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Research article

Good night, sleep tight: The effects of sleep deprivation on spatial associative learning in zebrafish

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ABSTRACT

Learning and memory are vital to an animal's survival, and numerous factors can disrupt cognitive performance. Sleep is an evolutionarily conserved physiological process known to be important for the consolidation of learning and memory. The zebrafish has emerged as a powerful model organism sharing organizational and functional characteristics with other vertebrates, providing great translational relevance. In our study, we used a simple spatial associative learning task to quantify the effects of sleep deprivation (partial vs. total) on learning performance in zebrafish, using an animated conspecific shoal image as a reward. Control animals maintained on a regular light:dark cycle were able to acquire the association between the unconditioned and conditioned stimulus, reinforcing zebrafish as a valid and reliable model for appetitive conditioning tasks. Notably, sleep deprivation did not alter the perception of and response to the conspecific image. In contrast, although partial sleep deprivation did not impair cognitive performance, total sleep deprivation significantly impaired performance on the associative learning task. Our results suggest that sleep is important for learning and memory, and that the effects of sleep deprivation on these processes can be investigated in zebrafish.

1. Introduction

Learning is an important process that is required for the acquisition of new skills and concepts based on past experiences (Amrein, 2015; Gould et al., 1999; Grafman, 2000; Kolb and Whishaw, 1998). The ability to modify behavioral patterns based on past experiences confers several advantages including foraging, courtship (Sison and Gerlai, 2010), and predator avoidance (Johnston, 1982). Moreover, the benefits associated with the predictive nature of learning may be enhanced when individuals retain long-term memories. However, a number of endogenous and exogenous factors can improve and/or impair learning (Levin et al., 2006; Luchiari et al., 2015; Sasson et al., 2007).

Among the number of factors that enhance learning and memory consolidation, sleep is one of the most commonly studied. Sleep can be defined as behavioral quiescence and is associated with an increased arousal threshold (Schmidt, 2014; Siegel, 2008). During sleep, the brain exhibits two types of electrical activity: 1) slow wave activity (NREM sleep, non-rapid eye movement) divided into four stages, and 2) desynchronized brain wave activity (REM sleep, rapid eye movement) represented by muscle atonia and wake-like brain activity (Carlson, 1986; Lent, 2004). While only endothermic animals exhibit REM sleep, ectothermic vertebrates also show sleep-like behavior (Carlson, 1986; Siegel, 2008).

Although the function of sleep in animals is still debated, the cumulative effects of sleep deprivation have been associated with negative health consequences including obesity, diabetes, stroke, and depression, along with a profound economic and societal impact (Colten and Altevogt, 2006). Research on sleep and sleep disorders have been increasing, however, diagnoses and treatments are still limited.

Research on the effects of sleep on learning and memory has been a focus of numerous studies in the field of behavioral neuroscience. Research in this area of study have focused on rodents and primates (Inostroza et al., 2013; Kelemen et al., 2014; Lo et al., 2004; Lyamin et al., 2008; Siegel, 2005). However, research on phylogenetically distant animals may identify evolutionarily conserved mechanisms regulating sleep behavior as well as learning and memory. Zebrafish have been a focus of attention because it is a tractable genetic model that shares organizational and functional characteristics with other vertebrates (Gerlai, 2011; Kalueff et al., 2014; Miklósi and Andrew, 2006; Panula, 2010). The main neurotransmitter systems regulating sleep in mammals are widely conserved in zebrafish, including monoaminergic (Holzschuh et al., 2001; Kaslin and Panula, 2001; McLean

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and Fetcho, 2004; Teraoka et al., 2004), cholinergic (Mueller et al., 2004) and hypocretinergic cell groups (Faraco et al., 2006; Kaslin et al., 2004; Prober et al., 2006; Yokogawa et al., 2007). A sleep-like behavioral state has been characterized in zebrafish (Zhdanova et al., 2001; 2006) and the pharmacological and genetic aspects of zebrafish sleep are similar to vertebrates including mammals (Chiu and Prober, 2013). One of the major advantages of the zebrafish model is the non-invasive nature of drug administration. Water soluble drugs can be added directly to the water which is then taken up by the immersed fish through its skin and gills. This feature has allowed researchers to test the effects of drugs that are known to alter sleep-like behavior such as alcohol (Williams and Salamy, 1972; Earleywine and Martin, 1993) and melatonin (Zhdanova et al., 2001; Gandhi et al., 2015), which respectively have known sedative and sleep promoting effects.

Although zebrafish have been increasingly used in learning and memory studies (Arthur and Levin, 2001; Bilotta et al., 2005; Lucon-Xiccato and Dadda, 2014; Pather and Gerlai, 2009; Sison and Gerlai, 2011a; Williams et al., 2002), the effects of sleep deprivation on learning performance remain unclear in zebrafish. In the present study, we examined the effects of partial and total sleep deprivation on learning performance with or without alcohol (ethanol) or melatonin administration. To quantify learning and memory, we used a spatial associative learning paradigm, in which a conspecific image was used as a reward due to the highly social nature of this species (Engeszer et al., 2007; Saverino and Gerlai, 2008).

2. Material and methods

2.1. Animals and housing

Zebrafish *(Danio rerio)* were obtained from a local fish farm (Natal, Rio Grande do Norte, Brazil) and acclimatized for one month prior to behavioral experiments. Adult zebrafish (3 months, mixed sexes) were transferred to 50 L tanks in a recirculating system with multistage filtration, including a mechanical filter, a biological filter, an activated carbon filter and a UV light sterilizing unit. Temperature, pH, and oxygen levels were measured regularly with fish density maintained at one animal/l.

Fish were kept on a light:dark cycle of 12 L:12D (12 h light:12 h dark), with zeitgeber time (ZT) 0 corresponding to lights-on at 7 a.m. and light intensity of 250 lx. Fish were fed twice daily with brine shrimp and a commercial diet. Animal use protocols were reviewed and approved by the Ethical Committee for Animal Use of Federal University of Rio Grande do Norte (CEUA 022/2012).

2.2. Experimental design

To determine the effect of sleep deprivation (SD) on learning performance, 210 zebrafish were randomly assigned to five different experimental groups. The sleep deprivation protocol used in the current study involved extending the light phase and/or exposing fish to pulses of light during the dark phase (sleep deprivation protocols are described in detail below).

The (1) *Control* group (no sleep deprivation) was kept on a 12 L:12D cycle (n = 30), with lights turning on at 7 a.m. (ZT0). The (2) *Partial sleep deprivation* group was kept on a 18 L:06D cycle (n = 45), with 18 h of light and only 6 h of dark (extended light phase with lights on from ZT0 to ZT18). The (3) *Total sleep deprivation* group was also maintained on 18 L:06D cycle, with light pulses applied for 1 min every 4 min during the entire dark phase (n = 45) (extended light phase with lights on from ZT0 to ZT18 and light pulses from ZT18 to ZT0). Two other groups were also maintained under total sleep deprivation conditions as described above and received additional drug treatments: (4) *Total sleep deprivation + Ethanol* group (1 h acute exposure to 0.5% v/v alcohol; n = 45) and (5) *Total sleep deprivation + Melatonin* group (10 days of chronic exposure to 100 nM melatonin; n = 45). The light:dark cycle manipulations were maintained for 72 h prior to behavioral testing.

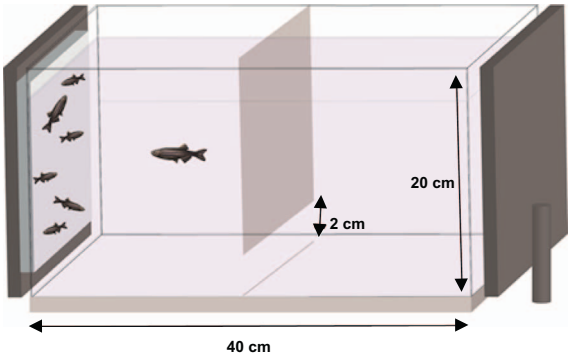

Fig. 1. Schematic overview of the spatial associative learning task. The tank (40 × 25 × 20 cm) was flanked with two LCD screens on each side. Screens presented an image of a group of zebrafish of similar size to the experimental test subject. The image was presented for 30 s, followed by 60 s of a blank screen (ISI), and this was repeated 20 times (20 trials) under three different presentation schemes: 1) one side only, 2) alternating sides and 3) random sides.

