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Time place learning and activity profile under constant light and constant dark in zebrafish (*Danio rerio*)

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ABSTRACT

The ability to learn about the signs of variability in space and time is known as time place learning (TPL). To adjust their circadian rhythms, animals use stimuli that change regularly, such as the light-dark cycle, temperature, food availability or even social stimuli. Because light-dark cycle is the most important environmental temporal cue, we asked how a diurnal animal would perform TPL if this cue was removed. Zebrafish has been extensively studied in the chronobiology area due to it diurnal chronotype, thus, we studied the effects of constant light and constant dark on the time-place learning and activity profile in zebrafish. Our data show that while under constant light and dark condition zebrafish was not able of TPL, after 30 days under the constant conditions, constant light led to higher activity level and less significant (robust) 24 h rhythm.

1. Introduction

The availability of food, sexual partners, predators and other biologically relevant stimuli vary both in time and in space (Carr and Wilkie, 1997). To process temporal and spatial information, animals use external cues that vary regularly, such as light and temperature, to adjust the internal clock and estimate time (Aschoff, 1954; Dunlap, 1999; Reebs, 2002; Kuhlman et al., 2015). The ability to learn about the variability of signs in the space and time is known as time place learning (TPL). According to Gallistel (1990), the occurrence of a biologically significant event promotes the formation of a memory code that includes the type of event, the time and place of the occurrence. This ability is related to the connection between the internal circadian system and associative memory (Anokhin, 1974).

Due to the 24 h duration of the sidereal day, light-dark cycle is the most remarkable zeitgeber (Kuhlman et al., 2015); the majority of the animals present photoreceptive cells, and thus can perceive light fluctuations throughout the day (Bell-Pedersen et al., 2005). However, under constant light conditions (24 h light or 24 h dark), the organisms still maintain rhythmicity, guided by endogenous regulators of the biological cycle (Johnson et al., 2004; Weger et al., 2013). Therefore, even in free running conditioning, a set of self-regulated molecular mechanisms generates the circadian rhythm through gene expression (Amaral et al., 2014), and allows organisms to predict and anticipate events that occur within a period of 24 h (Koukkari and Southern, 2006).

Among the studies on learning related to the circadian rhythm, bees were the pioneers to show TPL (Wahl, 1932; Finke, 1958), suggesting they possess a circadian oscillator that allows for monitoring time (Pittendrigh et al., 1958). After these studies, several others have found signs of both temporal and spatial learning linked to the endogenous clocks (Kramer, 1950; Boulos and Logothetis, 1990; Reebs, 1999; Gomez-Laplaza and Morgan, 2005;Heydarnejad and Purses, 2008). In addition to abiotic zeitgebers that favour rhythmicity and learning, recurrent visual, olfactory, auditory or tactile signals from one individual to another can entrain an animal activity/rest cycle, and thus be considered a social synchronizer (Rajaratnam and Redman, 1999). Several social species have their rhythms influenced by social cues, such as rodents (Crowley and Bovet, 1980; Mrosovsky, 1988) and primates (Erkert and Schardt, 1991; Melo et al., 2013). However, even being considered a synchronizer, there is no evidence the social stimuli can act lonely as a zeitgeber, without the most outstanding signal that is the light-

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dark cycle. Knowing that zebrafish (*Danio rerio*) is a highly social species (Pritchard et al., 2001; Larson et al., 2006; Gerlai, 2014) that presents TPL response based on social reward (Moura and Luchiari, 2016), the aim of this study was to test the TPL ability of zebrafish in the absence of light signals (constant light and constant dark). In this case, social stimuli would be the unique zeitgeber, and fish would have to use it to estimate time and adjust the circadian rhythm in the absence of light signals.

Zebrafish is considered a promising animal model, both for its high practicality of storage and maintenance as the high physiological and behavioural similarity to mammals (Ingham, 2009), allowing translational studies. In addition to these advantages, the zebrafish has been extensively studied in the chronobiology (Vatine et al., 2011) because of it diurnal activity pattern (Paciorek and Mcrobert, 2012), which favours its translational research, opposite to rodents that are nocturnal. In this sense, we use the zebrafish to study the effects of constant light or constant dark for the time-place learning, offering social stimulus as zeitgeber and reward. Our hypothesis are that (1) social stimulus can act as a synchronizer element, allowing rhythm, and (2) fish will show TPL due to the estimation on time given by the social stimuli and estimation of place given by the location of the stimuli.

2. Material and methods

2.1. Animals and procedures

Zebrafish *Danio rerio* (Hamilton, 1822) were obtained from a local fish farm (Natal, Rio Grande do Norte state) and kept in stocking tanks (2 fish/L) with aired and filtered water. Four 50L tanks make up one stocking unit in the closed water circulation system, with mechanical, biological and chemical filtration, in addition to UV disinfection. Water was maintained at $28 \pm 1\,^{\circ}C$, with pH 7.2 and low levels of ammonium and nitrite. The light cycle (fluorescent light, 150 Lux) was fixed at light-dark (12:12 LD), with the start of the light phase at 7 am. The fish were fed commercial pellets twice a day (38% protein, 4% lipids, Nutricom Pet) and *Artemia salina*.

Eighteen adult zebrafish of both sexes from the aforementioned stock were used to test time-place learning. All the procedures with the animals were authorized by the Animal Ethics Committee of Universidade Federal do Rio Grande do Norte (CEUA 039/2015).

