

**Up, Up, and Away: Melatonin and Diel Vertical Migration in *Daphnia pulicaria* and *Daphnia pulex***

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Matt Gregory

Advisor: Bret Coggins

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## **Abstract**

Recently, melatonin has been detected in *Daphnia*, a crustacean that is an important biological and ecological model organism. Evidence suggests that melatonin attenuates stress responses in *Daphnia* under acute stress conditions. For example, one study showed that melatonin attenuates morphological predator-defense responses in *Daphnia* induced by invertebrate kairomones. *Daphnia* that live in thermally stratified bodies of water (i.e., lakes) exhibit diel vertical migration (DVM) in response to stress. In other words, lake *Daphnia* spend most of the daylight hours at or near the bottom of the lake because it is dark and visual predators cannot successfully hunt them. At night, when visual predators are unable to hunt, *Daphnia* rise to the surface to feed safely on phytoplankton. DVM should not be adaptive in populations of *Daphnia* living in vernal ponds because their pond habitats are not thermally stratified and are not home to visual predators like fish. We set up thermally stratified experimental water columns and added melatonin treated and melatonin-free control pond species (*Daphnia pulex*) and lake species (*Daphnia pulicaria*) to these water columns. We recorded the distribution of the *Daphnia* in the experimental water columns after 30 minutes and found that *D. pulicaria* was more likely to be found at or near the surface than *D. pulex*.

## **Introduction**

Melatonin belongs to the indolamine family of neurotransmitters and is found in most organisms, including both prokaryotes and eukaryotes. Indolamines are substituted indole compounds that contain an amino group. Indole is an aromatic heterocyclic organic compound with the chemical formula  $C_8H_7N$ . Serotonin is another notable indolamine. Generally documented functions of melatonin include detoxification of reactive oxygen species, adjustment of the circadian clock to the environmental light regime, regulation and synchronization of cell

physiology, mediation of photoperiodic information, and the reduction of stress effects (Schwarzenberger, Christjani, & Wacker 2014).

In crustaceans, melatonin has several physiological purposes. Melatonin modulates locomotory activity, glucose/lactate levels, and neurotransmitter release in crayfish (Tilden et al. 2003). Melatonin has an influence on limb regeneration in the fiddler crab *Uca pugilator* (Tilden et al. 1997). Melatonin influences molting in the edible crab *Ozioltelphusa senex senex* (Sainath & Reddy 2010) and might play a role in antioxidant defense systems in the estuarine crab *Neohelice granulata* (Maciel et al. 2010).

Recently, melatonin has been detected in *Daphnia*, a crustacean that is an important biological and ecological model organism. The highest concentration of melatonin in *Daphnia* has been detected in the nervous system (Markowska et al. 2009) and its synthesis has been shown to result from rhythmic transcription of certain genes (Foulkes et al. 1997). This is similar to the process of melatonin production in mammals. In mammals, melatonin is produced in varying amounts by the pineal gland of the brain in a cyclical fashion depending on the time of day.

Several studies have focused on the effects of melatonin in *Daphnia* specifically. Kaas et al. (2009) documented a significant decrease in the heart rate of *Daphnia magna* resulting from the introduction of exogenous melatonin. Kashian and Dodson (2004) found melatonin has no effect on sex determination in *Daphnia*. Bentkowski et al. (2010) found that melatonin disturbed diel vertical migration (DVM) in male and female *Daphnia*.

DVM is a rhythmic pattern of movement that allows *Daphnia* to safely access its main food source, phytoplankton. DVM is influenced primarily by fish predation-pressure and ultraviolet radiation (Rhode, Pawlowski, & Tollrian 2001). Predation pressure on *Daphnia* is

lowest at night because many *Daphnia* predators, including fish, are visual hunters. Likewise, ultraviolet radiation levels are lowest at night due to reduced sunshine. As a result, *Daphnia* feed at the surface at night, when the risks of predation and ultraviolet radiation poisoning are lowest.