2.3. Sleep deprivation

The light pulse protocol utilizes short pulses of light during the dark phase of the light-dark cycle and has been previously shown to suppress sleep-like behavior without inducing excessive stress and/or perturbing learning and memory (Sigurgeirsson et al., 2013; Yokogawa et al., 2007). The sleep deprivation protocols (partial and total) used in the present study have also been previously validated by Pinheiro-da-Silva et al. (2017) by independently testing 1) the effects of an extended light phase and 2) light pulses during the dark phase. Briefly, to examine the effect of extending the light phase of the light-dark cycle, 3 groups of zebrafish (n = 8) were individually recorded in a 15 L tank during a 24 h period to quantify locomotor activity (average swimming speed) and the number of sleep episodes. Group 1 was kept on a 12 L:12D cycle (control), group 2 was subjected to partial sleep deprivation by extending the light phase (18 L:06D), and group 3 was maintained under constant conditions (24 L:00D). Pinheiro-da-Silva et al. (2017) found that fish in groups 1 and 2 were less active during the dark phase (i.e. reduced swimming speed – 1 = 1.96 ± 1.15 cm/s; 2 = 2.00 ± 0.05 cm/s) compared to fish in group 3 which were maintained under constant light (6.23 ± 0.83 cm/s). Moreover, sleep episodes (characterized by Yokogawa et al. (2007) as short periods of inactivity with the caudal fin dropped down, usually at the bottom or at the surface of the tank) were observed only for the control (16.92 ± 11.95 episodes/h of the dark phase) and partially (15.00 ± 4.10 episodes/h of the dark phase) sleep deprived groups, with the highest number of sleep episodes recorded for the control group during the dark phase (control group showed 35.5 ± 3.5 sleep episodes at the 7th hour of dark while partial group showed 20.0 ± 7.1 at the 1st hour of dark). To test the effect of the light pulses on sleep-like behavior, Pinheiro-da-Silva et al. (2017) examined the effect of light pulses during the night, as well as during the day to control for stress. The authors subjected 2 groups of zebrafish to the following conditions (1) 12 L:12D cycle with 6 h of light pulses (2 min on, 2 min off) during the waking period (light phase), and (2) 18 L:06D cycle and 6 h of light pulses (2 min on, 2 min off) during the entire dark phase. Fish behavior showed that light pulses do not imply any stress to the animal, and that this stimulus presented during the dark phase thwarted fish of sleeping (for more details see Pinheiro-da-Silva et al., 2017). Additionally, Yokogawa et al. (2007) showed that light induced sleep deprivation does not produce rebound, while electrical stimulation induces rebound.

In the present study, we extended the deprivation period to 72 h. This period was shown not to disrupt circadian rhythm and cause any

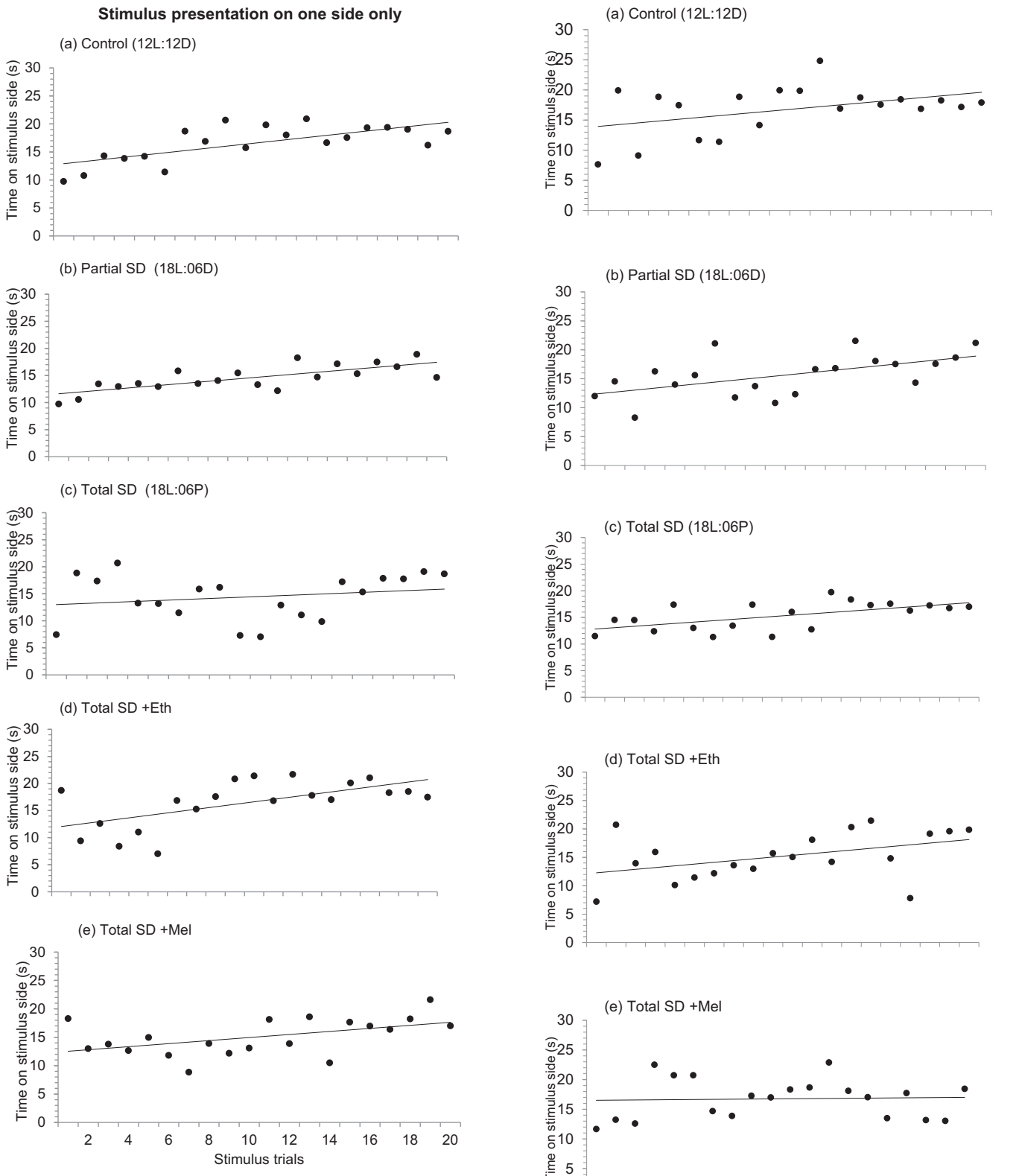

Fig. 2. Time spent on the stimulus side of the tank during the 20 trials when the stimulus was presented on one side only in an appetitive conditioning task using social stimulus as a reward. Five groups were tested: (a) control (12 L:12D; $n = 10$), (b) partial SD (18 L:06D; $n = 15$), (c) total SD (18 L:06D with light pulses; $n = 15$), (d) total SD + Eth (18 L:06D with light pulses + 60 min ethanol exposure before the onset of light pulses on the last day; $n = 15$), and (e) total SD + Mel (18 L:06D with light pulses + 10 days melatonin exposure including the 72 h of sleep deprivation; $n = 15$). Light:Dark cycle used was applied during 72 h for each group. In the graphs, black dots represent the time fish spent in the stimulus side and filled lines represent trends to the stimulus side. For further details of the results of statistical analysis see Results section.

