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Daily variation in the shoaling behavior of zebrafish *Danio rerio*

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Abstract Shoaling behavior provides numerous fitness benefits for fish, including enhanced access to mates, increased success in foraging and protection from predators. We were interested in determining whether shoaling intensity differed throughout the day. To do this we kept adult zebrafish *Danio rerio* in different lighting conditions for 10 days: "Normal" (12:12LD, lights on at 0800 hrs), "Reverse" (12:12LD, lights on at 2000 hrs), DD, or LL, and then observed the shoaling behavior at different times during the day. Our findings suggest that daily variations exist in shoaling behavior, with mean shoaling times for fish from the 'normal' group being the lowest at the mid–point of the dark phase in the fish's subjective day (00:00 hrs), then rising significantly throughout the day, reaching their highest intensity at 20:00 hrs (lights out). Fish from the "reverse" LD cycle (lights on at 20:00 hrs) showed differences in the mean shoaling times at different times of day, but did not show a gradual increase in shoaling throughout their subjective day. Fish from the DD and LL groups did not show significant differences in the mean shoaling values at different times of day, suggesting that the differences observed in LD fish may not represent circadian rhythms. Therefore, these results demonstrate the existence of daily variations in the shoaling behavior of fish and suggest that environmental cues in the form of light/dark cycles play an important role in regulating these variations [*Current Zoology* 58 (1): 129–137, 2012].

Keywords Shoaling behavior, Zebrafish, *Danio rerio*

Shoaling, or aggregation behavior, has become an important model system for the study of genetics, development and life history factors in fish. Shoaling has been demonstrated in a wide variety of fish species and confers advantages that include access to potential mates, increased foraging success, and enhanced defense against predators (for reviews see Krause and Ruxton, 2002; Brown and Laland, 2003; McRobert, 2004). By definition shoaling is simple grouping behavior, which can be demonstrated by placing an individual fish in the center compartment of a tank containing three compartments separated by clear glass. If one of the end compartments contains a group of fish and the other end compartment is empty, the test fish (in the center compartment) will, almost invariably, choose to spend more time swimming near the group than near the empty compartment. This simple procedure is the basis of all shoaling experiments and the results appear to indicate that social species of fish typically show an attraction to other fish.

For studies of shoaling, as in many other biological studies, the zebrafish *Danio rerio* has become an important model organism. Zebrafish are small, easy to maintain in the laboratory, and produce huge numbers of offspring, thus making them ideal for analysis of development and genetics. In studies on shoaling behavior, *D. rerio* have shown a strong propensity to shoal under experimental conditions (see Ruhl and McRobert, 2005). Furthermore, access to various mutant strains has provided the opportunity to study the effects of genes and development on shoaling behavior as shown in Engeszer et al (2004) in which wildtype *D. rerio* developed a shoaling preference for the mutant strains they were exposed to when young. Studies like this demonstrate the power of shoaling assays in a model organism like the zebrafish to ask questions about complex biological attributes.

Like many organisms, fish display circadian rhythms (Zhdanova and Reebs, 2006; Reebs, 2002), cycles of physiological and behavioral changes that occur throughout a given period of time. Under constant laboratory conditions these endogenous rhythms operate with a periodicity that is close to a 24 hr (or circadian) cycle. However, in the natural world these rhythms become entrained to specific environmental cues, commonly referred to as zeitgebers. The most common zeitgeber is light, and entrainment is mediated by photoreceptive organs responding to the variations that occur

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throughout the day (Bell-Pedersen et al., 2005). Daily adjustments to the circadian clock, caused by changes to the light-dark cycle, allow various biochemical, physical and behavioral processes to be executed at the times that optimize fitness. Changes in light/dark schedules can result in phase-shifts, or alterations in the normal cycle. This is commonly seen in the wake/sleep schedules of humans when travelling between various time zones. When organisms are subjected to constant conditions such as continuous light (LL) or darkness (DD), they can establish a free-running period, in which their activity patterns are controlled by their internal clock mechanisms without the aid of external zeitgebers to synchronize their daily rhythms. This may result in changes to the period, the length of time required to complete one rhythmic cycle (Koukkari and Sothern, 2006).