Melatonin levels in *Daphnia* peak around midnight when they are at the surface feeding, suggesting that melatonin might be a stress signal inhibitor since normal stress responses often prevent *Daphnia* from migrating to the surface (Bentkowski et al. 2010). However, DVM should not be adaptive for all *Daphnia*. DVM is primarily adaptive for *Daphnia* that live in lakes because lakes have a thermally stratified water column and are habitats for many visual fish predators. DVM is less likely to be adaptive in ephemeral ponds since these ponds lack visual fish predators and are thermally more homogenous than lakes.

The present investigation attempts to determine how melatonin affects the water column distribution of pond *Daphnia* relative to lake *Daphnia*. We tested the hypothesis that melatonin would have a significant effect on the water column distribution of lake *Daphnia* by encouraging them to migrate to the surface at times they would typically be found deeper in the water column while it would not have a significant effect on the water column distribution of pond *Daphnia* because it is not advantageous for pond *Daphnia* to migrate through the water column. Support for this hypothesis would suggest that melatonin is indeed acting as a stress signal inhibitor in lake *Daphnia*.

## **Methods**

Sample populations of pond and lake *Daphnia* were added to thermally stratified water columns that were 50 cm deep. The water temperature at the surface of each column was 24 °C. The water temperature at the bottom of each water column was 21.8 °C. Each experimental water column contained only pond *Daphnia* or only lake *Daphnia*. Four water columns were used in

each trial for the duration of the experiment. The water columns were filled half with pond water and half with lake water. For each trial, two of the water columns contained 20 pond *Daphnia* (*D. pulex*) and two of the water columns contained 20 lake *Daphnia* (*D. pulicaria*). Whether each column would contain pond *Daphnia* or lake *Daphnia* for a particular trial was determined randomly prior to each trial.

Ten *Daphnia* in each water column were treated with 50  $\mu$ L of 10 mM melatonin one hour prior to the beginning of the trial. Melatonin is not water soluble, so DMSO was used to dissolve the melatonin and make it accessible to the *Daphnia*. The remaining ten *Daphnia* in each water column were treated with DMSO—also one hour prior to the beginning of the trial—and used as a comparison group for the melatonin-treated *Daphnia*.

To differentiate between melatonin-treated and non-melatonin-treated *Daphnia*, the gut tracts of each were stained with unique food coloring. Each experimental population of 20 *Daphnia* was placed in a 50 ml conical vial with four drops of food coloring for 16 hours prior to each trial. The vials were filled with water appropriate to each species' typical habitat (i.e., *D. pulex* were stored and stained in pond water and *D. pulicaria* were stored and stained in lake water).

Two trials were conducted each day during the data collection period. The *Daphnia* for the first trial were treated with the appropriate melatonin/DMSO combination at 8 a.m. The *Daphnia* for the second trial were treated with the appropriate melatonin/DMSO combination at 11 a.m. Thus, the first trial began at 9 a.m. and the second trial began at 12 p.m. After placing treated *Daphnia* in the appropriate experimental water column, we waited 30 minutes before measuring water column distribution using the continuous variable of depth. After 30 minutes, the distribution of *Daphnia* in each water column was measured by visually scanning each water

column from top to bottom and recording the position of each *Daphnia* as it was encountered. At the conclusion of the 9 a.m. trial, we filtered the *Daphnia* out of the water columns in preparation for the 12 p.m. trial. After the 12 p.m. trial, the water columns were drained and refilled with fresh pond and lake water found on the University of Notre Dame Environmental Research Center property. We used pond water from a small vernal pond between the Hank Lab and the Wet Lab and lake water from Tenderfoot Lake to fill the water columns. The columns were aerated and heated overnight before each trial day.

We collected most of the *Daphnia* used in the experiment from two locations on property. We collected *D. pulicaria*—our lake *Daphnia*—from Tenderfoot Lake. We collected *D. pulex*—our pond *Daphnia*—from vernal pond N, which is just south of Plum Lake and Inkpot Lake. We kept the *Daphnia* used in the experiment on a natural light cycle by placing the conical vials in which we stored them on a windowsill. We prevented artificial light from interfering with the desired natural light regime using a blackout curtain.