2.2. Experimental design

The experimental animals were individually transferred to testing tanks ($100 \times 25 \times 25$ cm; length x width x height), divided horizontally into three same-size compartments (33 cm long): one central and two lateral (same procedure of Moura and Luchiari, 2016). The compartments were separated by opaque dividers, each with an 8 cm-diameter circular passage that allowed the fish to swim between the compartments. The passage was located on the right of the right side divider and on the left of the left side divider, such that the fish could not visualize more than two compartments at the same time, thereby preventing the stimulus placed in one of the side compartments from being seen when the animal was in the opposite side compartment. A cylindrical open-front receptacle (10 cm in diameter and 10 cm high) was fixed to the upper part of the lateral walls, and used to offer the stimulus (conspecific group) at specific times. The side compartments were randomly denominated morning compartment and afternoon compartment. Each tank was constantly aerated through an external filter (JEBO 50, 250L/h) located in the central compartment and air stones in each side compartment.

Animals were kept for 30 days under the above experimental conditions. A group of 5 zebrafish (same size and age) were introduced every day into the receptacle located in the morning compartment at 8 am and removed at 9 am, and into the receptacle of the afternoon compartment at 5 pm and removed at 6 pm, acting as a stimulus for the experimental fish to occupy the compartment where the group was placed. The group was introduced through a receptacle (500 ml) connected to a handle (2m) so that the experimenter could not be seen by the animals, which were separated by an opaque curtain. Food (artemia) was offered daily (once a day) at random times between 10 am and 4 pm, always in the central compartment so food would not be associated with any stimulus or time.

To verify if the TPL occurs in constant light conditions, two groups were tested: constant light group (LL; n = 8) and constant dark group (DD; n = 10). In constant light group, the animals were exposed to constant light (150 lux) during the 30-day experiment and behavioural was record. The constant dark group followed the same protocol, but with animals exposed to the total absence of light during the 30 days.

On days 15 and 30 of the experimental period, the behaviour of the animals was recorded on video for 1 h and 15 min, starting at 7:45 am and 4:45 pm, in order to observe animals for 15 min before the arrival of the group (stimulus) and during their entire presence. Behaviour on day 15 was recorded in the presence of the group to estimate the strength of this stimulus. However, on day 30, the animals were recorded without the presence of the group, in order to assess TPL in the experimental zebrafish.

For the video records, we used a handycamcorder (Sony DCR-SX45 Digital Video Camera Recorders) placed 1.5 m away and in front of the tanks. The behavioural analyses were conducted using the ZebTrack software, developed in MatLab. The following parameters were assessed: residence time and frequency of entry in the morning and afternoon compartments.

2.3. Activity registry

From the 8 fish under the LL condition and 10 fish under the DD condition, 4 fish of each group were also recorded during the last 6 days of the TPL experiment. Another 4 zebrafish from the stock condition were used to compose the LD condition, in order to have a control group. These 12 zebrafish were used to evaluate the effects of constant light conditions on the activity pattern. Fish held under light-dark (12:12 LD; n = 4) were also submitted to the TPL test, in order to have the same conditions of the other groups. The activity of each fish was recorded using Sony Kit infrared security cameras CCD, coupled to the DVR unit, for 144 h (the last 6 consecutive days of the 30 TPL days). The behaviour records were analysed using the ZebTrack software. We considered the average speed (cm/s) of each fish every 15 min of the 144 h. The data were plotted on diagrams of actogram, cosinor and waveform.

Actogram is a graphical representation of activity (average speed, y axes) along 24 h length of each plot line (x axes), and successive cycles are plotted below each other. The cosinor (Halberg et al., 1967) is a model to analyse the biological rhythms consisting of cosine curves with known periods (in our study, 24 h) to estimate rhythmic parameters and the pattern of the smooth rhythm. Each point of a cosinusoidal curve of a cosinor is a function of the average value of the variable of interest. These variables are: MESOR (M, Midline Estimating Statistic of Rhythm: the rhythm-adjusted mean that differs from the arithmetic mean when the data are not equidistant and/or do not cover an integer number of cycles), the amplitude of the oscillation (A), and the acrophase (φ, time at which the peak of a rhythm occurs) (Refinetti et al., 2007). The waveform is the prototypical cycle of a rhythm, defined by the amplitude (acrophase pairs of all harmonic terms included in the model to

account for the non-sinusoidality of the signal). Then, the waveform can be considered an extended cosinor analysis in inferential statistical chronobiology (Refinetti et al., 2007). The Sokolove-Bushell periodogram analysis was also developed to determine circadian rhythmicity.

2.4. Statistical analysis

Data were analysed for normality (Shapiro and Wilk, 1965; Doornik and Hansen, 2008) and homoscedasticity (Brown and Forsythe, 1974; Anderson, 2003) and parametric tests were used due to its normal and homoscedastic distribution. Behavioural data for residence time in the compartments and frequency of entry in each compartment were compared in the morning and afternoon, on days 15 and 30, using the paired student's T-test. We excluded the data from the central compartment, because it was a passage area and feeding site at random times, thus, the animal could visit this area to pass from the morning compartment to the afternoon or to search for food. The average speed data for activity registry for the last 6 days of TPL experiment were compared between LD, LL and DD groups using the one-way ANOVA. To verify the acrophase of each experimental group the Rayleigh test and Watson-Williams test were used (circular statistical analysis). The periodogram results were Bonferroni corrected, and one-way ANOVA was used to compare the groups.

3. Results

3.1. Constant light (LL)

On the day 15, during the 15 min before the group of conspecifics arrived, there was no significant difference in residence time between the morning and afternoon compartments in the morning (Student's t-test, $t = 1.20$ $p = 0.27$) or afternoon (Student's t-test, $t = -0.28$ $p = 0.79$), respectively (Fig. 1a). The frequency of entry into the compartments did not differ in the morning (Student's t-test, $t = 1.21$ $p = 0.26$; Fig. 1c), neither in the afternoon (Student's t-test, $t = -0.61$ $p = 0.56$; Fig. 1c).