(caption on next page)

Fig. 3. Time spent on the stimulus side of the tank during the 20 trials when the stimulus was presented on alternating sides in an appetitive conditioning task using social stimulus as a reward. Five groups were tested: (a) control (12 L:12D; $n = 10$), (b) partial SD (18 L:06D; $n = 15$), (c) total SD (18 L:06D with light pulses; $n = 15$), (d) total SD + Eth (18 L:06D with light pulses + 60 min ethanol exposure before the onset of light pulses on the last day; $n = 15$), and (e) total SD + Mel (18 L:06D with light pulses + 10 days melatonin exposure including the 72 h of sleep deprivation; $n = 15$). Light:Dark cycle used was applied during 72 h for each group. In the graphs, black dots represent the time fish spent in the stimulus side and filled lines represent trends to the stimulus side. For further details of the results of statistical analysis see Results section.

effect to the fish physiology. Yokogawa et al. (2007) showed that sleep deprivation of > 2 weeks is required to induce sleep rebound in zebrafish, and Moura et al. (2017) showed that despite continued light or dark conditions for up to 30 days zebrafish maintain the circadian rhythm (observed by Cosinor method - $t = 1440$).

2.4. Drug exposure

Zebrafish in group 4 (total sleep deprivation + ethanol) were exposed to 0.5% v/v ethanol for 1 h prior to the onset of the dark phase (from ZT17 to ZT18) on the last day of the sleep deprivation protocol. The ethanol concentration was achieved by diluting ethanol (99,8% P.A. – ACS, Dinâmica) with water to a final concentration of 0.5% v/v in a 15 L tank, a dose previously shown to be stimulatory (Gerlai et al., 2000). Fish were transferred to the tank containing ethanol solution and after a 60 min exposure, fish were returned to their home tank. We chose a 60 min exposure period based on previous studies demonstrating that this time period is sufficient for ethanol concentrations in the zebrafish brain to approach equilibrium with the external ethanol solution (Chatterjee and Gerlai, 2009; Dlugos and Rabin, 2003; Tran and Gerlai, 2013). A single acute ethanol exposure was used in this study because this drug was shown to promote sleep (Roehrs et al., 1999; Roehrs and Roth, 2001) but also to induce tolerance and lack the sleep-assistance effect after continued usage.

Zebrafish in group 5 (total sleep deprivation + melatonin) were exposed to 100 nM melatonin for 10 consecutive days, 24 h per day. Melatonin powder (cat#M5250, Sigma-Aldrich) was dissolved in ethanol to prepare a stock solution of final concentration of 10 μM. Then, the stock solution was added to the tank water to achieve a concentration of 100 nM.

The melatonin treatment continued for 10 consecutive days, 24 h per day. The tank water and drug concentration was replaced every day to maintain a constant drug dosage. Exogenous melatonin generates a peak of action around 2 h after exposure and returns to a basal level up to 2 h after that (Zhdanova et al., 2001, 2008). Also, it is relevant to highlight that melatonin promotes sleep-like state under appropriate sleeping conditions (Bunnell et al., 1992; Lavie, 2001) and acute doses applied could not generate sleep if the animals were not subjected to sleeping condition within 2 h of administration.

2.5. Appetitive conditioning task

The appetitive conditioning protocol was modified from Pather and Gerlai (2009). Individual zebrafish from each experimental group described above were transferred from their home tank to a testing tank (40 × 25 × 20 cm, width × depth × height). The testing tank was divided in half by a white partition with a 2 cm opening at the bottom that allowed the fish to swim from one side of the tank to the other. Two computer monitors (LG-Flatron E2011P-BN, 20″) connected to a desktop computer (Intel Pentium G3220 3.00GHZ) flanked the testing tank (Fig. 1). The computers ran an in-house software application that presented animated images of a zebrafish shoal at varying intervals on each monitor. Zebrafish are a highly social species and prefer to stay in close proximity to their conspecifics both in nature and in the laboratory (Engeszer et al., 2007). Zebrafish also respond in a similar manner to computer-animated conspecific images as to live conspecifics (Qin et al., 2014; Saverino and Gerlai, 2008). Ruhl and McRobert (2005) have previously shown differences in sex and body size preferences during shoal formation in zebrafish. Thus, we used an image of six zebrafish of mixed sexes and similar body sizes compared to the experimental fish to simulate naturally occurring shoals.

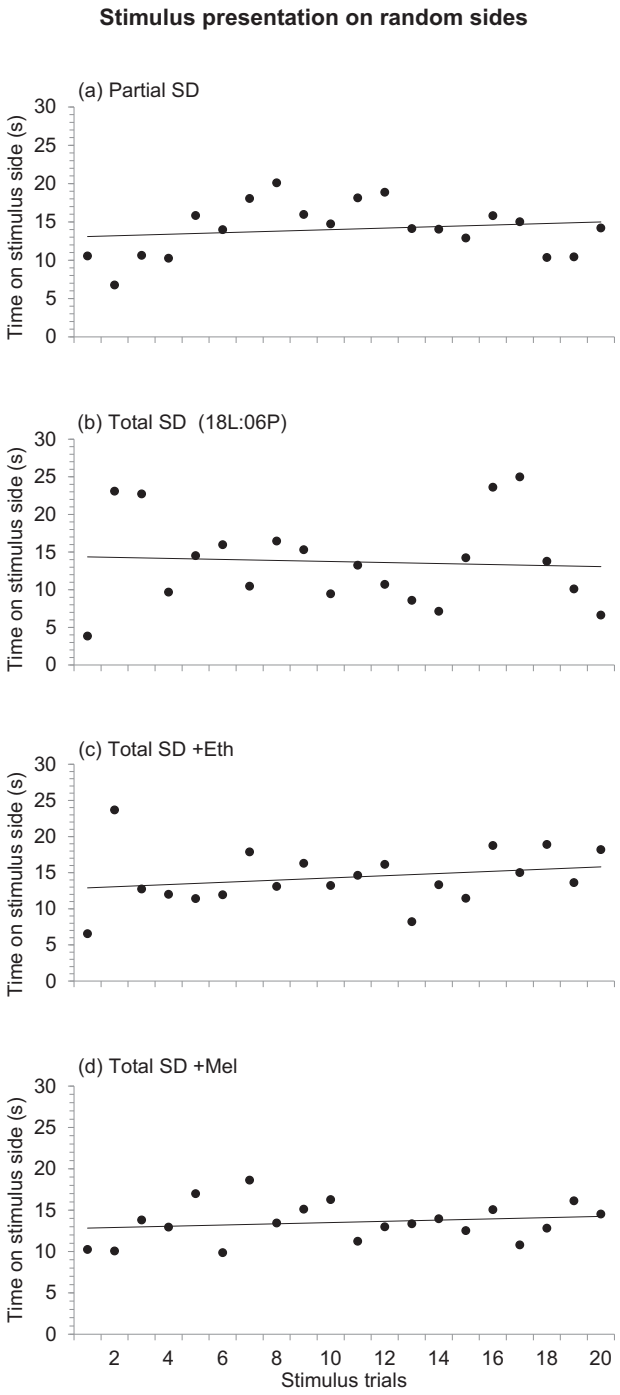

Fig. 4. Time spent on the stimulus side of the tank during the 20 trials when the stimulus was presented on random in an appetitive conditioning task using social stimulus as a reward. Five groups were tested: (a) control (12 L:12D; $n = 10$), (b) partial SD (18 L:06D; $n = 15$), (c) total SD (18 L:06D with light pulses; $n = 15$), (d) total SD + Eth (18 L:06D with light pulses + 60 min ethanol exposure before the onset of light pulses on the last day; $n = 15$), and (e) total SD + Mel (18 L:06D with light pulses + 10 days melatonin exposure including the 72 h of sleep deprivation; $n = 15$). Light:Dark cycle used was applied during 72 h for each group. In the graphs, black dots represent the time fish spent in the stimulus side and filled lines represent trends to the stimulus side. For further details of the results of statistical analysis see Results section.

The animated conspecific image was presented on: (a) one side only,

Table 1
Estimates of mixed effect model for time spent in the stimulus side during the stimulus presentation.

