Do Kairomones Influence Diel Activity Cycle in *Daphnia*?

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Introduction

The 24-hour period of day and night has been one of the most fundamental defining abiotic factors to influence any ecosystem on earth. Sunlight is the primary energy input to nearly every food web on earth, and the intermittent cycle of light and dark has influenced evolution and life history from the earliest single cellular life, to the most complex modern species. Many organisms have adapted to exploit the warmth of the sun or concealment of night (Helm et al. 2017). Development of behavior patterns follow, with animals specializing to complete unique tasks at specific times. To best take advantage of their adaptations, it is often useful for an organism to have an internal sense of passing time, to help anticipate the radical change brought to their habitat induced by the setting or rising sun (Weiss et al. 2019). The ability of an organism to keep time with an endogenous biological clock (and use that clock to inform its actions) is known as a circadian rhythm. Circadian rhythms can be used to inform cyclic gene expression, metabolism, and behavior.

When night gives way to day in aquatic ecosystems, phytoplankton in the water column begin to photosynthesize, releasing oxygen into the water (Gerhardt et al. 2006). Fish, which rely on sight to hunt, become more active. Crustaceans retreat to cover to wait out the day. Each of these organisms benefits from an innate knowledge of when the sun will rise (Ringelberg 1971). For example, phytoplankton that reach the surface before dawn will have the maximum duration to photosynthesize, and crayfish seek shelter under rocks for concealment from hungry bass once the fish can see (Helm et al. 2017). Between the fish and the phytoplankton in the food web is another organism that uses its circadian rhythm to inform its movements: the humble *Daphnia* (Gerhardt et al. 2006).

Daphnia is a type of zooplankter that filter-feeds on phytoplankton (Ringelberg 1971). It represents a crucial step on the food web linking primary producers to fish, but to perform its role, it must budget time carefully. *Daphnia*'s natural predators are usually planktivorous fish which primarily use vision to hunt, so during the day, *Daphnia* seek deeper, darker water to avoid predation (Cellier-Michel et al. 2003). They wait until night to swim to the epilimnion to feed in safety. This phenomenon is known as diel vertical migration (DVM). This investigation ascertains if this phenomenon is informed by an animal's endogenous circadian rhythm.

Daphnia rely on kairomones to detect the presence of predators in the water. Kairomones are the chemical byproducts of an animal's metabolism or activity, and can remain detectable in the environment for a long period of time (Joanna et al. 2020). In detecting the presence of kairomones in its environment, prey species can alter their behavior to best decrease the risk of predation (De Meester et al. 1997, 1999) (Nitta et al. 2019).

In this investigation, I ask: when removed from all external stimuli, will *Daphnia* under threat of predation maintain their diel activity cycle longer or more accurately than *Daphnia* that are not under threat? I hypothesize that while free running, *Daphnia* which are exposed to kairomones will exhibit a stronger and more consistent diel activity pattern than *Daphnia* in unadulterated water". I test this by tracking the average movement of a cohort of *Daphnia* in a well plate to observe changes in movement patterns between control and kairomone exposure.

Methods

For this experiment, I raised *Daphnia pulex* in 250mL cylindrical jars at 15 degrees Celsius. I subjected them to a 16-hour light, 8-hour dark photoperiod in FLAMES medium, as described by Celis-Salgado et al. (2008). I fed *Daphnia ad libitum* 3ml of resuspended *Cryptomonas ozolinii* every two to three days from *Cryptomonas* cultures growing in WC medium. I spun down *Cryptomonas* algae to a pellet, then resuspended into FLAMES medium for feeding purposes. I kept five *Danio rerio* in a 20L tank of FLAMES at 28 degrees C, with 40% water changes conducted weekly. A standard hang-on-back filter provided water flow and biological/physical filtration. I fed the fish a daily combination of pellets and live *Daphnia*.

I built the experiment inside a Percival growth chamber set at 15°C on a 16-hour light, 8-hour dark photoperiod. To design the well plate setup, I drilled out the bottom of each well in a 12-well plate, and affixed a 500-micron filter mesh to the bottom. This allows water to flow in and out of each well, but retains study subjects. The 12-well plate lies within an open-top acrylic tray, with an inflow and outflow hole drilled on opposite sides. A Rainin Dynamax RP-1 peristaltic pump generates flow between the acrylic tray and a 2L Erlenmeyer flask, to add to the effective volume the animals live in. I added a tube of resuspended *Cryptomonas* to the flask for feeding, and a stir plate and bar were employed to prevent the algae from settling.

For video recording, I mounted a Logitech BRIO camera on a tripod to record the acrylic tray from a vertical perspective. I custom modified the camera with a Logitech BRIO 4k Pro rework kit mk2 (Kurokesu UAB, Vilnius, Lithuania) to allow the attachment of CS lenses, and a longpass filter to block wavelengths below 850 nm, permitting infrared light. To illuminate the setup, a CMVision CM-IR110 infrared lamp is mounted beneath the tray, separated by a quarter-inch thick white acrylic sheet for light diffusion. The longpass filter blocks all light except infrared, allowing observation at day or night light levels. The video tracking is interpreted by a custom-written Python script written by Dr. Ian Woods using an openCV library (Bradski 2000).