During presentation of the group, residence time in the morning compartment was higher in the morning (Student's t-test, $t = 8.82$ $p < 0.001$), and in the afternoon it was higher in the afternoon compartment (Student's t-test $t = -4.99$ $p = 0.002$) (Fig. 1b). It was found higher frequency of entry in the morning compartment during the morning (Student's t-test, $t = 4.32$ $p = 0.003$), but it did not differ in the afternoon (Student's $t = -0.36$ $p = 0.73$) (Fig. 1d).

On the day 30, in the 15 min before the group was introduced into the tank, there was no difference between the time spent in each compartment in both the morning (Student's t-test, $t = -1.22$ $p = 0.26$) and the afternoon (Student's t-test, $t = -0.01$ $p = 0.99$; Fig. 2a). The frequency of entries was higher in the morning compartment in the morning (Student's t-test, $t = 3.75$ $p = 0.007$), but it did not differ in the afternoon (Student's t-test, $t = -1.22$ $p = 0.26$; Fig. 2c).

During the 60 min that the group should be presented (absence of the stimulus), the fish remained for a longer time in the afternoon compartment both in the morning (Student's t-test, $t = -3.41$ $p = 0.01$) and afternoon (Student's t-test, $t = -2.37$ $p = 0.05$; Fig. 2b) times. The frequency of entry did not differ in the morning (Student's t-test, $t = 0.97$ $p = 0.36$) or the afternoon (Student's t-test, $t = -1.87$ $p = 0.10$; Fig. 2d).

3.2. Constant dark (DD)

On the day 15, during the 15 min before the group of conspecifics arrived, there was no significant difference in residence time between the morning and afternoon compartments in the morning (Student's t-test, $t = 0.40$ $p = 0.70$) or afternoon (Student's t-test, $t = -0.05$ $p = 0.96$; Fig. 3a. The frequency of entry into the right compartments did not differ in the morning (Student's t-test, $t = -1.51$ $p = 0.15$), neither in the afternoon (Student's t-test, $t = 0.52p = 0.61$; Fig. 3c).

During presentation of the group, residence time in the compartments did not differ in the morning (Student's t-test, $t = -1.84$ $p = 0.08$), but it was higher in the afternoon compartment in the afternoon (Student's t-test $t = 2.85$ $p = 0.01$; Fig. 3b). With respect to the frequency of entry into the compartments, it was higher in the morning compartment in the morning (Student's t-test, $t = 2{,}54$ $p = 0.02$), but in the afternoon it did not differ (Student's t-test, $t = -0.80$ $p = 0{,}43$; Fig. 3d).

On the day 30, in the 15 min before the group presence into the tank, there was no difference between the time spent in each compartment both in the morning (Student's t-test, $t = -1.03$ $p = 0.31$) and in the afternoon (Student's t-test, $t = -1.40$ $p = 0.18$; Fig. 4a). The frequency did not differ in the compartments in the morning (Student's t-test, $t = -0.21$ $p = 0.83$), or afternoon (Student's t-test, $t = 0.02$ $p = 0.98$) (Fig. 4c).

During the 60 min that the group was expected (absence of the stimulus), the fish remained for a longer time in the morning compartment both in the morning (Student's t-test, $t = -2.33$ $p = 0.03$) and afternoon (Student's t-test, $t = -2.07$ $p = 0.05$; Fig. 4b). However, there were no differences in the frequency of entries in the compartments in the morning (Student's t-test, $t = -0.31$ $p = 0.76$) or the afternoon (Student's t-test, $t = 0.10$ $p = 0.93$; Fig. 4d).

3.3. Activity registry

During the last 6 days of the experimental period for TPL, the animals under constant light (LL), constant dark (DD) and light-dark cycle (LD) showed circadian rhythm and their activity profile is represented by the actogram in Fig. 5. The activity (average speed) mean values statistically differed between the three tested conditions (One way ANOVA $F = 4.35$ $p = 0.04$; Fig. 6a–c; Table 1): animals under LL showed higher activity than the animals under DD, but none of them differed from LD. While, animals under LD (Rayleigh $r = 0.997$ $p = 0.007$) and DD (Rayleigh $r = 0.938$ $p = 0.017$) groups showed significant directionality in the acrophase distribution (within a 24 h circle), the same did not happen for LL group (Rayleigh $r = 0.477$ $p = 0.427$; Fig. 6d–f), however the acrophase distribution did not differ between the groups (Watson-Williams test $F = 0.575$ $p = 0.585$) (Table 2). The centre of gravity was also significant different between the groups (One way ANOVA $F = 4.83$ $p = 0.04$; Table 1). There was no significant difference between the groups regarding the total area under the curve (One way ANOVA $F = 3.92$ $p = 0.06$). The periodogram analysis showed that animals under LD had stronger circadian rhythmicity (Sokolove-Bushell periodogram with Bonferroni correction, showing higher percentage of the total variance) than the animals under LL and DD, but there were no difference between LL and DD (One way ANOVA $F = 8.17$ $p < 0.05$).

Regarding the subjective light phase (7am to7pm), both the mean interval (I-m (w)) (One way ANOVA $F = 3.96$ $p = 0.06$) and the area under the curve (I-a (w)) (One way ANOVA $F = 3.94$ $p = 0.06$) did not differ between the groups (Table 1). The percentage of activity, as measured by percentage of the total area (I-a (w)%), showed significant difference between the conditions (One way ANOVA $F = 10{,}60$ $p = 0.004$; Table 1).

On the last day of the experiment (probe day), in which the stimulus was not presented to the experimental animals, the activity of the groups was significantly different (One way ANOVA $F = 29.98$ $p < 0.001$). The animals under LL and LD showed higher activity than the animals under DD (Fig. 7).