Circadian rhythms have been demonstrated in both embryonic and adult *D. rerio*. Hurd and Cahill (2002) observed that circadian rhythms can influence locomotor behavior in larval zebrafish, and that embryos require long-term exposure to LD cycles post-fertilization in order to maximize locomotor activity as juveniles. Behaviors possibly associated with circadian rhythms have also been described in adult zebrafish. As diurnal organisms, zebrafish tend to have bouts of high activity during the day and reduced activity at night, at which point they engage in activity analogous to sleep. In this state, swimming activity is reduced, as well as some visual and cognitive functions. Cahill (2002) observed rhythms associated with locomotor activity in both larval and adult zebrafish which confirm these findings. Age has also been shown to have an effect on particular aspects of circadian activity, with older zebrafish producing less melatonin, a chemical involved in circadian rhythm maintenance, resulting in noticeable reductions in general cognitive behavior, and decreases in sleep behavior (Zhdanova et al., 2008).

In this study we investigated the possibility that shoaling behavior follows a circadian rhythm. To do this we utilized a simple shoaling assay in which test fish were given the opportunity to shoal by swimming near a small group of conspecifics at different points during the day. At each time period we determined whether shoaling was occurring and monitored the intensity of shoaling behavior. Test fish for this study were taken from one of four light entrainment groups to determine whether light/dark cycles prior to testing affected the possible rhythms of shoaling behavior. Our prediction was that shoaling intensity would differ at different times during the subjective day of the fish. Furthermore, we considered the possibility that fish from the Normal (12:12 LD cycle with lights on at 08:00 hrs) and Reverse (12:12 LD cycle with lights on at 20:00 hrs) might show opposite trends in shoaling activity.

1 Materials and Methods

1.1 Fish stocks and housing

Adult, wild type *D. rerio* were obtained from a local fish distributor (Seven Star Tropical Fish, Philadelphia, PA) and housed in groups of 50 to 100 fish in four 76 liter tanks at 28°C. The walls of each holding tank were covered with black construction paper and each tank was housed under a black plastic cover to prevent exposure of the fish to external light cues. Light for each tank was provided by fluorescent lamps (with 20 Watt, full-spectrum bulbs) controlled by timers and maintained under one of four different conditions: 1). "Normal" = 12:12 LD cycle with lights on at 08: 00 hrs and off at 20: 00 hrs; 2). "Reverse" = 12:12 LD cycle with lights on at 20:00 hrs and off at 08:00 hrs; 3). LL = 24:0 LD cycle with the tank exposed to constant light; and 4). DD = 0:24 LD cycle with the tank exposed to constant darkness). Additional tanks were set up under these conditions as backups. Upon arrival in the lab, fish were randomly assigned to one of the four holding tanks and were allowed to acclimate to their specific lighting cycle for 10 days prior to testing. Fish were fed three days a week between 09:00 and 10:00 hours. Housing and testing of fish occurred during the months of May and June, 2011.

1.2 Shoaling assays

Test tanks were created by dividing 76 liter (74 cm × 31 cm × 31 cm) tanks into three separate compartments. The two end compartments (18 cm × 31 cm × 31 cm) were separated from the central compartment (38 cm × 31 cm × 31 cm) by clear glass partitions held in place with aquarium caulking. Preference zones within the central compartment were delineated by lines drawn on the front glass 6.35 cm (approximately two *D. rerio* body lengths) from each glass partition.

For each shoaling assay, a single test fish, taken from one of the holding tanks, was placed into the central compartment and allowed 15 minutes to acclimate prior to the start of the test. During the acclimation period the lights in the test tanks were set to match the housing environment of the test fish (i.e. if the test fish would be experiencing light at the time of the test, then the test tank lights would be on. If the test fish would be experiencing darkness at the time of the test, then the test tank lights would be off). However, once the shoaling

test began, the test tank lights were turned on to more accurately observe shoaling behavior. During a shoaling assay one of the end compartments contained a shoal of five *D. rerio* from holding tanks at Saint Joseph's University (raised in 12:12 LD at 28°C) while the other compartment remained empty. These fish were replaced with new shoals between light conditions. Equal numbers of assays were run with the target shoal in the right compartment and the left compartment to reduce the risk of tank effects. Each shoaling assay lasted 600 seconds, during which the total time the test fish spend in either preference zone was recorded. Shoaling assays began at 8 am EST (08:00 hrs) for each lighting condition, and were performed at four different time periods: 12 am EST (00:00 hrs), 8am EST (08:00 hrs), 12 pm EST (12:00 hrs), and 8 pm EST (20:00 hrs). The sample size consisted of 20 assays for each housing group at each time period. Each test fish was used only once. Two assays per time period were performed each day so as to complete each test condition within 10 days.