Explanatory variable	Stimulus presentation scheme								
	One side only			Alternate sides			Random sides		
	Chi-squared	*p*-value		Chi-squared	Pr(> chisq)		Chi-squared	Pr(> chisq)	
Stimulus Trials	29.88	< 0.01*		23.57	< 0.01*		3.13	0.07	
Groups	7.18	0.12		4.86	0.30		8.03	0.04*	
Pairwise comparison	lsmeans ± sem	t-value	*p*-value	lsmeans ± sem	t-value	*p*-value	lsmeans ± sem	t-value	*p*-value
Control vs Partial SD	0.29 ± 0.18	1.63	0.48	0.07 ± 0.06	1.10	0.80	–	–	–
Control vs Total SD	0.39 ± 0.18	2.15	0.21	0.10 ± 0.06	1.68	0.45	–	–	–
Control vs Total SD + Eth	0.03 ± 0.18	0.16	0.99	0.10 ± 0.06	1.60	0.49	–	–	–
Control vs Total SD + Mel	0.26 ± 0.18	1.40	0.62	0.002 ± 0.07	0.04	1.00	–	–	–
Partial SD vs Total SD	0.09 ± 0.18	0.52	0.98	0.03 ± 0.06	0.56	0.98	0.33 ± 0.17	1.94	0.22
Partial SD vs Total SD + Eth	− 0.26 ± 0.18	− 1.47	0.58	0.03 ± 0.06	0.48	0.98	− 0.10 ± 0.18	− 0.58	0.94
Partial SD vs Total SD + Mel	− 0.04 ± 0.18	− 0.19	0.99	− 0.07 ± 0.07	− 0.94	0.88	− 0.05 ± 0.17	− 0.27	0.99
Total SD vs Total SD + Eth	− 0.36 ± 0.18	− 1.99	0.28	− 0.004 ± 0.06	− 0.08	1.00	− 0.44 ± 0.17	− 2.51	0.07
Total SD vs Total SD + Mel	− 0.13 ± 0.18	− 0.71	0.95	− 0.10 ± 0.07	− 1.45	0.59	− 0.38 ± 0.17	− 2.26	0.12
Total SD + Eth vs Total SD + Mel	0.23 ± 0.18	1.25	0.72	− 0.09 ± 0.07	− 1.38	0.64	0.05 ± 0.17	0.33	0.98

SD: sleep deprivation, sem: Standard error of the mean, St Dev: Standard Deviation.

(b) alternating sides, and (c) random sides of the tank. Fifteen fish from each of the 5 groups (control, Partial SD, Total SD, Total SD + Eth, Total SD + Mel) were presented images as described above. The random side presentation condition was not applied to the control group. For the one side only presentation condition, half of the experimental fish received the stimulus on the left side and half on the right side of the tank to control for side bias. We expected that when images were presented only on one side, zebrafish would prefer to stay on the stimulus presentation side. In contrast, when images were presented on alternating sides, zebrafish were expected to learn to shuttle back and forth due to the rewarding nature of the stimulus, allowing the quantification of learning performance. Similarly, when images were presented on random sides, we expected zebrafish to prefer to stay on the side of the stimulus presentation but will be unable to anticipate the location of the next stimulus presentation (Pather and Gerlai, 2009).

After being introduced to the testing tank, experimental fish were shown a blank screen for 2 min (habituation period). An image of conspecifics was subsequently presented for 30 s always starting on the left side of the tank followed by an interval of 60 s without the stimulus on both screens, henceforth referred to as the inter stimulus interval (ISI). The presentation protocol (stimulus + ISI) was repeated 20 times with subsequent images presented either on the same side, alternating sides, or random sides depending on the presentation condition. Therefore, there were a total of 20 stimulus presentations and 20 ISI. Fish were tested individually and their behavior was recorded using a handycam (Sony Digital Video Camera Recorder; DCR-SX45) positioned 1.5 m away from the front of the testing tank. The behavioral tests were conducted between 9 am and 4 pm.

2.6. Behavioral analysis

The video recordings were analyzed using ZebTrack, a video tracking software developed in MatLab, previously described by Pinheiro-da-Silva et al. (2017). We quantified the amount of time zebrafish spent on each side of the tank during the 30 s stimulus presentation and during the 60 s ISI for all 20 trials. We also analyzed other behavioral parameters including average speed, maximum speed, total distance traveled and freezing.

2.7. Statistics

To apply inferential statistics, we first evaluated the data using an exploratory analysis in consideration of potential problems, such as outliers, heterogeneity of variance, normality, zero inflation, collinearity and variable independency, as suggested by Zuur et al. (2010).

To develop a model of time spent on the correct side of the tank (response variable) and the explanatory variable (stimulus or ISI trials and treatments), we used a mixed effects model analysis for longitudinal data. The term longitudinal is related to repeated measures of a response variable over time (Zuur et al., 2010). The mixed model present random effect factors (represented by the variation within zebrafish behavior), fixed effect factors (represented by the influence of the explanatory variables: stimulus trials and groups) and error.

The data used on the model was “time on each side during the stimulus presentation”, ranging from 1 to 20, and the different experimental groups. The response variable was calculated based on the time probability values *(pi),* in relation to the total stimulus presentation time *(ni)*. In this model, *i* represents any one of the 20 stimulus presentations. Thus, it is reasonable to assume that *yi* follows the binomial distribution error β*in (ni, pi)* with logit link function (according to Zuur et al., 2010). Lastly, the formulation of the logistical model for probability *pi* can be represented below, as in Wood (2006):

$$log\left(\frac{pi}{1-pi}\right) = \beta_0 + \beta_1 x_i$$

pi = time probability on the correct stimulus side.
β_0 = Model's linear coefficient.
β_1 = Stimulus trial's angular coefficient.
χi = Trial.

To develop the mixed effects model, we used the glmmPQL function from the MASS package (Venables and Ripley, 2003) of the R software (Team, 2015). We decided to use this algorithm due to the abnormal distribution and over dispersed nature of the residuals in the response variable detected during the exploratory analysis. Moreover, the response variable was discrete quantitative data that varied from 0 to 30 (stimulus trials) or 0 to 60 (ISI), which may present a binomial distribution error (according to Zuur et al., 2010). The glmmPQL function was effective in this case because it presents mixed generalized models with a ‘quasi’ distribution, adequate for over dispersed data.

For each model (one side only, alternating sides and random sides) the *p* values for explanatory variables (stimulus or stimulus-to-be trials) were obtained through the Wald chi-squared Test, with the “car” package using R software (Fox and Weisberg, 2011). The post-hoc comparisons between treatments, of each model, were made using the Tukey test in “lsmeans” package (Lenth and Hervé, 2014).

Average speed, maximum speed, freezing and distance traveled were also compared between the groups after pooling data from the 3 different stimulus presentation conditions using One-Way ANOVA. For all comparison, the probability level considered for significance was