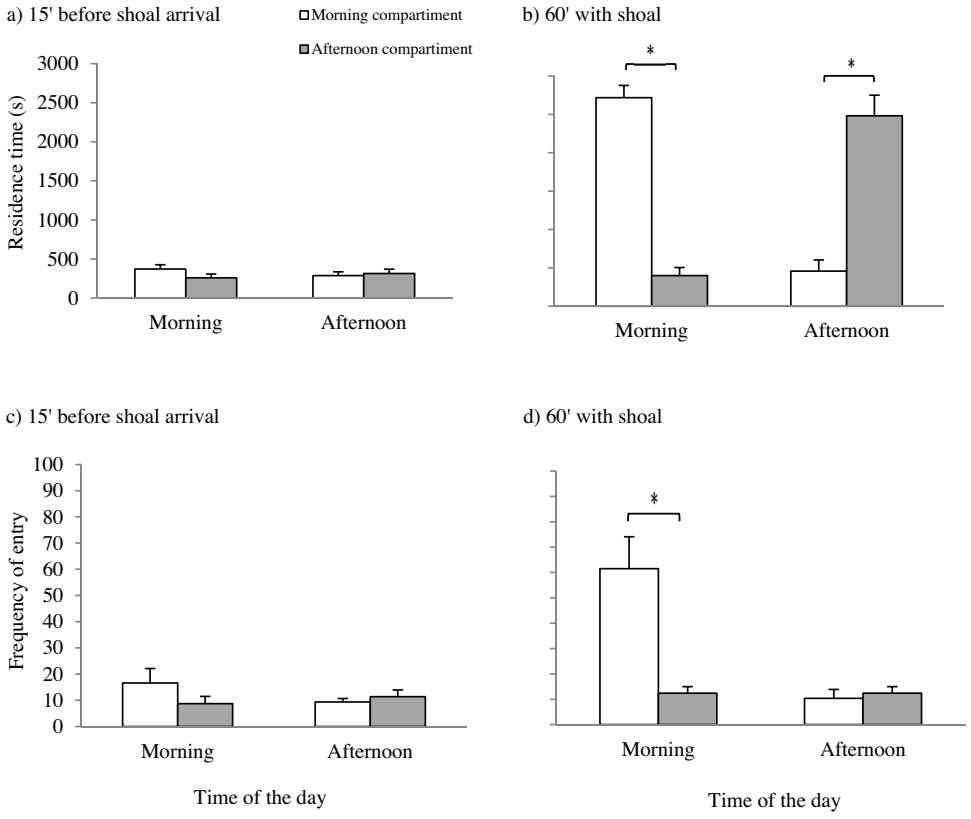

Fig. 1. Zebrafish residence time (a and b) and frequency of entry (c and d) in the morning and afternoon compartments on day 15 of the TPL test (n = 8) under constant light. Observations were made between 7:45 and 9:00 am, and 4:45 and 6:00 pm The first 15 min of observation indicate their ability to anticipate the arrival of the social stimulus (a and c). During the following 60 min, the social stimulus (group with 5 conspecifics) was maintained inside the experimental tank (b and d). * indicates statistical significance (Student's *t*-test, p < 0.05) between the compartments corresponding to each experimental period.

Table 1
Activity variables measured in zebrafish submitted to light-dark cycle (LD), constant light (LL) and constant dark (DD).

	Mean activity	Center of gravity	Total area under the curve	Mean interval	Area under the curve	Percentage of total area
LD	$3.94 \pm 0{,}11^{ab}$	740.90^{ab}	364.63	5.00	245.35	64.29^{a}
LL	$4.17 \pm 0{,}39^{a}$	724.45^{b}	401.07	4.35	213.45	52.66^{c}
DD	$3.20 \pm 0{,}12^{b}$	761.82^{a}	307.41	3.76	184.62	59.45^{b}

Different letters indicate statistical differences between the groups in the same variable evaluated (One way ANOVA, $p < 0.05$).

Table 2
Cosinor summary of the zebrafish submitted to light-dark cycle (LD), constant light (LL) and constant dark (DD).

	Animals	Mesor	Amplitude	Acrophase	%Ve(total)
LD	1	4.11: 4.05–4.17	1.83: 1.72–1.93	797.23: 783.94–810.53	97.20
	2	4.04: 3.98–4.10	1.52: 1.41–1.63	807.38: 791.02–823.74	96.88
	3	4.027: 3.96–4.090	1.46: 1.35–1.57	823.06: 805.42–840.70	96.65
	4	3.60: 3.51–3.70	1.36: 1.19–1.54	783.08: 754.07–812.09	90.81
LL	1	3.75: 3.68–3.82	0.65: 0.53–0.77	908.43: 865.19–951.67	95.25
	2	3.96: 3.90–4.020	0.309: 0.20–0.41	784.82: 706.22–863.43	96.83
	3	5.32: 5.25–5.40	0.60: 0.47–0.73	970.31: 921.28–1019.34	97.37
	4	3.67: 3.58–3.75	0.30: 0.15–0.464	241.75: 118.32–365.18	91.98
DD	1	3.52: 3.35–3.68	1.19: 0.90–1.48	920.57: 864.73–976.4	76.94
	2	3.01: 2.88–3.15	0.89: 0.64–1.14	884.12: 818.77–949.47	76.24
	3	3.27: 3.12–3.42	1.23: 0.96–1.50	832.051: 781.49–882.61	76.83
	4	2.99: 2.88–3.12	0.35: 0.14–0.57	1051.76: 902.03–1201.5	80.28

Period analysed: 1440 min.