1.3 Data analysis

The amount of time the test fish spent in each preference zone was compared using a paired *t*-test. Shoaling intensity across the different time periods was compared using a One-Way ANOVA followed by a post hoc Tukey HSD test.

2 Results

With the exception of a single assay, fish from all housing groups performed shoaling behavior, spending significantly more time swimming near a shoal of conspecifics than near an empty chamber, at all time periods tested**.** For fish from the "Normal" housing group: 00:00 hrs, $P<0.001$, $t = 4.711$. 08:00 hrs, $P<0.001$, $t = 5.690$; 12:00 hrs, $P<0.001$, $t = 6.697$; 20:00 hrs, $P<0.001$, $t = 9.858$ (Fig. 1). For fish from the "Reverse" housing group: 00:00 hrs, $P<0.001$, $t = 5.270$; 08:00 hrs, $P<0.001$, $t = 5.585$; 12:00 hrs, $P = 0.028$, $t = 2.383$; 20:00 hrs, $P<0.001$, $t = 6.042$ (Fig. 2). For fish from the DD housing group: 00:00 hrs, $P = 0.016$, $t = 2.641$; 08:00 hrs, $P = 0.010$, $t = 2.846$; 12:00 hrs, $P = 0.051$, $t = 2.080$; 20:00 hrs, $P = 0.035$, $t = 2.264$ (Fig. 3). For fish from the LL housing group: 00:00 hrs, $P < 0.001$, $t = 4.446$; 08:00 hrs, $P < 0.001$, $t = 4.965$; 12:00 hrs, $P < 0.001$, $t = 4.310$; 20:00 hrs, $P < 0.001$, $t = 5.728$ (Fig. 4).

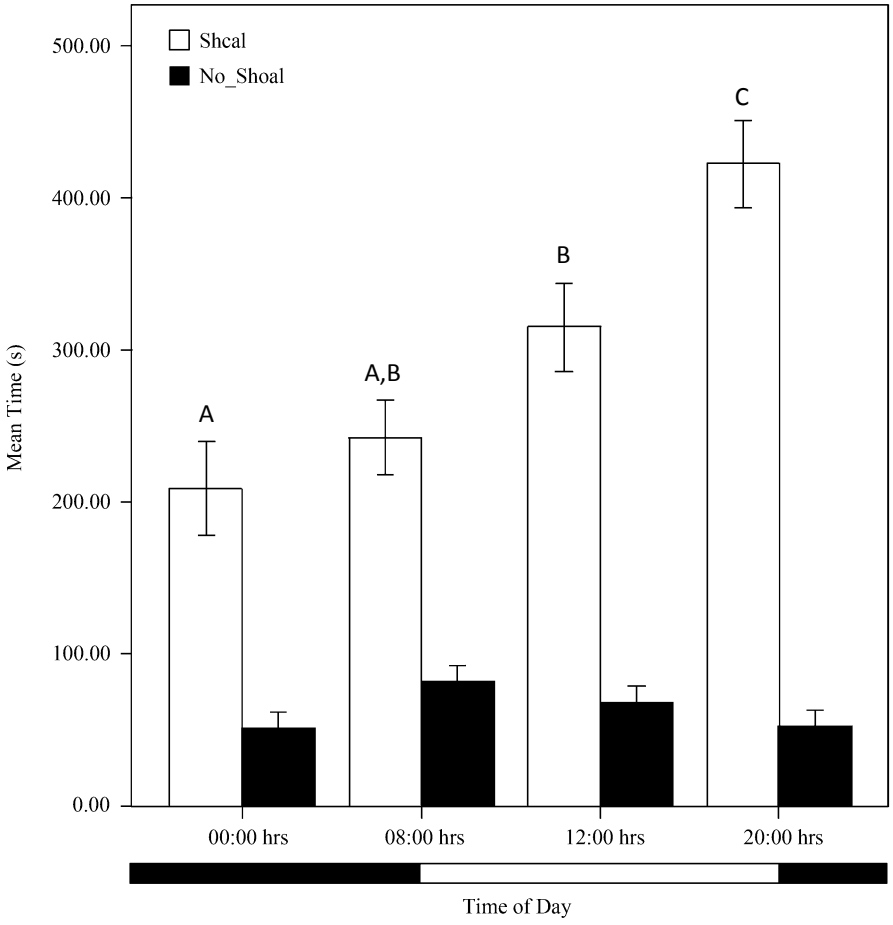

Fig. 1 Average shoaling behavior for fish from the "Normal" light regimen ±1 *SE* (*n* = 20)
White bars represent time spent near the target shoal. Black bars represent time spent near the empty target chamber. Bars with the same letter are not significantly different from each another.

