behaviour displayed by zebrafish larvae in response to light-dark stimuli was consistent with the phenomenon of habituation, and the decrease in the hyperactivity was not due simply to motor exhaustion.

Final conclusions

We have shown that zebrafish larvae have different locomotor activity levels at different developmental stages. However, there was no effect of time of day. We also showed that zebrafish larvae display a locomotor activity of higher magnitude following onset of sudden dark which is waned and become stable after certain period of time. The zebrafish locomotor activity in dark remains always higher than activity in the light. ISI plays a vital role in the habituation. Longer ISIs result in either the absence of habituation, or in poor habituation. In contrast, shorter ISIs result in a rapid decline in locomotor activity and hence in rapid habituation. The response to the same stimulus after allowing a period of recovery, also results in fast decline in the activity and hence a stable level is reached much earlier than in the phase before recovery. An increase in the locomotor activity of zebrafish larvae in response to a different stimulus (sound) after a stable level was reached, suggests that the decline in activity in response to darkness was due not to exhaustion but to habituation. More work is required to see whether compounds which influence learning and memory can result in rapid habituation or complete loss of this phenomenon.