4. Discussion

According to our results, after 30 days under constant light or dark conditions, zebrafish lost the ability to show TPL based on social stimulus (Figs. 2 and 4), however it maintained 24 h rhythm in both conditions (Table 1). Although zebrafish were trained for 30 days to find a conspecific shoal at different time and place have remained longer in only one of the places during the morning and the afternoon, fish from the dark condition searched for the group on the opposite side of the fish from the light condition. Further-

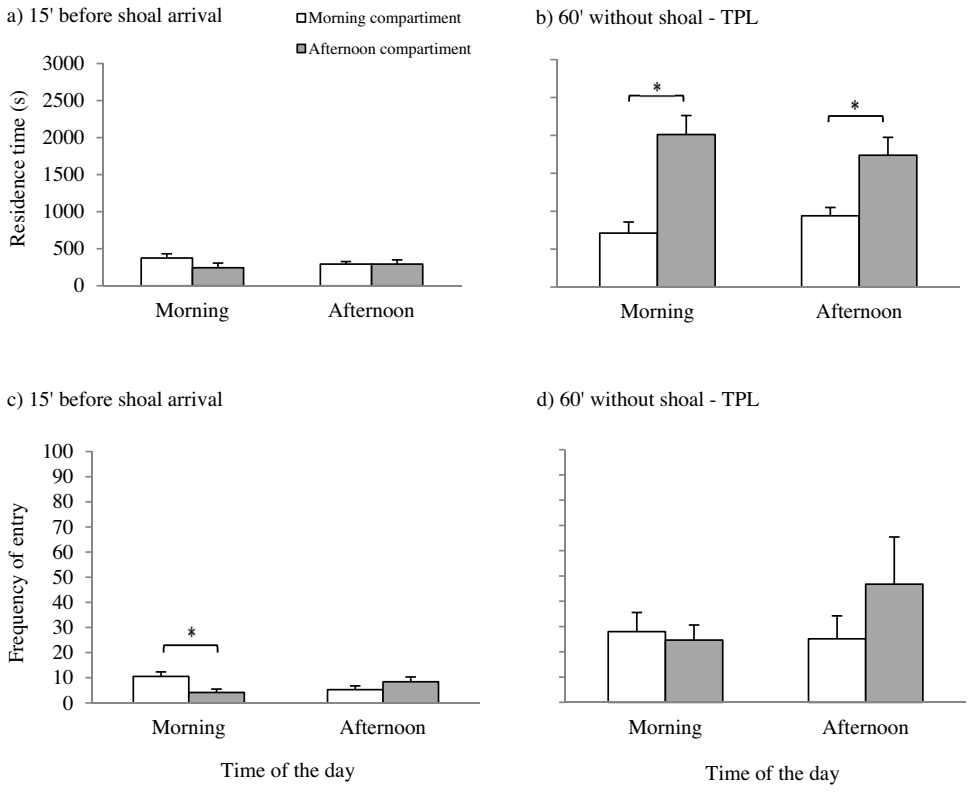

Fig. 2. Zebrafish residence time (a and b) and frequency of entry (c and d) in the morning and afternoon compartments on day 30 of the TPL experiment (n = 10) under constant light. Observations were made from 7:45 to 9:00 am, and from 4:45 to 6:00 pm The first 15 min of observation indicates the ability to anticipate the arrival of the social stimulus (a and c), while the next 60 min indicates the ability to learn time and place of the stimulus presentation (b and d). * indicates statistical significance (Student's *t*-test, $p < 0.05$) between the compartments in each experimental period.

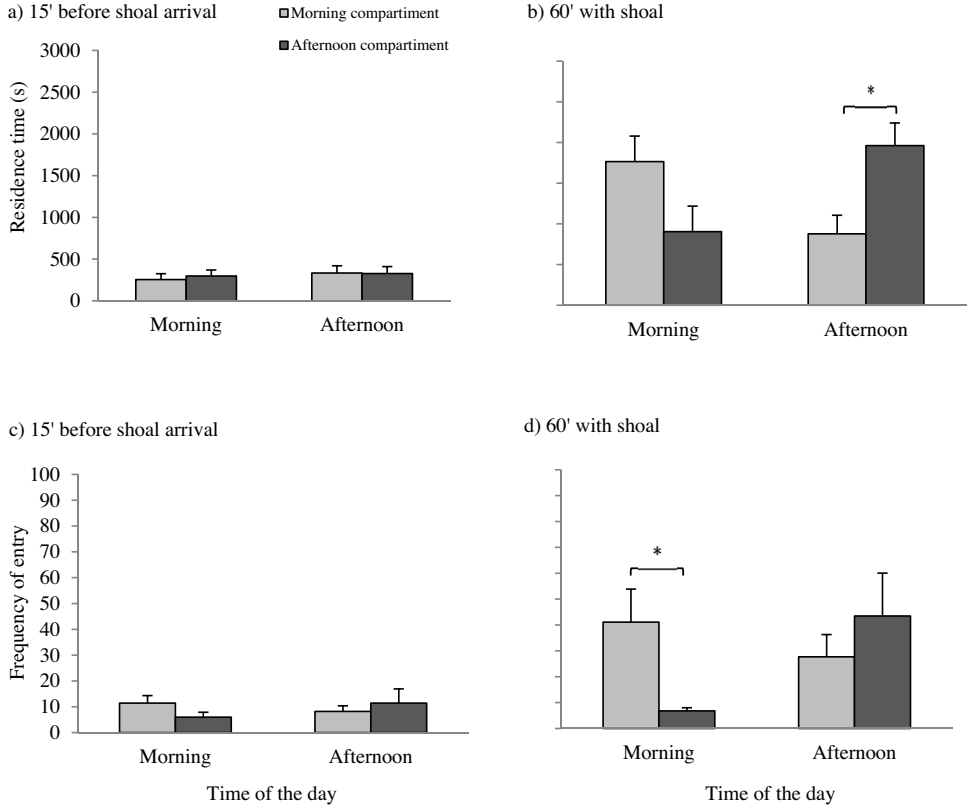

Fig. 3. Zebrafish residence time (a and b) and frequency of entry (c and d) in the morning and afternoon compartments on day 15 of the experiment (n = 8) under constant dark. Observations were made between 7:45 and 9:00 am, and 4:45 and 6:00 pm a and c represent the 15 min before shoal arrival, c and d represent the 60 min in which the social stimulus was present. * indicates statistical significance (Student's *t*-test, $p < 0.05$) between the compartments corresponding to each experimental period.