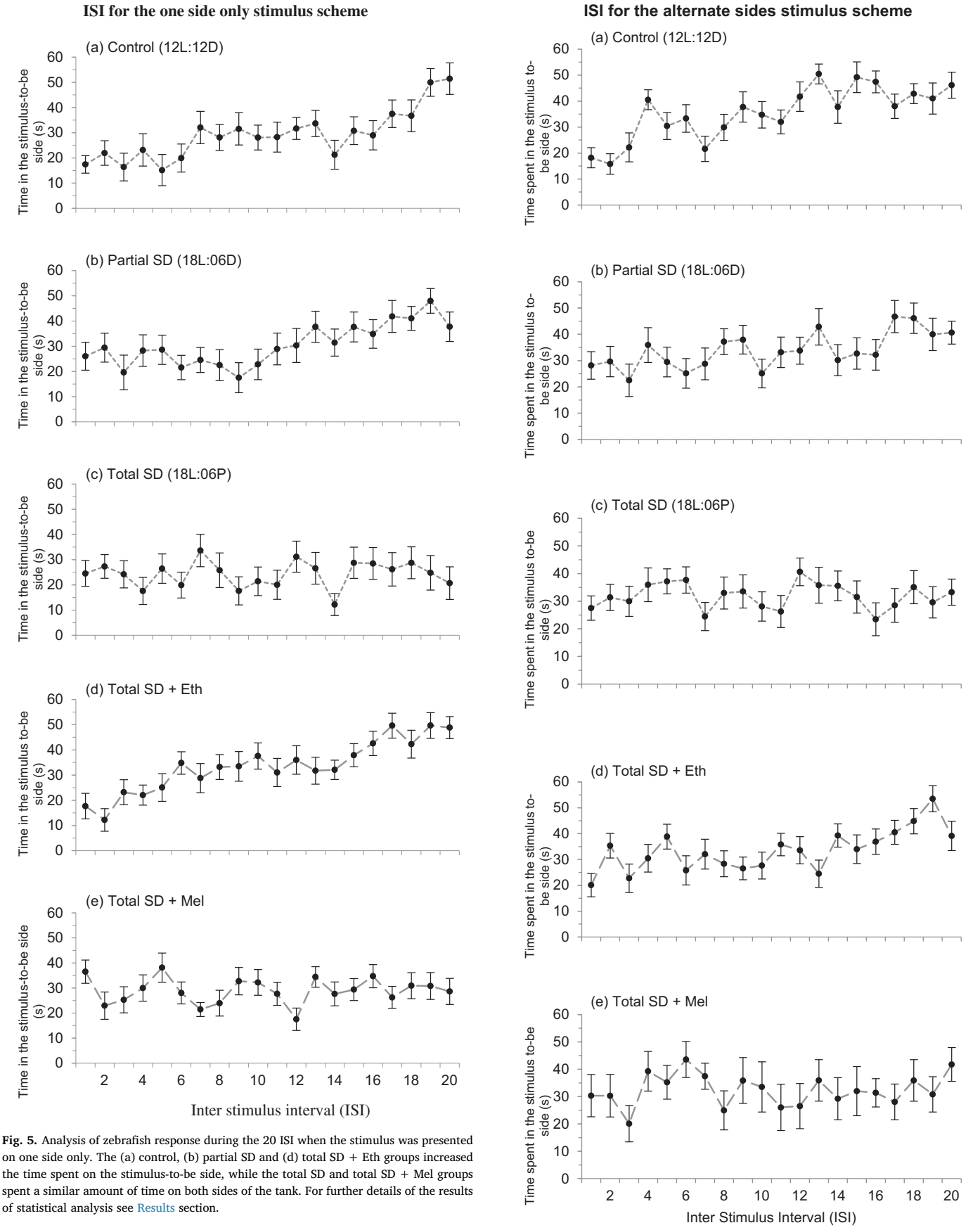

Fig. 5. Analysis of zebrafish response during the 20 ISI when the stimulus was presented on one side only. The (a) control, (b) partial SD and (d) total SD + Eth groups increased the time spent on the stimulus-to-be side, while the total SD and total SD + Mel groups spent a similar amount of time on both sides of the tank. For further details of the results of statistical analysis see Results section.

(caption on next page)

Fig. 6. Analysis of zebrafish response during the 20 ISI when the stimulus was presented on alternate sides. The (a) control, (b) partial SD and (d) total SD + Eth groups increased the time spent on the stimulus-to-be side, while the total SD and total SD + Mel groups spent a similar amount of time on both sides of the tank. For further details of the results of statistical analysis see Results section.

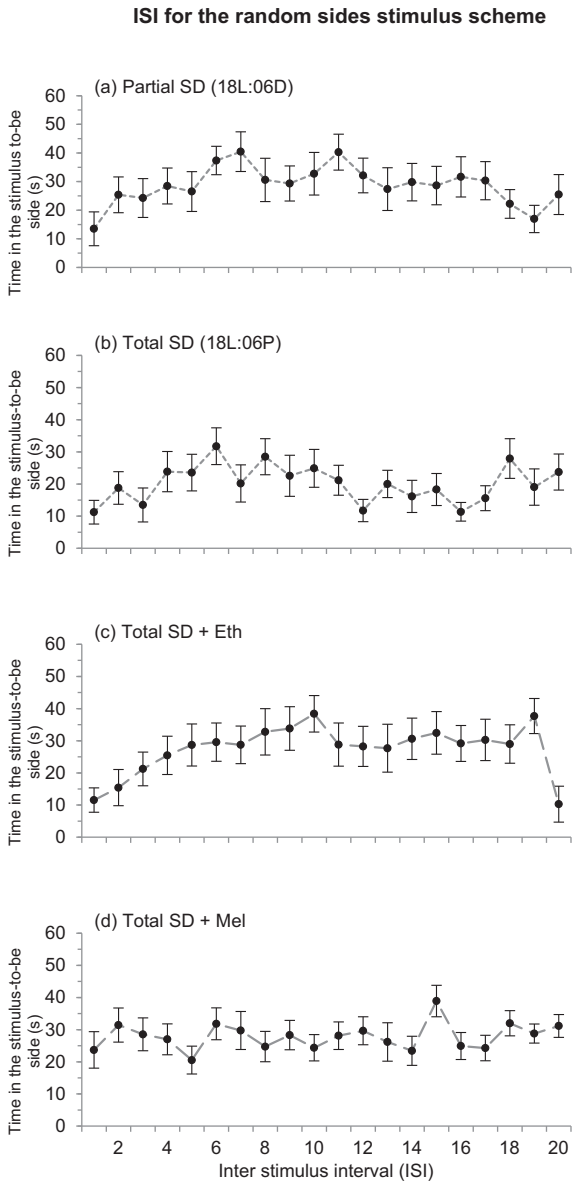

Fig. 7. Analysis of zebrafish response during the 20 ISI when the stimulus was presented on random sides. The (a) control, (b) partial SD and (d) total SD + Eth groups increased the time spent on the stimulus-to-be side, while the total SD and total SD + Mel groups spent a similar amount of time on both sides of the tank. For further details of the results of statistical analysis see Results section.

$p < 0.05$.

3. Results

Three types of behavior were analyzed: (1) response to stimulus (behavior during the stimulus presentation period), (2) learning performance (behavior during the ISI), and (3) locomotor responses (motor responses averaged over the entire testing session).

Figs. 2, 3 and 4 show the amount time zebrafish spent on the stimulus side of the tank during the stimulus presentation (i.e. response to stimulus) for each experimental group that received the stimulus presentation on one side only, alternating sides and random sides, respectively. The mixed model comparison showed that during the stimulus presentation period, none of the experimental groups differed in the amount of time zebrafish spent on the stimulus side of the tank (Table 1). Post-hoc comparison tests (lsmeans) between groups for each stimulus presentation condition are shown in Fig. 8. The Wald chi-squared test indicates that with an increasing number of trials, all groups spent an increasingly significant amount of time on the stimulus side of the tank during the presentation period when the stimulus was presented on only one side of the tank (GLMM, $\chi^2 = 29.88$, df = 1, $p < 0.01$; Table 1), but the amount of time spent on the stimulus side did not differ between groups (GLMM, $\chi^2 = 7.18$, df = 4, $p = 0.12$; Table 1). Similarly, when the stimulus was presented on alternating sides, with an increasing number of trials, all groups spent an increasingly significant amount of time on the stimulus side (GLMM, $\chi^2 = 23.57$, df = 1, $p < 0.01$; Table 1), but the time spent on the stimulus side did not differ between groups (GLMM, $\chi^2 = 4.86$, df = 4, $p = 0.30$; Table 1). In contrast, when the stimulus was randomly presented, the time spent on the stimulus side did not differ over subsequent trials (GLMM, $\chi^2 = 3.13$, df = 1, $p = 0.07$; Table 1), however, one of the groups exhibited a differ patter compared to the others (GLMM, $\chi^2 = 8.03$, df = 4, $p = 0.04$; Table 1).