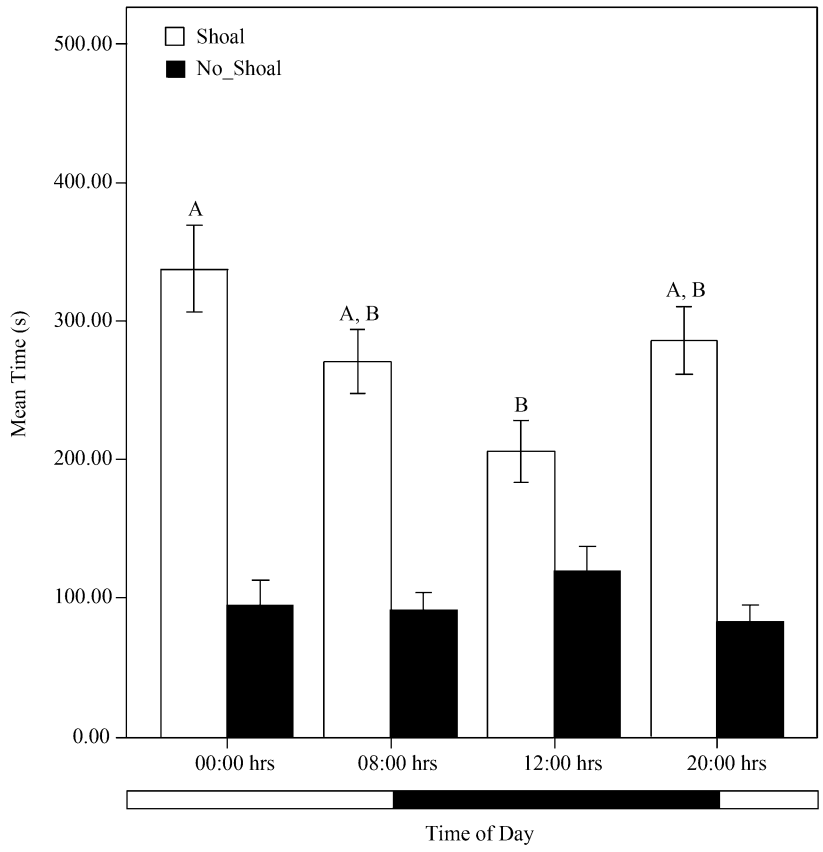

Fig. 2 Average shoaling behavior for fish from the "Reverse" light regimen ±1 *SE* (*n* = 20)

White bars represent time spent near the target shoal. Black bars represent time spent near the empty target chamber. Bars with the same letter are not significantly different from each another.