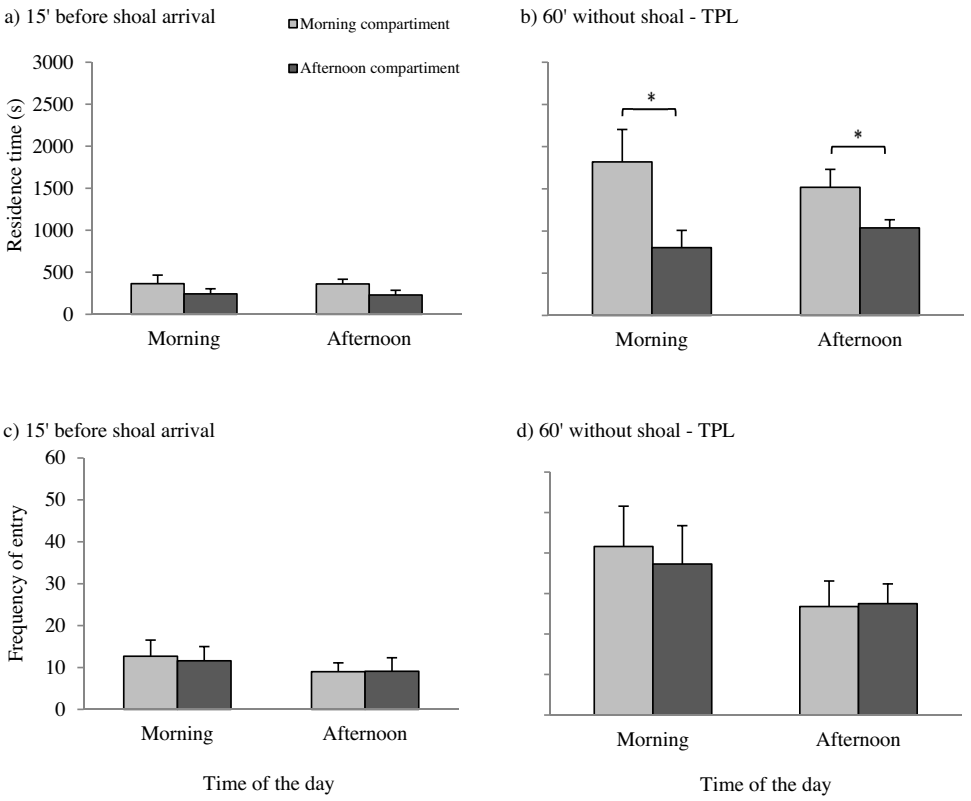

Fig. 4. Zebrafish residence time (a and b) and frequency of entry (c and d) in the morning and afternoon compartments on day 30 of the experiment (n = 10) under constant dark. Observations were made from 7:45 to 9:00 am, and from 4:45 to 6:00 pm The first 15 min of observation indicates the ability to anticipate the arrival of the social stimulus (a and c) and the following 60 min indicates time and place association with the reward (b and d). * indicates statistical significance (Student's *t*-test, $p < 0.05$) between the compartments corresponds to each experimental period.

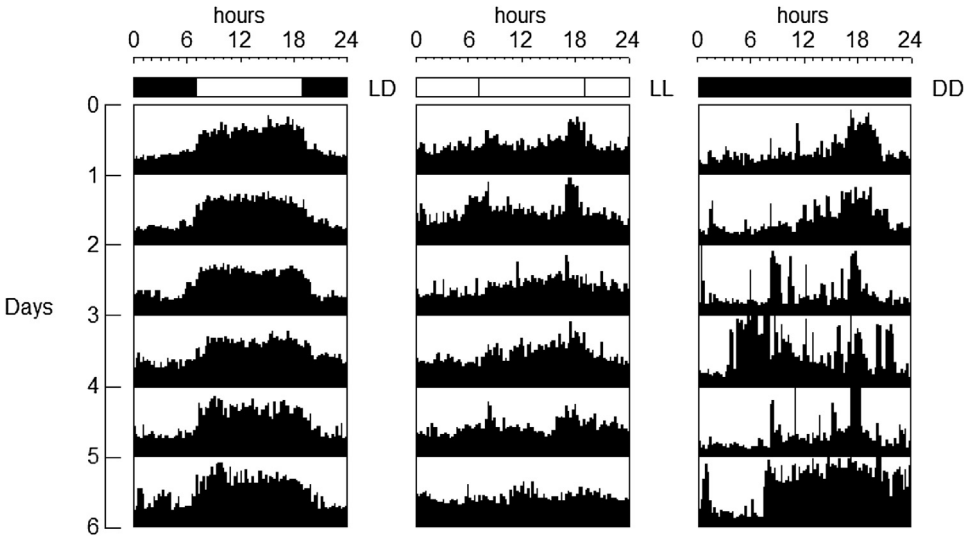

Fig. 5. Representative actogram (average speed) of zebrafish submitted to LD: light dark cycle, LL: constant light, DD: constant dark during the lasts 6 days of the 30-day TPL experiment.

more, fish under constant dark decreased overall activity, while the fish under constant light did not change activity level but it was more homogeneously distributed throughout the 24h-day period, despite the observed significant 24-h rhythms.