Figs. 5, 6 and 7 show the amount of time zebrafish spent on the side of the tank that the stimulus will be presented next (henceforth referred to as the "stimulus-to-be side) during the ISI for all groups receiving the stimulus presentation on one side only, alternating sides and random sides, respectively. Analysis of time spent on the stimulus-to-be side over the 20 ISIs using the mixed model is shown in Table 2. Analysis revealed that when the stimulus was presented on one side only or on alternating sides, the total SD group spent less time on the stimulus-to-be side compared to the control and partial SD groups. Post-hoc comparisons (lsmeans) are presented in Table 2 and Fig. 8, and indicate statistical differences during the ISI for different stimulus presentation conditions. The Wald chi-squared test indicates that with an increasing number of ISI trials, zebrafish spent an increasingly significant amount of time on the stimulus-to-be side and the amount of time spent on the stimulus to-be side also differed between groups when the stimulus was presented on one side only (trials: $\chi^2 = 39.33$, df = 1, $p < 0.01$; groups: $\chi^2 = 29.00$, df = 4, $p < 0.01$; Table 2), alternating sides (trials: $\chi^2 = 31.08$, df = 1, $p < 0.01$; groups: $\chi^2 = 19.14$, df = 4, $p < 0.01$; Table 2), and random sides presentation scheme (trials: $\chi^2 = 4.36$, df = 1, $p = 0.03$; groups: $\chi^2 = 14.17$, df = 4, $p = 0.002$; Table 2).

Analysis of locomotor responses during the entire testing session revealed that maximum speed was significantly higher for the partial SD group compared to all other groups (One-Way ANOVA, F = 11.28 $p < 0.001$, Fig. 9a), while average speed was significantly higher for the partial SD and total SD + Eth groups compared to the control group (One-Way ANOVA, F = 12.31 $p < 0.001$, Fig. 9b). One-Way ANOVA found no significant differences in freezing between the different groups (F = 1.85 $p = 0.12$, Fig. 9c). The total distance traveled was higher for the control, partial SD and total SD + Eth and lower for total SD and total SD + Mel groups (One-WayANOVA, F = 11.71 $p < 0.001$, Fig. 9d).

4. Discussion

In the current study, we demonstrated that sleep deprivation impairs learning performance in a spatial associative learning task in zebrafish *(Danio rerio)*. Although our partial sleep deprivation protocol did not impair learning and memory, total sleep deprivation for 72 consecutive hours was shown to interfere with behavioral performance in the learning task. However, when total sleep deprived zebrafish were treated with either melatonin or ethanol prior to behavioral testing, the learning impairment was rescued. Our results demonstrate that ethanol (a single acute exposure) and melatonin (10 day chronic exposure) treatment was sufficient to counteract the learning impairment induced

Table 2
Estimates of mixed effect model for time spent in the stimulus-to-be side during the inter stimulus interval (ISI).

Explanatory variable	Stimulus presentation scheme								
	One side only			Alternate sides			Random sides		
	Chi-squared	*p*-value		Chi-squared	*p*-value		Chi-squared	*p*-value	
Stimulus Trials	39.33	< 0.01*		31.08	< 0.01*		4.36	0.03*	
Groups	29.00	< 0.01		19.14	< 0.01*		14.17	0.002*	
Pairwise comparison	lsmeans ± sem	t-value	*p*-value	lsmeans ± sem	t-value	*p*-value	lsmeans ± sem	t-value	*p*-value
Control vs Partial SD	− 0.001 ± 0.09	− 0.01	1.00	0.05 ± 0.05	1.01	0.84	–	–	–
Control vs Total SD	0.33 ± 0.09	3.57	0.005*	0.24 ± 0.05	4.19	0.001*	–	–	–
Control vs Total SD + Eth	− 0.13 ± 0.08	− 1.50	0.56	0.09 ± 0.05	1.74	0.42	–	–	–
Control vs Total SD + Mel	0.13 ± 0.09	1.47	0.58	0.11 ± 0.06	1.69	0.44	–	–	–
Partial SD vs Total SD	0.33 ± 0.09	3.58	0.005*	0.18 ± 0.05	3.17	0.02*	0.37 ± 0.14	2.51	0.07
Partial SD vs Total SD + Eth	− 0.13 ± 0.08	− 1.49	0.57	0.04 ± 0.05	0.71	0.95	− 0.14 ± 0.15	− 0.95	0.77
Partial SD vs Total SD + Mel	0.13 ± 0.09	1.48	0.57	0.05 ± 0.06	0.81	0.92	− 0.04 ± 0.14	0.31	0.99
Total SD vs Total SD + Eth	− 0.46 ± 0.09	− 5.06	< 0.0001*	− 0.14 ± 0.05	− 2.50	0.10	− 0.52 ± 0.15	− 3.45	0.006*
Total SD vs Total SD + Mel	− 0.19 ± 0.09	− 2.03	0.26	− 0.13 ± 0.06	− 1.98	0.28	− 0.42 ± 0.14	− 2.89	0.02*
Total SD + Eth vs Total SD + Mel	0.27 ± 0.09	2.94	0.03*	0.01 ± 0.06	0.19	0.99	0.10 ± 0.15	0.66	0.91

effect of sleep deprivation in zebrafish.

Our findings confirm previous studies by demonstrating that zebrafish are capable of appetitive reinforcement-based learning in a spatial alternation task (Pather and Gerlai, 2009; Williams et al., 2002), as well as other associative learning tasks (Al-Imari and Gerlai, 2008; Braubach et al., 2009; Chacon and Luchiari, 2014; Colwill et al., 2005; Gómez-Laplaza and Gerlai, 2010; Karnik and Gerlai, 2012; Luchiari and Chacon, 2013; Pittman and Lott, 2014; Santos et al., 2016; Yu et al., 2006). We also showed that sleep deprivation did not impair behavioral responses to the animated conspecific image, even when receiving alcohol or melatonin treatment (Figs. 2, 3 and 4).

The stimulus we used in our learning task was a computer animated image of a zebrafish shoal. Zebrafish are a highly social species that prefers swimming in groups called shoals (Pitcher, 1983), similar to other shoaling fish species (Krause et al., 1996; Pollock et al., 2006). Zebrafish recognize conspecifics and exhibit preference for groups with similar characteristics. Shoaling may reduce predation risks, facilitate foraging and boost reproductive success (Saverino and Gerlai, 2008; Sison and Gerlai, 2011b). Qin et al. (2014) have previously shown that live conspecifics (inside or outside the tank) are as equally effective as animated computer images (2D or 3D) for inducing robust shoaling behavior in zebrafish. Although we found a more robust shoaling response when the stimulus was presented on one side only compared to when the stimulus was presented on alternating sides, our results show that fish from all experimental groups responded to the stimulus in a similar manner and exhibited a preference for the stimulus presentation (Fig.8).

Although behavioral responses to the conspecific image did not differ between groups, sleep deprivation significantly altered locomotor parameters (Fig. 9). Partial SD animals and the total SD + Eth animals showed higher average speed and total distance traveled suggesting increased activity during the tests, while the total SD and total SD + Mel were similar to the control group. These findings are similar to those reported by Yokogawa et al. (2007) and Pinheiro-da-Silva et al. (2017), who showed that short-term sleep deprivation reduced activity levels, whereas prolonged SD increased locomotor activity, similar to our partial and total sleep deprivation groups.

Recurrent sleep deprivation increases sleep debt which has physical and psychological implications: simple mental tasks may become more difficult and higher cognitive processing becomes compromised (Alhola and Polo-Kantola, 2007; Killgore, 2010; Prince and Abel, 2013; Raidy and Scharff, 2005; Yu et al., 2006), including learning and memory. The spatial associative learning task used in this study consisted of a 30 s stimulus presentation followed by a 60 s inter stimulus interval (ISI). Zebrafish exhibited a preference for the stimulus demonstrated by an increased in the amount of time spent near the conspecific image during the presentation period. However, we expected zebrafish to learn the pattern of presentation and anticipate where the stimulus would appear next, and respond by moving to the “stimulus-to-be side” during the next ISI. When the stimulus was consistently presented on one side only, the control, partial SD and total SD + Eth groups increased the time spent on the stimulus side during both the presentation period and the ISI, a response not observed in the total SD group (Figs. 5 and 8d).

