# THE EFFECTS OF FEEDING ON ZEBRAFISH *DANIO RERIO* CIRCADIAN-DEPENDENT ACTIVITY PATTERNS

A Report of a Senior Study

by

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ABSTRACT

In most studies using the zebrafish *Danio rerio*, researchers do not feed the fish due to fears that feeding will induce increased activity and metabolism of some fish. In this experiment, fish were divided into 4 groups: fed during daylight, fed during nighttime, not fed with marbles, and not fed without marbles. It was hypothesized that (1) fish fed during daylight hours would exhibit increased daytime activity compared to those fed during nighttime hours, (2) fish fed during nighttime hours would exhibit unaltered behavior compared to other groups, and (3) fish that were not fed would display different activity compared to those that were fed. The fish were maintained at a 14h light:10h dark cycle. After 9 days of observation three times a day, the first and third hypotheses were supported, and the second was rejected. In this study, the unfed, no marbles group displayed usual behavior and served as the control, as they were able to feed on oviposited eggs each morning. The unfed, with marbles group appeared to be the most stressed, decreasing activity over the 9 days of observation. The group fed once during the light hours and once during the dark hours exhibited a slight increase in the observed behaviors of response to feeding, level of aggression, swimming intensity, and amount of foraging in the marbles or along the bottom of the tank. Aggression was lowest in the not fed, no marbles tank. For level of aggression, there was a significant decrease at 8:00 am from day 1 to day 9 ($p$=0.0003) and at 5:00 pm ($p$=0.0015), but not at 10:00 pm ($p$=0.6006). For swimming intensity, there was a significant difference among the

four tanks at 8:00 am ($p$=0.0062), at 5:00 pm ($p$<0.0001), and at 10:00 pm ($p$=0.0003). For response to food at 8:00 am, there was a significant increase in response to food for the light fed (LF) tank compared to the dark fed (DF) tank ($p$<0.0001). For level of foraging, there was a significant difference among the four tanks at 8:00 am ($p$=0.0195) and at 5:00 pm ($p$=0.0066), but not at 10:00 pm ($p$=0.2290). Thus, in this study of the effects of feeding on circadian-dependent behaviors of zebrafish *Danio rerio*, it was found that feeding does have an effect on circadian-dependent behaviors.

TABLE OF CONTENTS

## List of Figures



## List of Tables

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# Chapter I

## INTRODUCTION

Biological organisms experience time in many different ways. These include with a clock, the sun and moon, and also indirectly and involuntarily. Many organisms have developed an intrinsic time-keeper referred to as a biological clock. This "clock" is entrained by photoperiod and is affected by temperature, population density and interaction, and hormone levels (Hurd, Debruyne, Straume, Cahill, 1998). This natural clock controls an organism's circadian rhythm, a rhythm maintained by environmental factors that is a cycle of one day (Dunlap, Loros, DeCoursey, 2004). However, some organisms have periods that are different from one day; circalunar, circannual, and circatidal cycles consist of a period length of one month, one year, or one tidal cycle (12.4 h), respectively. This feature of many organisms has been studied extensively, and yet there are many unanswered questions about the mechanism, regulation, and function of chronobiology at different stages during the life cycle. Figure 1 shows a generic model for circadian rhythmicity.

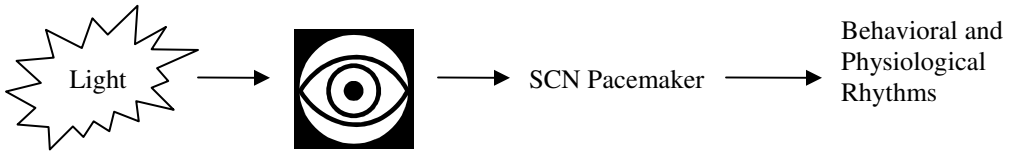



**Figure 1.** The circadian rhythm model of vertebrates and invertebrates. In the process, light stimulates a photosensory source, which activates the superchiasmata nucleus (SCN) pacemaker. The output of that cycle is behavioral and physiological rhythms such has activity level and melatonin production.