On the 15th day of behaviour registry, neither groups (LL or DD) showed differences in time spent or frequency of entry in the compartments 15 min before the stimulus presentation (Figs. 1 a, c and 3 a, c). These results suggest that fish could not anticipate the social stimulus arrival. Under 12:12 LD cycle, zebrafish shows a weak behaviour of anticipation on the 15th training day (Moura and Luchiari, 2016), and thus one would expect that after only 15 days of constant light conditions fish would present much difficulty to forecast the stimulus event. During the 60 min of the conspecific shoal presence, zebrafish under LL remained significantly longer near the group both in the morning and afternoon (Fig. 1b), but animals under DD only shoal with the conspecific group in the afternoon

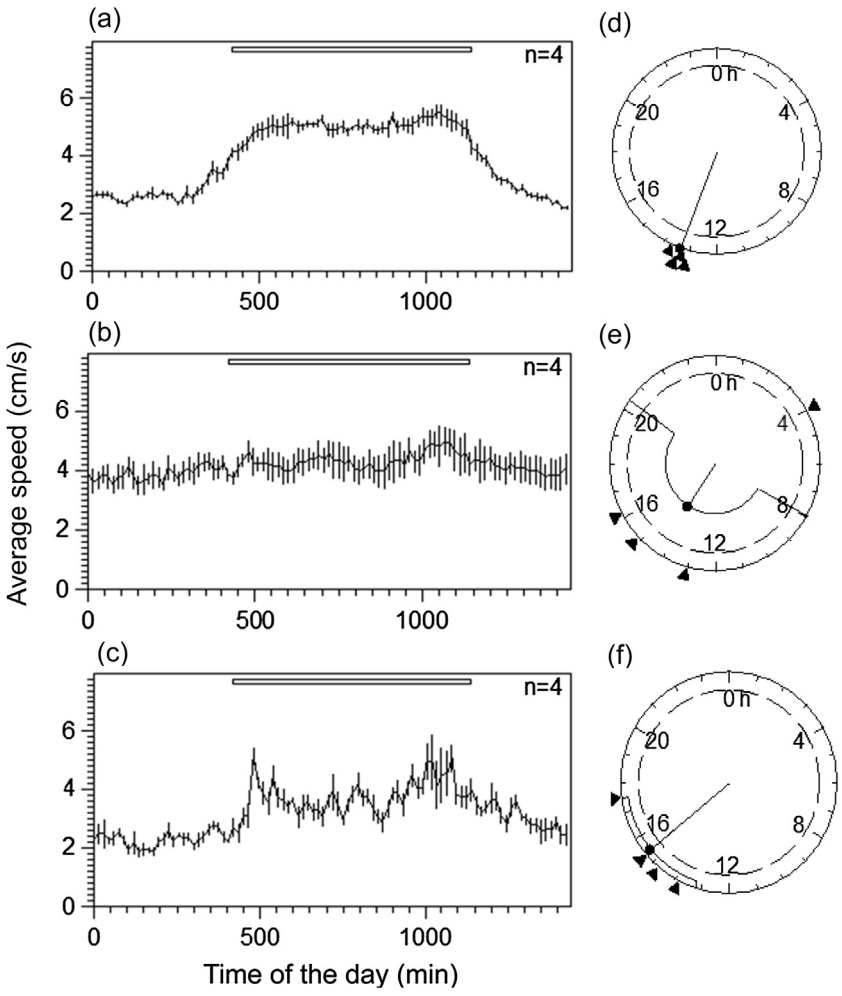

Fig. 6. Waveform of the activity (a–c) for each group, and representative cosinor showing the acrophase (d–f) of the animals under LD (light-dark cycle), LL (constant light) and DD (constant dark) conditions on the last 6 days of the TPL test. LD group showed significant difference in acrophase between 12am and 2pm (Rayleigh, $p < 0.05$). DD group showed significant difference in acrophase between 2am and 4 am (Rayleigh, $p < 0.05$). LL group had no significant difference in acrophase (Rayleigh, $p > 0.05$).

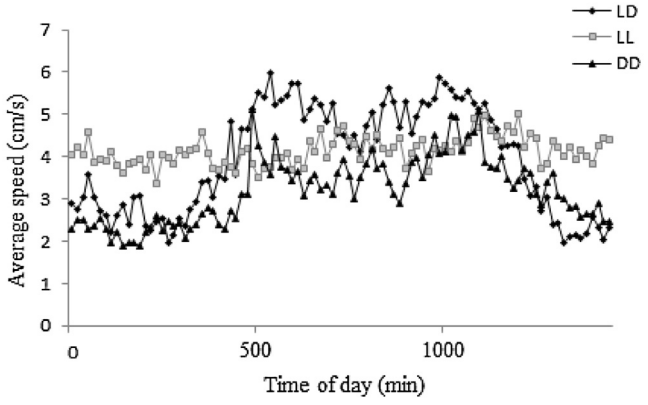

Fig. 7. Activity (average speed) of the animals under LD (light-dark cycle), LL (constant light) and DD (constant darkness) on the probe day (30th day) of the test for TPL test. LL and LD groups showed significant higher activity than DD group (One way ANOVA, $p<0.05$).

(Fig. 3b). Due to the social nature of zebrafish (Pritchard et al., 2001; Larson et al., 2006; Gerlai, 2014; Luchiari et al., 2015), the presence of a shoal is a strong stimulus to drive a single fish towards it. Thus, we expected the fish to join the group upon its presence, what did not happen in the DD condition probably because the animals had no visual cues, but chemical and mechanical cues to locate the group into the tank. Zebrafish is highly responsive to light (Tamai et al., 2007; Moore and Whitmore, 2014) and its visual system is a very accurate sense, presumably the most efficient in terms of stimuli detection (Fleisch and Neuhauss, 2006). While chemical cues quickly disperse into the water and mechanical cues may not have passed through the compartments, we believe the experimental zebrafish struggle finding the stimulus in the dark.