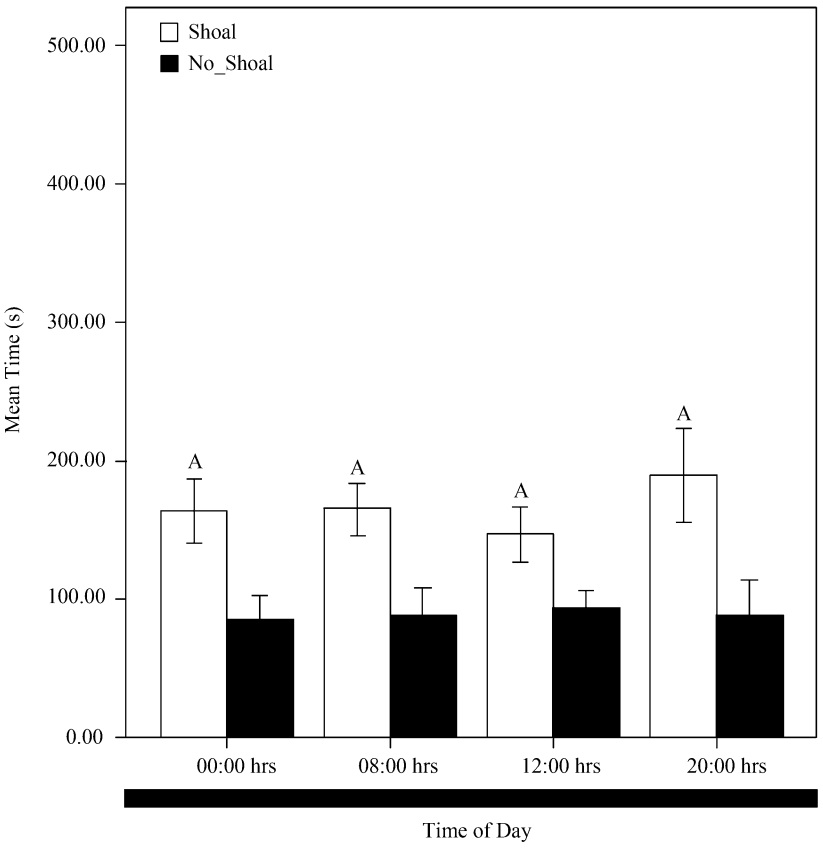

Fig. 3 Average shoaling behavior for fish from the DD regimen ±1 *SE* (*n* = 20)

White bars represent time spent near the target shoal. Black bars represent time spent near the empty target chamber. Bars with the same letter are not significantly different from each another.

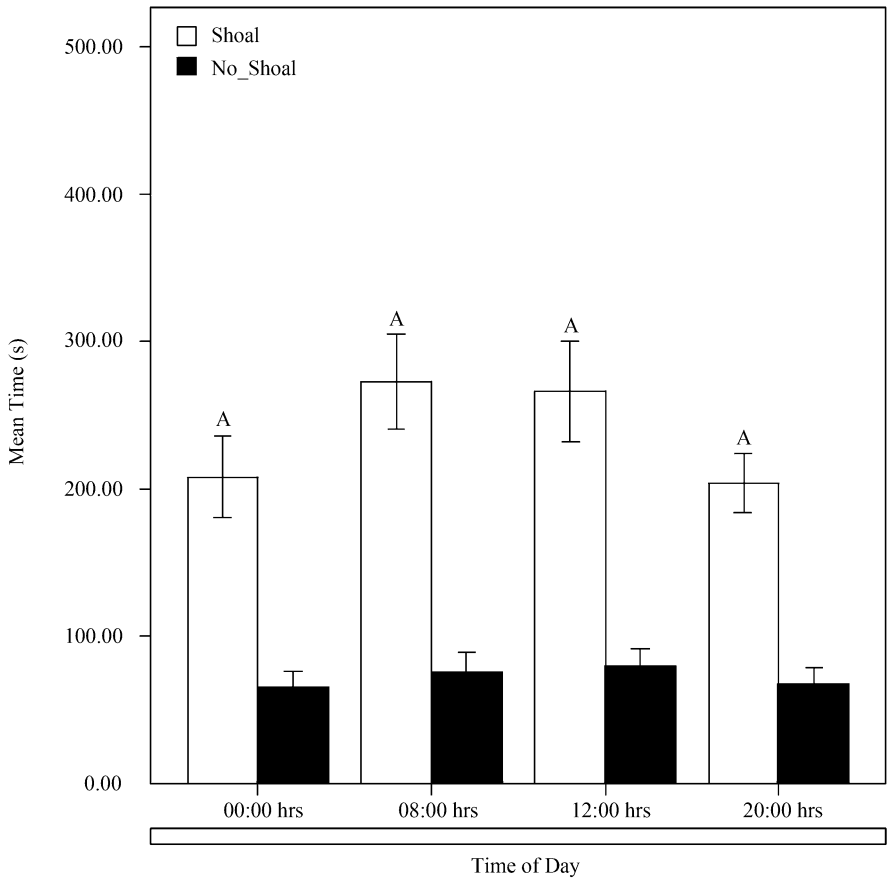

Fig. 4 Average shoaling behavior for fish from the LL regimen ±1 *SE* (*n* = 20)

White bars represent time spent near the target shoal. Black bars represent time spent near the empty target chamber. Bars with the same letter are not significantly different from each another.

When comparing mean shoaling times (time spent near the group of fish in the end compartment of the test tank), significant differences were found among tests at different times of day in some entrainment groups but not in others. For fish from the "Normal" group, mean shoaling time significantly increased throughout the day (P<0.001, $F_{3, 19}$ = 11.184, ANOVA). Mean shoaling in tests performed at 00: 00 hrs (208 ± 30.7sec) was significantly lower than mean shoaling in tests at 0800 hrs (241 ± 24.5 sec), which was significantly lower than mean shoaling in tests at 12: 00 hrs (314 ± 29.1sec), which was significantly lower than mean shoaling at 20: 00 hrs (422 ±28.6 sec).