## **Results**

The data we collected did not meet the assumptions necessary to run an ANOVA. Levene's test for homogeneity of variance revealed that our data was heteroscedastic ( $df = 1$ ,  $F = 24.332$ ,  $p = 1.309e-06$ ). Transformations failed to rectify the non-normality of the collected data. Despite the inappropriate nature of using a parametric test in this situation, we ran a 2-way ANOVA anyway. The 2-way ANOVA indicated that type of *Daphnia* (*D. pulicaria* or *D. pulex*) had a significant effect on depth in the water column ( $df = 1$ ,  $F = 8.551$ ,  $p = 0.0037$ ). Treatment type (melatonin and DMSO or just DMSO) also had a significant effect on depth in the water column ( $df = 1$ ,  $F = 35.034$ ,  $p = 8.43e-09$ ). The interaction between *Daphnia* type and treatment

was significant as well ( $df = 1$ ,  $F = 6.090$ ,  $p = 0.0141$ ). We performed all statistical analyses using the statistical software R (R Core Team 2017). Our data is summarized in **Figures 1-3**.

## **Discussion**

We expected that *D. pulicaria* would demonstrate diel vertical migration in response to the melatonin treatment while *D. pulex* would not respond to the melatonin treatment and would be distributed randomly throughout the water column. Our statistical tests revealed that this was not necessarily the case, as both *D. pulicaria* and *D. pulex* appeared to respond to the melatonin treatment on occasion. However, Figures 1-3 do suggest that melatonin might have had a more significant impact on *D. pulicaria* than *D. pulex*. This means that lake *Daphnia* might be more sensitive to the presence of exogenous melatonin than pond *Daphnia*, which aligns with our original hypothesis that lake *Daphnia* will respond more strongly to the melatonin treatment than pond *Daphnia* because DVM is advantageous for lake *Daphnia* and not advantageous for pond *Daphnia*. Further experimentation and subsequent sound statistical analyses could validate the trend hinted at by our data.

Despite our expectation that *D. pulicaria* and *D. pulex* would exhibit differential responses to the melatonin treatment, we must mention that it is intriguing that such similar species would show differential responses to a treatment that operates on a pathway that is likely fairly conserved between the two species. In the northeastern United States, researchers often encounter *Daphnia* that are intermediate in form between *D. pulicaria* and *D. pulex*, suggesting that the species might be able to hybridize. Future researchers should keep the similarity of these two species in mind when conducting experiments and should consider the similarity itself an avenue for possible future research.

Our results are similar to the findings in Schwarzenberger et al. (2014) in that they suggest that melatonin attenuates *Daphnia* stress responses under acute stress conditions. Schwarzenberger et al. (2014) showed that melatonin disturbs the predator-avoidance behavior of *Daphnia* by demonstrating that melatonin attenuates morphological predator-defense responses induced by invertebrate kairomones. Here, we show that melatonin might attenuate *Daphnia* stress response by demonstrating the melatonin can encourage *Daphnia* to migrate to the water's surface at times which would normally be dangerous to them, namely during the day when the Sun is shining and visual predators are hunting.

We suggest that our results be viewed as a mere suggestion that melatonin encourages *Daphnia* to alter its DVM in this fashion because of the limitations of our experimental design and statistical approaches. *Daphnia* depth distribution in the water column was recorded with the naked eye, meaning these measurements were prone to error. Each water column was observed top to bottom. As *Daphnia* were encountered, their position in the water column was recorded. Since there were 20 *Daphnia* in each column, there should have been 20 observations for each column. However, sometimes we did not observe all 20 *Daphnia*. When this happened, we assumed that the unobserved *Daphnia* were at the bottom of the water column since there was a small portion of the bottom of the column that was impossible to observe directly. As a consequence, we had a large number of data points that indicated that *Daphnia* were at the bottom of the water column. The paucity of non-bottom observations likely contributed to the gross and unavoidable heteroscedasticity of our data. The fact that it was difficult (though importantly not impossible) to distinguish between stained *Daphnia* further complicated things.