Many other studies have already demonstrated TPL in fish (Reebs, 1993, 1996, 1999; Gómez-Laplaza and Morgan, 2005; Delicio et al., 2006; Barreto et al., 2006; Delicio and Barreto, 2008; Heydarnejad and Purser, 2008; Ebrahimi et al., 2013; Brannas, 2014), all of them using food as the reward. The TPL protocol used herein was a learning test based on social reward. In a previous study, we (Moura and Luchiari, 2016) have shown that live conspecifics were effective to induce robust TPL behaviour in zebrafish. However, recurrent lack of luminosity signals to indicate day and night may have TPL implications: zebrafish under LL and DD did not seek for the correct place in the morning and in the afternoon in order to get the social reward.

On the probe day (day 30; Figs. 2 and 4), both time spent and frequency of entry in the correct compartments in the 15 min before the stimulus did not differ in the LL and DD groups, showing the animals could not anticipate the event even after 30 days of training. During the 60 min that the group was expected to be present, zebrafish under LL remained in the afternoon compartment at both

testing times (Fig. 2b), while fish under DD settle in the morning compartment at both testing times (Fig. 4b).

It is possible that in the absence of the LD cycle, which functions as cue to predict time, the ability of orientation had been impaired, since light-dark cycle is one of most relevant zeitgebers for the guidance of individuals (Hastings, 1991). However, it is worth to notice that fish seem to show some temporal association because it spent most time in a specific compartment at both tested times, but did not discern the correct side in the correct time, in other words, there was no time-place association. Tasks involving appetitive/aversive events, in which the individual needs temporal perception, implicate on interval timing and circadian rhythm as well as associative learning of predictive cues (Ralph et al., 2013). According to Cain et al. (2004), time memory can be explained by the circadian oscillator action, which is modulated by significant experiences. Thus, in the absence of light zeitgebers (strong cue), the organisms are dependent on weaker cues (such as temperature and social cue), and the endogenous clock. While our zebrafish seem not to display free-running ($t = 1440$; Table 1 and 2), interval timing to predict time and place was not observed. Indeed, learning to associate time with spatial location is not an easy task (Biebach, 1989), and depending on the species it may require a significantly strong zeitgeber to show TPL, for instance the LD cycle.

Despite constant light conditions (LL and DD), the activity registry on the last 6 days of the 30-days test showed that zebrafish maintained circadian rhythm (as detected by the Cosinor method – $t = 1440$). It may have occurred due to the presence of daily and fixed times of stimulus presentation, reinforcing the strength of the social cue to circadian rhythm species (Mrosovsky, 1988). The LL group showed higher activity (average speed) than the DD group, but similar to the LD group (Fig. 6 and 7, Table 1). We believe this pattern was related to the diurnal chronotype of the zebrafish (Hurd et al., 1998) that may have induced the LL group to maintain light responsiveness. Additionally, although it was not possible to observe a lengthening effect of light on the activity phase of animals exposed to LL, the increase in overall locomotor activity level is in accordance with the circadian rule, which stands that the intensity of the light stimulus is positively correlated with locomotor activity level in diurnal animals (Enright, 1980). While the Aschoff's rule was designed mainly for mammals, it presents statements to describe and predict an animal circadian behaviour when housed under constant light conditions. For instance, this rule predicts that nocturnal animals under constant dark would have periods in free-course smaller than under constant light and periods in free-course that increase with the increasing light intensity, and vice-versa for diurnal animals. In a study by Elbaz et al. (2013), zebrafish kept under LL cycle became more active and lost circadian rhythm, although our fish under LL showed higher speed, activity was more distributed over the day time (not concentrated in the interval of the subjective day) and they have maintained the circadian rhythm probably due to the social cue presented. Our results, therefore, seem to indicate that the zebrafish circadian rhythm needs stronger cue, such as the light-dark cycle, but other environmental cues precisely repeated overtime might be used to maintain their rhythmicity. However, weaker zeitgebers such as the social stimuli used herein may not be effective to predict time, what might have affected the zebrafish ability of TPL.

According to Yokogawa et al. (2007), under prolonged constant conditions, adult zebrafish sleep overnight in both LD and DD cycle, but sleep-wake rhythm is deleted under LL and only returns after about seven days in this condition. Under constant dark, zebrafish display rhythmic activity and increase it during the subjective day (Cahill et al., 1998; Hurd et al., 1998). Following the same path, we showed that only animals under LD and DD had significant acrophase with higher activity occurring between 12am and 4pm (Fig. 6).

Although zebrafish has been recently used as an effective model in cognitive studies, no data associating LD cycles influence on learning has been provided to date. In this paper we applied a previously validated protocol to test TPL under constant light conditions, reaching negative results both for constant light or constant dark, thus refuting our hypothesis. To demonstrate TPL, an animal must learn to associate different times of the day at different locations of an event (Reebs, 1996). We also observed that constant dark leads to decreased but more concentrated activity of the animals than constant light condition.

Behavioural studies represent an important method to identify neurofunctional changes. The finding that constant light conditions impair TPL implies that light is more than only an environmental cue to adjust life rhythm. Moreover, the zebrafish represents a useful vertebrate model to fulfil many scientific gaps regarding the learning processes, leading an opportunity to research about the molecular mechanisms involved in the maintenance of the circadian rhythm. However, our study presents some faults, such as the need for more observation days beyond 15th and 30th days, a longer period of 24 h activity registry in order to find out changes in behaviour due to the imposition of an altered light regime, and other LD cycles to test TPL (e. g. 16:08 and 18:06). Even though other studies in this area are still needed to the better understanding of light-dark cycle role on learning, we presented here robust results in respect to the negative effects of constant light conditions to TPL. Furthermore, this paper recommends zebrafish as an appropriate model for chronobiology, as well as suggests further investments on the relation between light cycles, clock genes expression and learning.

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