For fish from the "Reverse" entrainment group, mean shoaling time was also shown to be significant among test times (P = 0.005, $F_{3, 19}$ = 4.585, ANOVA). Mean shoaling time for tests performed at 00:00 hrs (337 ±31.1 sec) were significantly higher than mean shoaling time for tests performed at 12:00 hrs (205 ± 22.1 sec). However, no other significant differences were found among mean shoaling times performed at other times of day. There were no significant differences in mean shoaling times for fish from the DD entrainment group (P = 0.681, F = 0.503, ANOVA) or for fish from the LL entrainment group (P =0.199, $F_{3,19}$ = 1.589, ANOVA).

Finally, significant differences were found when comparing total shoaling behavior (TSB = mean time spent near the shoal of fish over the entire day of tests). TSB for fish from the "Normal" entrainment group (297.0 ± 16.7 sec) was significantly greater than TSB in both the DD entrainment group (165.9 ± 12.2 sec, P < 0.001) and the LL entrainment group (237.5 ± 14.8 sec, P = 0.019), but not significantly different from the "Reverse" entrainment group (274.7 ± 13.5 sec, P = 0.693). TSB for fish from the "Reverse" group was significantly greater than TSB for fish from the DD group (P < 0.001), and TSB for fish from the LL group was significantly greater than TSB for fish from the DD group (P = 0.003). All results summarized in Fig. 5.

3 Discussion

In this study we examined the shoaling behavior of zebrafish *D. rerio* that had been maintained in four different light regimens: 12:12 LD, with lights on at 08:00 hrs ("Normal"), 12:12 LD, with lights on at 20:00 hrs

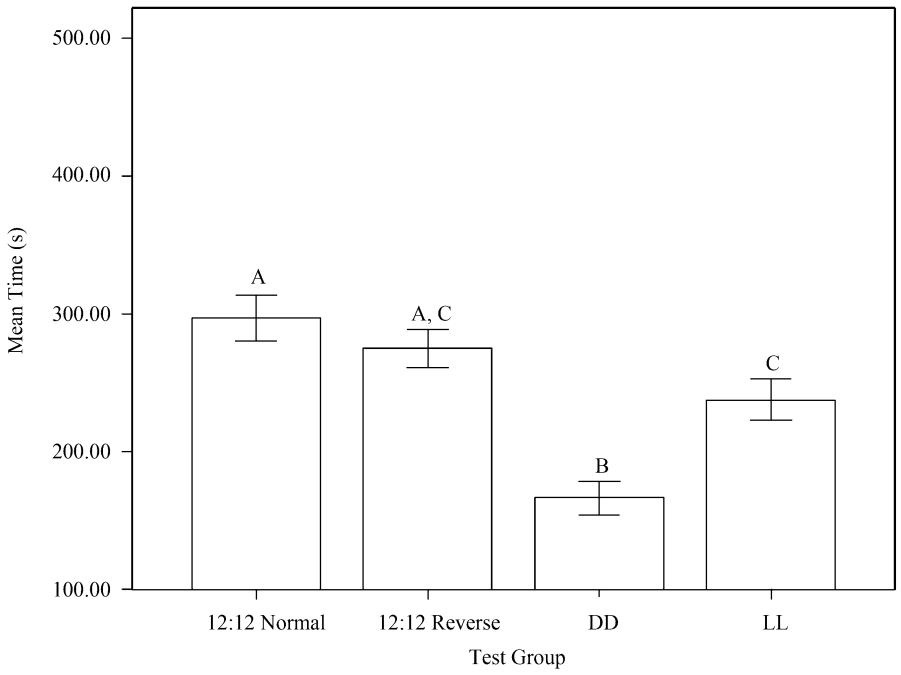

Fig. 5 Total shoaling behavior for fish from all light regimens ±1 *SE* (*n* = 80)

Bars represent total time spent near the target shoal across each treatment. Bars with the same letter are not significantly different from each another.