To raise the animals, I separated cohorts of *Daphnia* neonates from their mothers at 2-3 day intervals, so that all animals in any run are coeval. I did this to prevent neonates from appearing and interfering with motion tracking, as well as preventing subjects from escaping through the mesh. The ideal age to begin a run is 7-10 days old. Each run consists of a 12-animal cohort subjected to motion tracking over three days of day/night cycling, then three days of 24-hour light. I fed the experiment daily at irregular times during the day, using the same procedure as standard culture conditions. I never fed at the same time twice to prevent creating a rhythmic schedule identifiable by the *Daphnia*. I conducted the control run using 2L of standard FLAMES medium, and the trial run using 2L of FLAMES collected from the zebrafish tank (Celis-Salgado et al. 2008). Both runs follow the 16-hour light, 8-hour dark photo period at first, but after five days I set the chamber to 24-hour light.

I used the Rethomics R package (Geissmann et al. 2019) to refine the individual XY movements into averages across the entire 12-animal group. The daytime hours data collection were omitted as animals acclimate to their new surroundings. I then compared the average pixels moved in each 30-minute period in the control treatment to the average pixels moved in the kairomone treatment to see if the animals maintain their free-running activity cycle longer when they are under duress of predation via chemical signaling.

Results

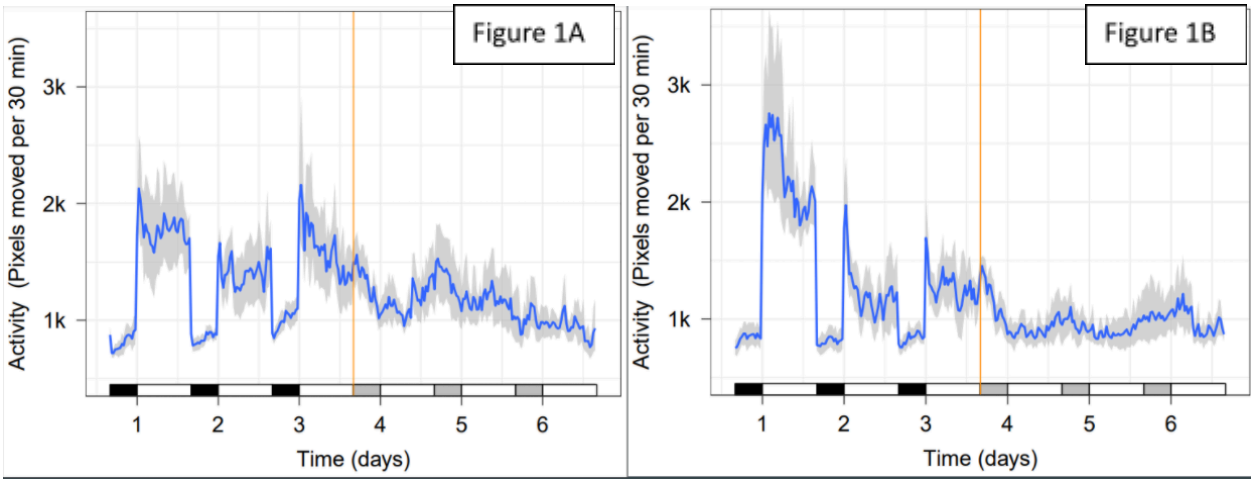


Figure 1: Combined movement data from each of 12 animals in run 1A (left) and 1B (right). Graphs represent the average pixels traveled for 12 animals in each 30-minute time slot in 1A) control run and 1B) kairomone treatment run. Y axes represents average number of pixels traversed during a 30-minute period, and the X axes plots time over the 7 day runs, with shaded regions indicating bootstrapped 95% confidence intervals. Shaded boxes indicate lighting conditions in the chamber, with black = lights out, white = lights on, and grey = lights on (at objective night), and amber line represents the start of 24-hour light.