Circadian rhythms of many animals are regulated physiologically by melatonin levels dependent on light-dark cycles (Dekens, Santoriello, Vallone, Grassi, Whitmore, Foulkes, 2003). Melatonin is a hormone produced by the pineal gland and is secreted during periods of darkness. Elevated melatonin causes a decrease in activity inducing a "sleep-like state" in vertebrates (Zhdanova, Wang, Leclair, Danilova, 2001). Not only the pineal gland, but the retinas of many vertebrates also produce and secrete melatonin. However, the specific influence that retinal melatonin has in altering activity state has yet to be determined (Cahill, 1996).

Phyletic conservation of the biological clock and circadian rhythms has allowed non-human animals to be used as models for human rhythmicity. Natural rhythms of life were first noticed in insects. For example, the life cycle of a butterfly relies heavily on environmental factors. The mechanism that controls this is unknown. Even unicellular organisms have been studied based on circadian rhythms. Prokaryotes such as the cyanobacterum *Synechococcus* have been shown to have circadian regulation and temperature compensation. The eukaryotic dinoflagellate *Gonyaulax* is the most studied of all unicellular eukaryotic species. Researchers have shown that in this organism, at least four different events are influenced by circadian rhythmicity: photosynthesis, flashing, glowing, and cell division. Among more derived species, an obvious circannual rhythm is that of hibernation. In hibernating organisms, body temperature decreases, activity level is practically depleted, and metabolic demand is much less than normal. To maintain bodily functions during the long, cold winters, hibernating animals have a pacemaker that "wakes them" just long enough to maintain a certain body temperature (Dunlap *et al.*, 2004).

Researchers have discovered that zebrafish (*Danio rerio*) provide a simple vertebrate animal model to elucidate the importance of melatonin on rhythmicity. Zebrafish are an excellent model for studying circadian rhythms because this diurnal organism is active during the day, rests at night, and shows clear circadian patterns of melatonin secretion (Zhdanova *et al.,* 2001). Studying these effects in zebrafish may allow new medicines to be developed for humans and may reveal new information that could better human understanding of the complexity of the biological clock. Further, embryonic and larval zebrafish display great sensitivity to melatonin levels, even increasing the level of cell proliferation and development (Danilova, Krupnik, Sugden, Zhdanova, 2004). Moreover, circadian rhythmicity is conserved between genders in zebrafish, as it is in humans (Hurd *et al.,* 1998).

As mentioned, melatonin is the hormone that is thought to regulate the natural clocks of many organisms. As can be seen in Table 1, most organisms have clock mechanisms; however, the phylogenetically primitive organisms do not produce melatonin. Zebrafish, the focus of this experiment, produce melatonin for regulating circadian rhythm.

**Table 1.** Presence of melatonin in a range of organisms and its role, if any.

| Organism | Melatonin Present | Clock | Source |
|---|---|---|---|
| *Synechococcus* | N | Y | Dunlap, Loros, DeCoursey; 2004 |
| *Neurospora* | N | Y | Dunlap, Loros, DeCoursey; 2004 |
| *Drosophila* | Y | Y | Tamai, Carr, Whitmore; 2005 |
| Hawk moth | Y | Y | Lampel J, Briscoe AD, Wasserthal LT.; 2003 |
| Zebrafish | Y | Y | Cahill; 1996 |
| African clawed frog | Y | Y | Wiechmann AF, Vrieze MJ, Dighe R, *et al.*; 2003 |
| Green Anole | Y | Y | Norris, 1997 |
| House Sparrow | Y | Y | Foster, Kreitzman; 2004 |

| | | | |
|---|---|---|---|
| Humans | Y | Y | Dunlap, Loros, DeCoursey; 2004 |

Melatonin structure has been well conserved among organisms. This hormone is an amine hormone, merely a modification of the amino acid tryptophan (see Figure 2). Melatonin (N-acetyl-5-methoxytryptamine) is synthesized by the hydroxylation of tryptophan, decarboxylation of serotonin, and methylation of the N-acetylserotonin metabolite, as can be seen in Figure 3 (Agency for Healthcare Research and Quality, 2004).