("Reverse"), LL and DD. Standard shoaling tests, providing test fish with a choice between an empty compartment and a compartment containing five conspecifics, were run at four different times of day (00:00 hrs, 08:00 hrs, 12:00 hrs and 20:00 hrs). Fish from all light regimens demonstrated shoaling behavior, spending significantly more time swimming near the conspecfic shoal than near the empty chamber, at each of the four different time periods (with a single exception, fish from the DD group at 12: 00 hrs, $P = 0.051$, which we believe was a statistical tendency). These results add to the growing body of literature emphasizing the robust nature of shoaling, and support the thought that shoaling behavior is a critical life history parameter in fish. Shoaling is thought to provide numerous benefits, including enhanced access to mates (Nordell and Valone, 1998), increased feeding opportunities (Ruxton et al., 1995) and reduced risk of predation (Foster and Treherne, 1981; Godin et al., 1988; Krause, 1993). The fact that shoaling was shown to occur at all times during the day, even in the middle of the subjective night for the two groups acclimated to a 12:12 LD cycle, suggests that zebrafish will locate and attempt to join a shoal whenever they find themselves alone. This result also suggests that shoal formation may provide benefits, most likely related to anti–predatory behavior, even during the phase of the day when fish are likely to be sleeping (see Zhdanova, 2006).

In addition to the demonstration that fish from four different light regimens performed shoaling behavior at different times during the day, our results also showed a daily variation in shoaling behavior. Fish from the "Normal" light regimen showed significant differences in the level of shoaling behavior performed at different times throughout the day. For these fish, the mean time spent swimming near the target shoal of conspecifics was lowest at 00:00 hrs (midpoint of the dark-phase of their subjective day), increased significantly at 08:00 hrs ("lights on" in their subjective day), increased again at 12:00 hrs (midpoint of the light phase of their subjective day), and finally reached its highest level at 20:00 hrs (the end of the light–phase of their subjective day). These results show that while shoaling behavior occurs throughout the day, levels of shoaling intensity differ from time to time. At 00:00 hrs, where the lowest levels of shoaling were detected, it is possible that the fish were sleeping when removed from their holding tank for testing. Locomotor activity might be expected to be at its lowest level during this part of the day (for both test fish and stimulus fish, which were maintained on similar cycles) and it has been shown that lowered activity levels (in stimulus fish) lead to lowered shoaling preferences (Pritchard et al., 2001). Locomotor activity, which was not monitored in this study, might be expected to increase at the beginning of the light–phase of the day as fish begin to forage and search for mates. We have shown that levels of shoaling behavior increase at this time, then rise significantly throughout the day, reaching their highest level just before dark, when the fish possibly

settle down with other conspecifics for the night.

If the gradual increase in shoaling activity we observed in fish from the "Normal" light regime represented circadian rhythms, we might have expected such variations to be demonstrated by fish maintained under constant conditions. In addition, if these variations were circadian rhythms we thought that it might be possible for fish from the "Reverse" light regime to demonstrate a similar shoaling pattern, but with their lowest level of shoaling occurring at 12:00 hrs, then gradually rising to its highest level at 08:00 hrs. This pattern did not occur, although we did observe the lowest mean shoaling intensity for fish tested at 12:00 hrs, which was significantly lower than mean shoaling intensity for fish tested at 00:00 hrs. Therefore, fish from both the "Normal" and "Reverse" light regime groups showed significant differences in mean shoaling time at different points during the day, and fish from both groups demonstrated their lowest mean shoaling activity during the dark phase of the subjective day. In both groups, the time period at which the fish demonstrate their lowest mean shoaling activity might be related to the decreased activity level for fish that are being tested in the midst of their respective 'nights." With respect to the fact that fish from the "Reverse" light regime did not demonstrate the gradual increase in mean shoaling activity throughout the day might be related to acclimation time within the different light regimes prior to testing. Fish in the "Reverse" group experienced the effects of their subjective day for a relatively short period of time prior to testing while fish in the "Normal" light regime were placed into a subjective day that was actually quite similar to the conditions they had been experiencing before the experiment began. It is possible that phase–shifting of a circadian pattern of shoaling behavior requires a longer period of acclimation to a specific light/dark cycle.