To avoid these observation issues in the future, we recommend that future researchers rely on camera equipment to take a still image of the water column, so the distribution of



*Daphnia* can be recorded more accurately. Color correction software might also be helpful when distinguishing between stained *Daphnia* in still images.

Despite the limitations of our experiment, our results still provide some evidence that melatonin does indeed affect water column distribution in *Daphnia*. This supports the hypothesis presented in Schwarzenberger et al. (2014) that melatonin acts as a stress signal inhibitor in *Daphnia*. Future experiments should attempt to further tease apart the differential responses of pond and lake *Daphnia* to melatonin. We also suggest that future researchers include predation as a factor in their experiments. One easy way to accomplish this would be to add *Chaoborus* kairomones—*Chaoborus* is a *Daphnia* predator—to the experimental water columns. Typically, *Daphnia* hide in deep, dark water to avoid *Chaoborus*. We would expect that they would do the same in experimental water columns containing *Chaoborus* kairomones unless the addition of exogenous melatonin inhibited the stress response that drives *Daphnia* to the depths in the presence of predators. If future researchers find that *Daphnia* do rise to the top of the water column—even in the presence of predators—when dosed with melatonin, then we would have still more evidence for the stress-signal-inhibiting effects of melatonin suggested by Schwarzenberger et al. (2014).

## Figures

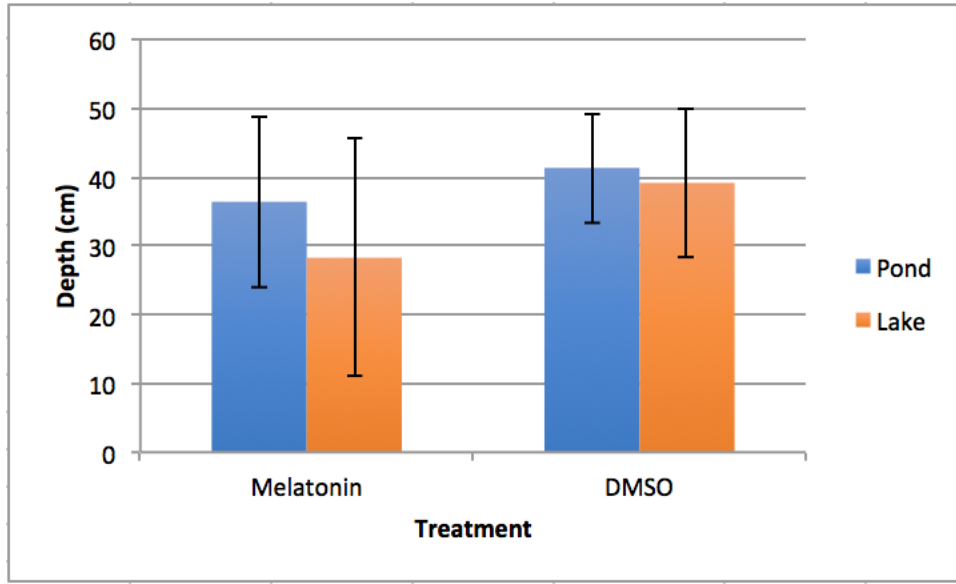


Figure 1. *Daphnia* water column distribution for all trials. Generally, lake *Daphnia* appeared in shallower parts of the water column than pond *Daphnia*. It appears that melatonin had a stronger effect on lake *Daphnia* than pond *Daphnia*. Because of our statistical limitations, further experimentation would be necessary to corroborate this apparent trend.