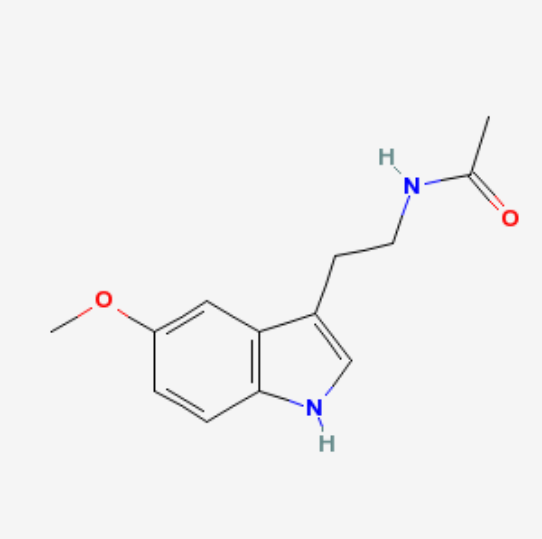



**Figure 2.** Structure of melatonin (NCBI, PubChem, 2006).

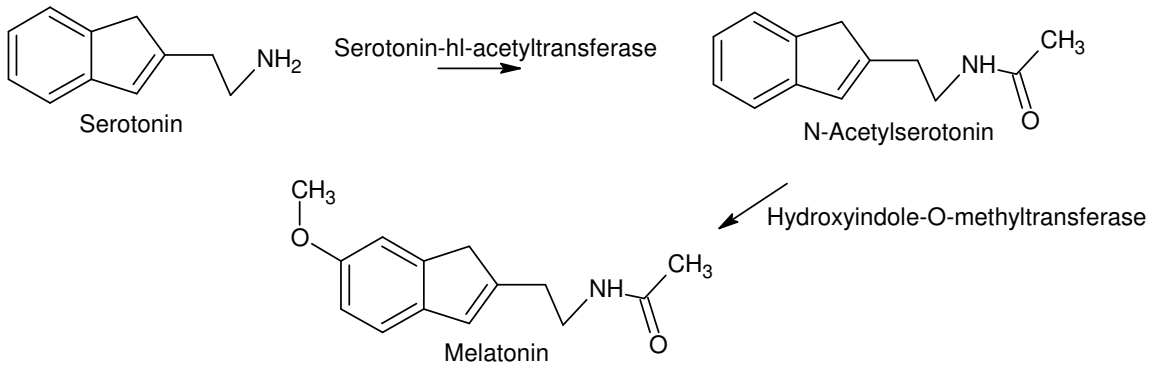



**Figure 3.** Synthesis of melatonin.

Melatonin is important in many biological processes including sleep, reproduction, and mood. Whereas the structure of melatonin is the same in humans and zebrafish, the stimulus for melatonin production is somewhat different between the two vertebrates. Mammals rely primarily on involuntary neural signals to synthesize melatonin and are stimulated by photosensory elements. Zebrafish, on the other hand, rely simply on photoreception and an established pattern (Dunlap *et al.,* 2004).

Zebrafish circannual rhythmicity and melatonin production are primarily regulated by photoperiod and temperature. Regulation of circadian rhythms is marked by the ability to maintain physiological functions without the appropriate cues. Photoperiod is the main regulation factor and has the strongest influence in embryos and recently hatched juveniles. Zebrafish tissues and cells are directly light-responsive, meaning exposing explanted tissue and cell lines to light or darkness will stimulate clock gene expression. Researchers have found that melatonin production, stimulated by periods of darkness, incites a higher rate of cell proliferation (Danilova *et al.,* 2004). This higher rate of cell proliferation is especially evident during the periods of darkness, and it is thought that this has evolved to prevent DNA damage from UV-light. In other words, the embryos and developing juveniles grow more during darkness and avoid potential mutation opportunities during periods of light (Dekens *et al.,* 2003). The mechanism by which this occurs is thought to be similar to that found in other organisms. Cells enter into "S" phase of mitosis more frequently at the end of the day or early night. Therefore, photoperiod must partially control the cell cycle in zebrafish and other organisms. Further, temperature has also been found to regulate circadian rhythms of zebrafish. Hurd *et al.* found that the majority of the fish in the study were mostly active at 25 ℃

during the light portion of a 12h light: 12h dark cycle. In addition, they found that the optimal temperature for the most activity during constant conditions to be 21 °C. This suggests that the circadian rhythm of zebrafish is temperature compensated.