Unlike the "Normal" and "Reverse" groups, *D. rerio* raised in DD and LL conditions showed no significant differences in shoaling intensity across the time periods when shoaling tests occurred. Both groups of fish displayed mean shoaling times that remained consistent regardless of the time of the test. These results suggest that fish from the DD and LL groups may have entered a free–running period, which could result in the lengthening or shortening of a circadian rhythm. Organisms in this state become desynchronized from normal circadian rhythms due to the removal of the standard LD cycle. When exposed to continuous conditions, LL or DD, organisms may also experience "damping", where the strength of the circadian rhythm is lessened over time (Koukkari and Sothern, 2006). Reebs (2002) reviews circadian rhythmicity in various cyprinid species and noted fluctuations in activity within constant conditions, suggesting that circadian rhythms are not necessarily rigid in terms of diurnal or nocturnal activity, and that circadian rhythms may play a larger role in anticipatory responses, rather than just strictly activity. Arrhythmia has been noted in several species raised in constant conditions. Pill bugs raised under DD conditions showed weaker rhythms of locomotor activity compared to those raised under LD conditions (Renfinetti, 2000). Similarly, mice housed under LL conditions were shown to display a disruption in circadian activity in the SCN, leading to arrhythmic activity patterns (Ohta et al., 2005) and Binkley (1977) observed arrhythmic behavior and increased locomotor activity under LL conditions in sparrows. Results of this experiment seem to suggest that zebrafish also enter a state of arrhythmia in constant conditions, which appears to influence shoaling intensity throughout the day. Overall, this data may further support the possibility of the existence of a circadian rhythm associated with shoaling behavior, and suggest that light/dark cycles act as a zeitgeber for the shoaling cycle.

While light is generally referred to as the dominant zeitgeber, additional factors in the entrainment of circadian rhythms suggest that light may not be the only zeitgeber at work. Feeding regiments in test organisms have also been linked to circadian rhythms. Stokkan et al. (2001) showed that food availability was able to affect liver function in rats, causing peripheral circadian oscillators in rat livers to entrain to various food cycles and cause changes in locomotor activity based on anticipation of food availability. While *D. rerio* in the present study were placed on a feeding schedule (three times a week in the morning), it is unclear as to whether this had any effect on their behavior, though it is possible that a feeding regiment such as this may cause entrainment via anticipatory response and alter behavior. Temperature has also been closely associated with circadian rhythms. In particular, several studies have speculated that *D. rerio* circadian rhythms are temperature-dependent (Hurd et al., 1998; Lopez-Olmeda et al., 2006). Temperature cycles have been shown to act as the primary zeitgeber for zebrafish embryos when light is abolished, and promotes transcriptional regulation and expression of certain clock genes (Lahiri et al., 2005). Spieler et al. (1978) noted that changes in water temperature were shown to cause shifts in the rhythms of the hormone prolactin in gulf killifish *Fundulus*

grandis. However, these changes in water temperature were made over a 24-hour period from 20°C to 28°C. Zebrafish in the present study were maintained and tested at a constant temperature (28°C) in order to prevent potential changes to the expression of circadian rhythms. However, it is possible that fluctuations in temperature may influence circadian entrainment and shoaling tendency in fish.

Circadian rhythms are known to exist for a wide variety of biological processes, both physical and behavioral. In the present study, we monitored the effects of light/dark cycles on the shoaling behavior of fish and examined the possibility that shoaling behavior follows a circadian rhythm pattern. Overall we have shown that *D. rerio* engaged in shoaling behavior throughout the day, regardless of light/dark conditions under which they were maintained prior to testing. In addition, for fish raised under 12:12 LD cycle, with lights coming on at 08:00 hrs (our "Normal" group), we saw a distinct pattern of change in shoaling intensity throughout the day, with the lowest level occurring at the midpoint of the dark phase (00:00 hrs) and highest level occurring at end of the light phase (20:00 hrs). Fish raised under a 12:12 LD cycle with lights on at 20:00 hrs (our "Reverse" group) showed differences in shoaling activity at two points during the day, but did not demonstrate the pattern seen in the "Normal" group, which may reflect a need for a longer acclimation time under this light regime. Fish subjected to continuous conditions (LL or DD) showed no significant changes in shoaling behavior throughout the day, suggesting that the removal of an LD cycle may abolish the rhythm associated with shoaling behavior. We suggest that the pattern of change in the shoaling activity of *D. rerio* from our "Normal" light regime represents a daily variation in shoaling behavior, which has not been previously described. Indeed, we wonder whether this observation may lead to studies that will demonstrate that shoaling behavior actually follows a circadian rhythm. We acknowledge, however, that the information presented here is not sufficient for such a claim. Future studies will be directed at analyzing shoaling behavior over multiple 24-hr cycles, lengthening the acclimation times at different light regimes, examining locomotor activity as well as shoaling behavior, examining the possible effects of secondary zeitgebers, and looking for rhythms that may exist in more complex shoaling scenarios.

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