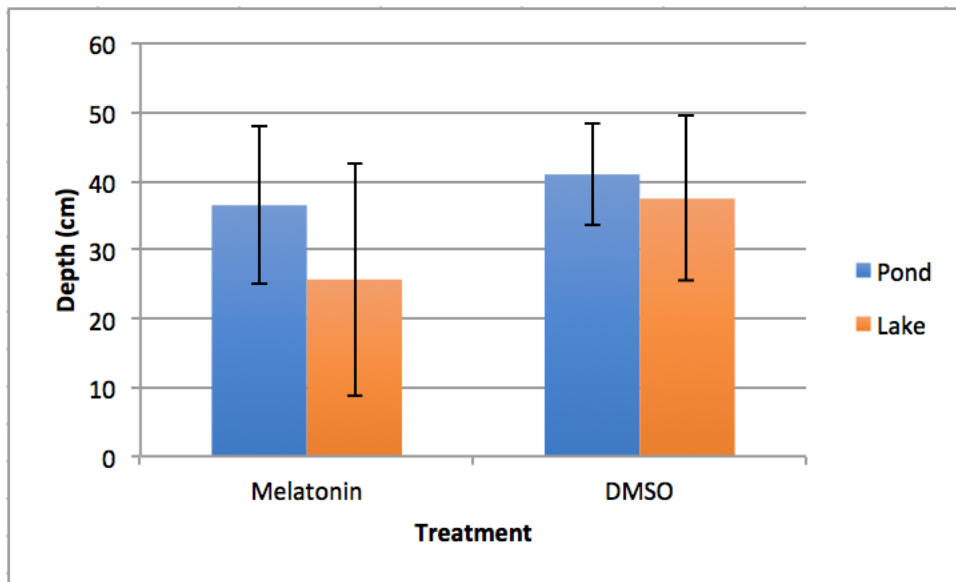


Figure 2. *Daphnia* water column distribution for only 9 AM trials. As with the overall results, lake *Daphnia* appear in shallower parts of the water column than pond *Daphnia*. Again, it appears that melatonin had a stronger effect on lake *Daphnia* than pond *Daphnia*.

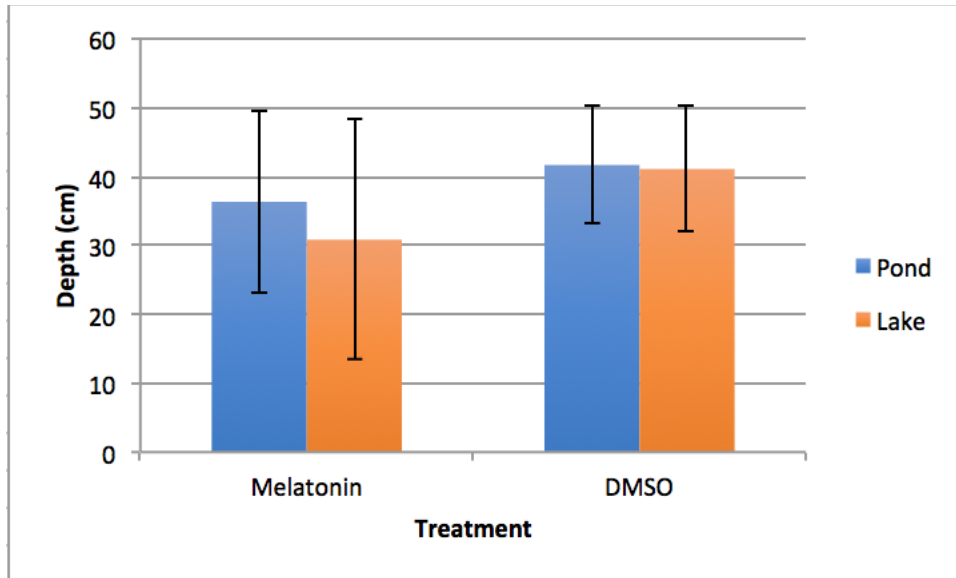


Figure 3. *Daphnia* water column distribution for only 12 PM trials. Consistent with the overall results and the 9 AM results, lake *Daphnia* appear in shallower parts of the water column than pond *Daphnia*. Once again, it seems that melatonin had a stronger influence on the distribution of lake *Daphnia* than pond *Daphnia*.

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