As previously mentioned, there are two sources of melatonin synthesized in most organisms, the pineal gland and retina. In the wrasse, retinal melatonin rhythm was thought to be regulated by either light or a clock mechanism. Under constant dark conditions, the ocular melatonin levels were consistent; however, under constant light conditions, it was low. It was found that the ocular melatonin levels were regulated, in fact, by a retinal circadian clock mechanism that is entrained to a light-dark cycle by way of photoreception factors (Iigo, Ikeda, Sato, Kawasaki, Noguchi, Nishi, 2006). In zebrafish, Cahill (1996) found that retinal melatonin levels decreased in constant dark conditions, while pineal melatonin levels were persistent under the same conditions. Therefore, one can be confident that the bulk of zebrafish melatonin is produced by the pineal gland of the brain.

Zebrafish provide a useful model organism for humans because both function on a 24-hour rhythm. The oscillation is involved in biochemical and physiological processes that control behavior and involuntary hormone synthesis, with light-dark cycles serving as the main influence on the oscillating cycle. For example, jet lag is actually a human response from the change in the light-dark cycle. Zebrafish also display a form of "jet lag" when their light-dark cycle is suddenly altered (Cahill, 1996). Further, both humans (and all other mammals) and zebrafish have retinal and pineal melatonin synthesis that are regulated by central and peripheral clocks. Some of the same structures are responsible for the circadian rhythm of the organism. In humans, these structures are the

retina, SCN, and pineal gland. In zebrafish, these are the retina and pineal gland. Zebrafish lack an SCN, and nothing of the like has been identified in this organism. Also, zebrafish have three *Per* genes that correlate to those in mammals. However, there are some differences in expression of these genes between zebrafish and mammals. All of these similarities, among others, give justification for zebrafish to be used as a model organism for humans and mammals.

During most of the aforementioned zebrafish experiments, the fish were not fed during the study due to the fear that feeding increases the level of activity and cellular processes in fish, which could alter the results of the study (Hurd et al, 1998). Indeed, rats increase their level of activity up to 2 hours before feeding (Ritcher, 1922). Other mammals and birds display such anticipatory behavior. However, the direct effects of feeding on zebrafish circadian-dependent activity patterns have not been investigated.

Serotonin is the starting material in the synthesis conversion of melatonin. Orchard (2005) found that in *Rhodnius prolixus*, serotonin levels increased in the haemolymph, increasing heart rate, saliva secretion, and stimulating muscle contraction of the digestive tract. It was concluded that serotonin coordinates feeding behavior in this insect. Given that serotonin is a precursor of melatonin (Figure 3, next page), it is expected that increased serotonin levels would increase the production of melatonin, resulting in a decrease in activity level after feeding. This study will examine food-associated post-feeding activity in zebrafish. It was hypothesized that (1) fish fed during daylight hours would exhibit increased daytime activity compared to those fed during nighttime hours, (2) fish fed during nighttime hours would exhibit unaltered behavior

compared to other groups, and (3) fish that were not fed would display different activity compared to those that were fed (see Figure 4).