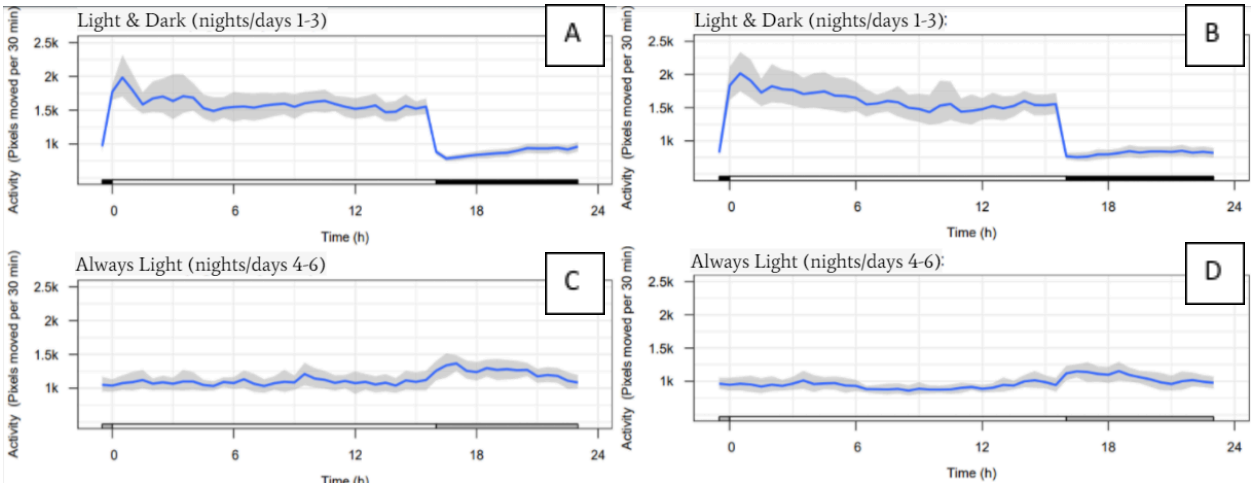


Figure 2: Wrapped data showing activity averaged by time of day in A,B) the first three days in light/dark and C, D) the final three days of 24-hour light. 3A and 3C are control, and 3B & 3D represent kairomone treatment.

These data show before 24-hour light began, *Daphnia* had a high activity level during their objective daytime, and a low activity level during objective nighttime (Fig. 1). Upon beginning 24-hour light, the animals did not display different levels of activity between objective daytime and subjective daytime (Fig. 1, Fig. 2). Similarly, the *Daphnia* did not show substantial differences in activity between control treatments and kairomone treatments. During light/dark, there a spike in activity right before dawn, but not in 24-hour light (Fig. 2).

Discussion

I began this investigation with prior knowledge that *Daphnia* have an endogenous circadian clock (Cellier-Michel et al. 2003). *Daphnia* have been demonstrated to have endogenous circadian gene expression (Coldsnow et al. 2017), and other studies have documented circadian DVM using vertical motion tracking (De Meester et al. 1999). In my own investigation, I wanted to determine if exposure to kairomones from planktivorous fish would cause *Daphnia* to follow a daily activity pattern, indicating the animal is attempting DVM.

Daphnia were active during daytime, and inactive at night, but only before entering 24-hour light (Fig. 1). In almost all cases, across all runs, the first day of recording was the most active period for the subject animals. Upon beginning 24-hour light, the animals quickly lost their previously robust daily activity pattern. If the *Daphnia* had been following an endogenous clock, they should have maintained their normal level of daytime activity during objective daytime, and settle down to low activity during objective night, even after the lights transition to 24-hour light; however, this was contrary to the observed trend. Instead of keeping their previous activity pattern from light/dark, the animals mostly continued their daytime activity into the objective night, and tapered off to normal subjective night activity levels as objective dawn broke. This tapering-off in activity is best evident at the night between day 3 and 4 (Fig. 2).

When considering the persistence of the daily activity pattern past LD into 24-hour light, the light/dark activity pattern tightly adheres to the lighting conditions (Fig. 1). After the transition to 24-hour light, there is some evidence of cyclic activity patterns to be seen in the peaks and subsequent troughs, but they are not synchronized with the changes between objective night and day. Overall, this data is not strong evidence in favor of *Daphnia* maintaining a diel activity cycle based on their endogenous circadian clock, but it does not necessarily refute the hypothesis or suggest the role kairomones play.

When comparing the control treatment to the kairomone treatment, there was no observable effect of the kairomone treatment on the animal's activity patterns (Fig. 1, Fig. 2). The control runs showed the result that I was expecting to see, but I had anticipated that the kairomone treatment data would have shown a robust activity pattern that would maintain its integrity into the 24-hour light section. I feel that although unsupported, the hypothesis is not refuted.

A suspected error in the mode of data collection is that, despite studying vertical migration, the animals were not allowed vertical motion. Activity of the *Daphnia* in their wells was anticipated to equate to times when the *Daphnia* would be trying to adjust their depth. However, the only time this performed as a reliable indicator was at dawn, during LD. In subsequent experimentation, modifications to the experimental setup may improve the results. The well tray the animals lived in could be replaced with a series of vertical glass tubes, with the camera tracking laterally, so the animals are free to move vertically. This would address the movement restriction to the animals, and it would

permit the script to measure vertical movement, so the data could better indicate when an animal is changing depth.

Another improvement to the kairomone treatment could be to ensure a constant source of kairomone by replacing the 2L flask of extra water volume with the fish tank. A possibility for the lack of response to the treatment is because the kairomones from the fish attenuated or broke down over time, so once 24-hour light began there was not sufficient concentration in the water for detection. Ensuring a constant, fresh source of kairomone may provide a stronger sense of danger to the *Daphnia* in the experiment. If the danger signal is stronger, the *Daphnia* may be incentivized to follow a robust rhythm into 24-hour light.

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Citations

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