By presenting the stimulus on alternating sides, we examined the fish's ability to learn a slightly more complex presentation pattern. Fig. 6 shows that animals learned the presentation pattern by significantly increasing the time they spent on the stimulus-to-be side by the second half of the trials. The results indicate that the control and partial SD groups learned to anticipate the presentation of the stimulus on the correct side of the tank. In contrast, zebrafish in the total SD group were unable to learn to anticipate where the next stimulus presentation would appear. Notably, we found that when fish in the total sleep deprivation condition were treated with either ethanol or melatonin, there was an increase in learning performance.

By presenting the stimulus on random sides, we confirmed that the increased time zebrafish spent near the “stimulus-to-be side” did not simply reflect a side bias. As expected, since the presentation of the stimulus does not predict the location of the next stimulus presentation, none of the groups exhibited a preference for the stimulus-to-be side during the ISI (Fig. 7). Overall, our results are in line with Pather and Gerlai (2009), which suggests associative learning performance in this task is driven by an animal's motivation to join groups.

Although the total SD groups (total SD, total SD + Eth, total SD + Mel) perceived the conspecific images as rewarding and showed preference for the stimulus, we observed that total SD decreased total distance traveled, as shown in Fig. 9d, similar to the effects of SD on rest-activity rhythm (Tobler et al., 1998; Moura et al., 2017). Sleep deprivation was also shown to cause changes in daytime locomotor activity as well as enhance arousal thresholds on the following day (Zhdanova et al., 2001).

In addition to the negative effects of total SD on learning performance, we also examined the effects of two drugs known to affect sleep: ethanol and melatonin. Ethanol has been shown to induced behavioral changes in zebrafish (Tran et al., 2015), impair coordination and swimming, as well as alter fear and anxiety-like responses (Gerlai et al., 2000).

We found that the total SD + Eth group exhibited a preference for the stimulus and learned to anticipate the presentation of the stimulus when it was presented on one side only and on alternating sides, similar to the control group. Ethanol is classified as a depressant (Charness et al., 1989) with sedative effects (Roehrs et al., 1999) and exposure on the last night of sleep deprivation may have promoted sleep-like

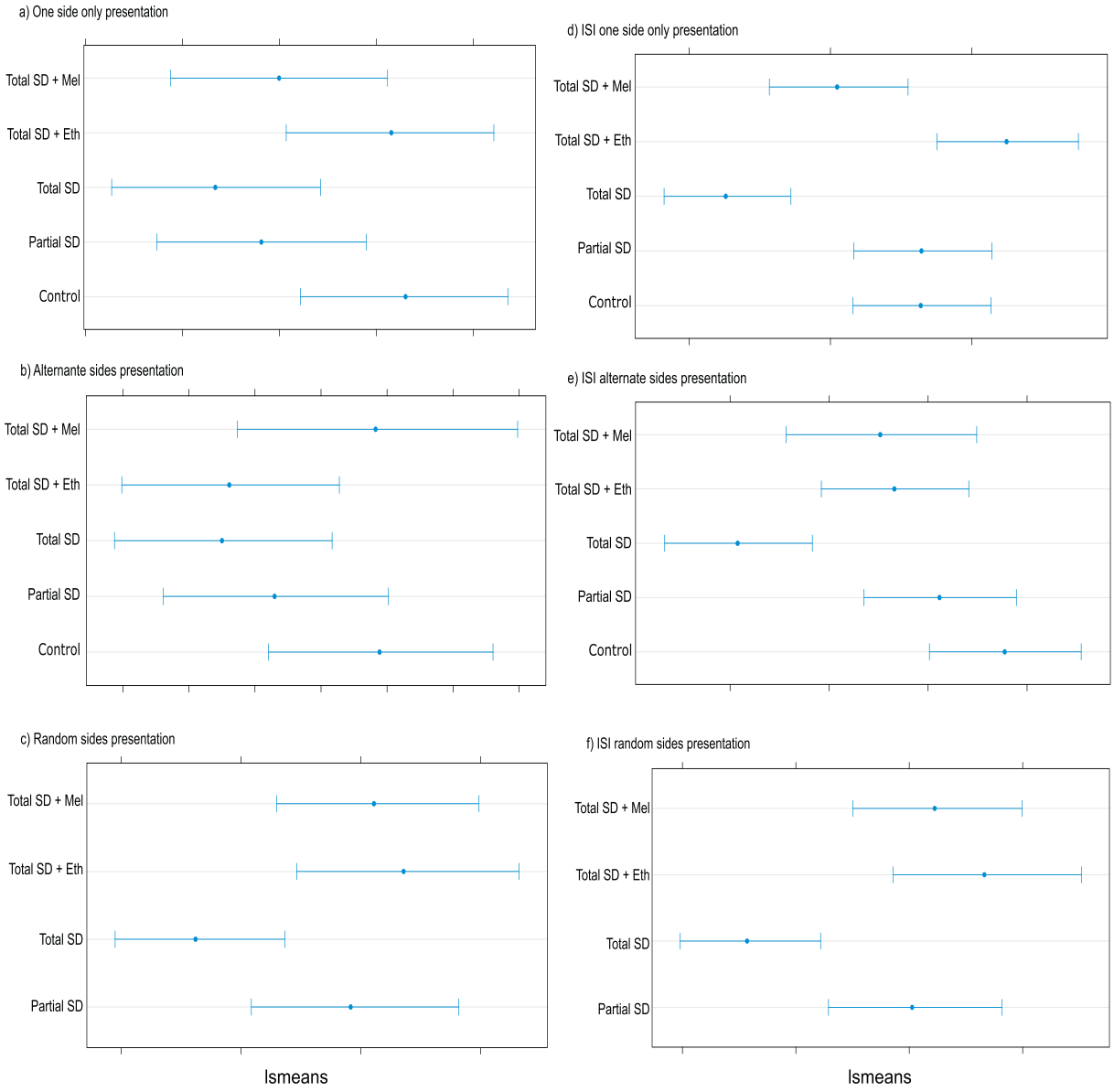

Fig. 8. Confidence intervals for LSmeans for time spent on each side of the test tank during the 20 trials when the stimulus was presented on (a) one side only, (b) alternate sides and (c) random sides in the appetitive conditioning task, and confidence interval LSmeans for zebrafish response during the 20 ISI when the stimulus was presented on (d) one side only, (e) alternate sides and (f) random sides of the appetitive conditioning task. The groups tested were kept for 72 h under different light:dark cycles and then associative learning took place. The five groups were: control (12 L:12D; $n = 10$ for each presentation scheme), partial SD (18 L:06D; $n = 15$ for each presentation scheme), total SD (18 L:06D with light pulses; $n = 15$ for each presentation scheme), total SD + Eth (18 L:06D with light pulses + 60 min ethanol exposure before the onset of light pulses on the last day; $n = 15$ for each presentation scheme), and total SD + Mel (18 L:06D with light pulses + 10 days melatonin exposure including the 72 h of sleep deprivation; $n = 15$ for each presentation scheme). For further details of the results of statistical analysis, see Tables 1 and 2.

behavior and may have improved memory consolidation. This hypothesis is supported by Roehrs and Roth (2001) and Williams and Salamy (1972) who found that ethanol changes sleep structure, in addition to its sedative and sleep-promoting effects. Similarly, studies in humans have shown that an ethanol dose of 0.16 g/kg reduced sleep latency and increased sleep time (Roehrs and Roth, 2001; Stone, 1980). While zebrafish and humans are phylogenetically distant, it is worth exploring potential links between sleep and ethanol consumption.

Studies in humans have shown that an ethanol dose of 0.16 g/kg reduced sleep latency and increased sleep time (Roehrs and Roth, 2001; Stone, 1980). While zebrafish and humans are phylogenetically distant, it is worth exploring potential links between sleep and ethanol consumption. However, we should take into account that ethanol is a drug and (1) may cause tolerance and dependence (Chacon and Luchiari, 2014; Ford and Kamerow, 1989; Weissman et al., 1997), (2) under uncontrolled use results in the disruption of sleep architecture and continuity (Brower, 2003), (3) chronic heavy consumption leads to neural damage (Chacon and Luchiari, 2014), (4) there is no effective treatment for alcoholism (Vengeliene et al., 2008) and (5) sleep disorders may persist even after the cessation of ethanol consumption (Drummond et al., 1998). Therefore, although we report that alcohol exposure may have allowed the learning performance in sleep deprived fish, additional research still needs to be conducted.

In contrast to the effects of ethanol on sleep deprivation, we found that melatonin treatment did not completely rescue the learning impairment. Melatonin is a pineal-produced hormone shown to promote sleep and entrain circadian rhythmicity under appropriate conditions (Brzezinski et al., 2005; Zhdanova et al., 2001). We treated sleep deprived fish with melatonin for 10 days before the test (total SD + Mel) and although this group responded to the stimulus presentation in way

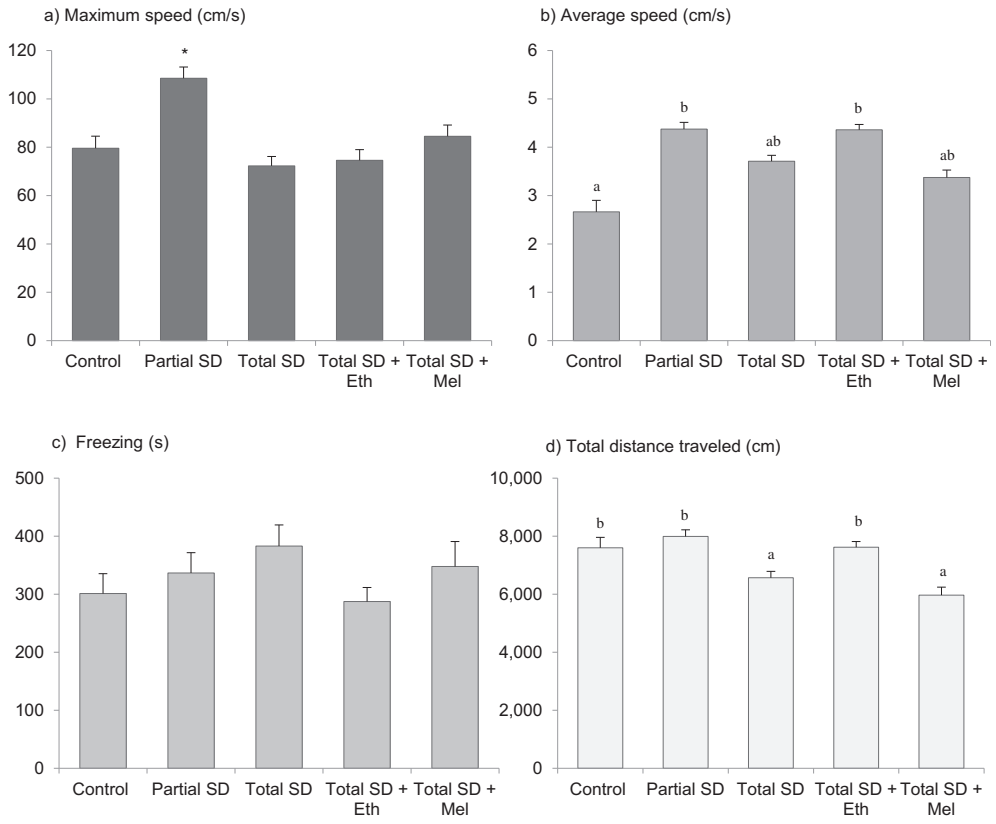

Fig. 9. Behavioral parameters analyzed for (a) maximum speed swimming, (b) average speed swimming, (c) freezing behavior and (d) total distance traveled by the zebrafish. Five groups of fish were kept for 72 h under different ligh:dark cycles and then tested for associative learning. The parameters presented correspond to the behavior of fish for 30 min during which the learning task had been tested. The groups tested were: control (12 L:12D; $n = 30$), partial SD (18 L:06D; $n = 45$), total SD (18 L:06D with light pulses; $n = 45$), total SD + Eth (18 L:06D with light pulses + 60 min ethanol exposure before the onset of light pulses on the last day; $n = 45$), and total SD + Mel (18 L:06D with light pulses + 10 days melatonin exposure including the 72 h of sleep deprivation; $n = 45$). Data were analyzed by tracking software (ZebTrack). (*) and different letters indicate statistical significance, One-Way ANOVA $p < 0.05$).

comparable to the other groups (Fig.8), learning performance was slightly different from the control and partial sleep deprivation groups. Studies have shown that exogenous melatonin treatment facilitates daytime and nighttime sleep, without altering sleep structure and duration (Arendt and Skene, 2005; Gandhi et al., 2015; Rajaratnam et al., 2004; Stone et al., 2000). Our findings suggest that exogenous melatonin administration may have promoted sleep in zebrafish that were undergoing total SD which may have contributed to the observed response to the conspecific stimulus and learning performance (Fig. 3e).

However, melatonin treatment did not alter behavioral parameters such as average speed, freezing and total distance traveled (Fig. 9). Light is known to suppress melatonin synthesis (Scheer and Czeisler, 2005)and is an important in the regulation of sleep (Bunnell et al., 1992; Lavie, 2001). Although Rawashdeh et al. (2007) have shown that melatonin can suppress memory consolidation during learning tasks, our results support the effects of melatonin as a sleep promoter that, for this reason, may have allowed learning.

Finally, although we have previously shown that our sleep deprivation protocol can suppress sleep-like behavior in zebrafish (Pinheiro-da-Silva et al., 2017), the effects of light on circadian rhythms cannot be ruled out. Light has direct masking effects on behavior (Colwell et al., 1990; Weger et al., 2011), can disrupt the molecular clock through light-dependent mechanisms (Cahill, 2002; Kaneko et al., 2005; Pando and Sassone-Corsi, 2002) and is a known cue for the entrainment of circadian rhythms (Armstrong, 1989; Duffy and Wright, 2005; Skene et al., 1999; Wang et al., 2014; Whitmore et al., 2000). For example, the delayed onset of darkness (e.g. extended light phase) can phase-shift the circadian rhythm (Honma et al., 1987; Minors et al., 1991; Tamai et al., 2007) and disruptions to the circadian rhythm has been shown to impair learning (Drummond et al., 2000; Graves et al., 2003; Killgore, 2010; Ruskin et al., 2004). Similarly, light can also interfere with the circadian regulation of melatonin synthesis (Armstrong, 1989; Bunnell et al., 1992; Lima-Cabello et al., 2014), which has also been implicated in learning and memory (Arendt, 2003; Mintz et al., 1998; Rawashdeh et al., 2007). Although we and others have shown that constant light and dark conditions does not abolish circadian activity in zebrafish (Hur et al., 2012; Moura et al., 2017; Sigurgeirsson et al., 2013), the direct effects of light on behavior and circadian rhythms should be noted.

Although zebrafish have recently been used as an effective animal model for studying learning and memory, the effect of sleep deprivation on learning performance has been unknown in this species. In this experiment, we utilized a previously validated protocol to quantify the effects of SD on cognitive performance in a spatial associative learning task. We found that totally sleep deprived animals exhibited reduced cognitive performance.

Behavioral studies represent an important method for identifying neuropathology. The finding that SD impairs learning performance implies that sleep deprivation affects brain function in fish similar to mammals (Graves et al., 2003; Rasch and Born, 2013; Ruskin et al., 2004). Therefore, our results show that the zebrafish represents a useful vertebrate model that can be used to investigate the molecular mechanisms regulating sleep, learning, and their interaction. Although research on the effects of sleep deprivation on cognitive function in zebrafish is still in its infancy, we now demonstrate the negative effects of total SD on a simple and complex learning task, as well as the effects of alcohol and melatonin exposure on learning performance in sleep deprived fish. Overall, our results reinforce the utility of zebrafish as a useful animal model for the proposed analysis.

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SD: sleep deprivation, sem: Standard error of the mean, St Dev: Standard Deviation.

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