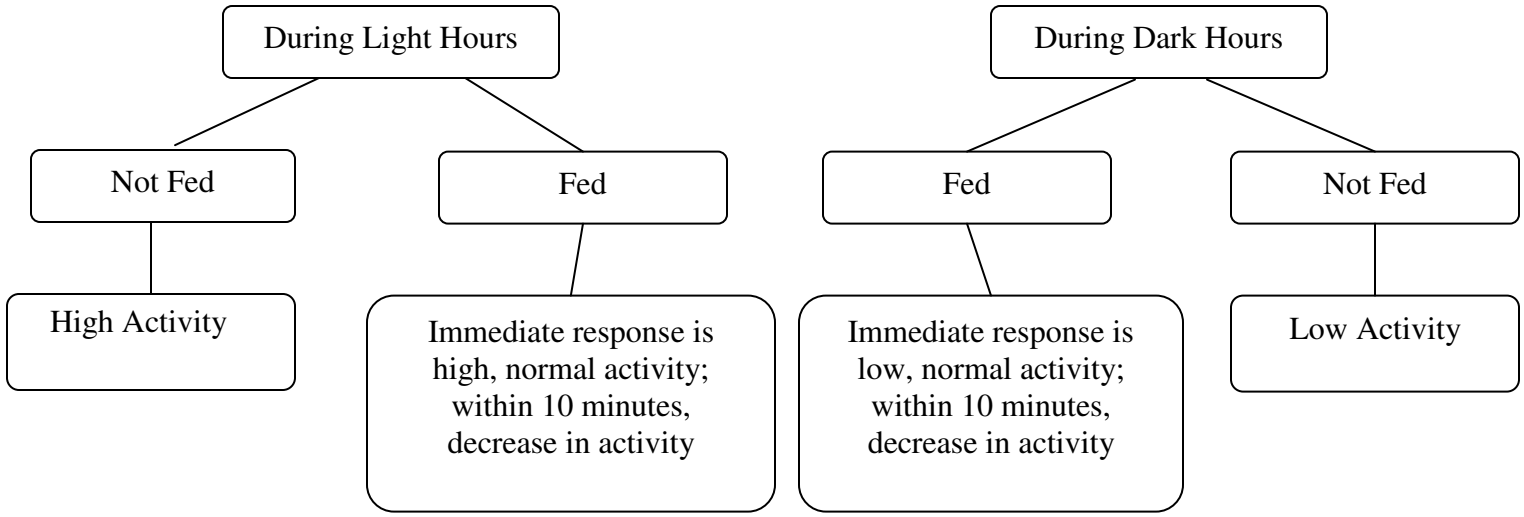



**Figure 4.** Hypothesized response of zebrafish to feeding during both the light and dark hours. It was hypothesized that (1) fish fed during daylight hours would exhibit increased daytime activity compared to those fed during nighttime hours, (2) fish fed during nighttime hours would exhibit unaltered behavior compared to other groups, and (3) fish that were not fed would display different activity compared to those that were fed.

# Chapter II

## MATERIALS AND METHODS

Four Plexiglas fish tanks were washed and fully assembled as follows. Forty gallons of tap water was dechlorinated using Jungle Start Right dechlorinator (1 tsp. per gallon) that removes chlorine and chloramines. The prepared water was allowed to stand for 30 minutes, and then 10 gallons of treated water were added to each of the four clean tanks. Clear and pink marbles were cleaned and placed in three of the tanks. Also, one green plastic aquarium plant was placed in each tank. One Tetra Whisper Heater for 10-30 gallon tanks was placed in the corner of each tank, along with a Regent Aqua-Tech 5-15 gallon power filter with Regent Aqua-Tech EZ-Change Filter Cartridges. After purchasing 36 unsexed zebrafish (*Danio rerio)* from Carolina Biological Supply Company, with extras provided, 13 randomly chosen fish were placed in each tank. Each zebrafish was massed using the Mettler-Toledo Delta Range balance to verify that each tank contained a relatively even distribution of fish; this was determined by doing statistical analysis (ANOVA) using *Microsoft Excel*.

The zebrafish were slowly acclimated to the 21 ℃ water once the water temperature was stable. The study began after the fish were in the tanks for three days and an ethogram of their behaviors had been completed. Further, during the second day in the tanks, all the fish were fed as it was noticed that many of them were dying. The

fish were fed frozen brine shrimp eggs (San Francisco Bay Brand). The room the fish were housed in was set on a 14h light: 10h dark cycle.

By the beginning of the experiment, there were 10 fish per tank. The fish were observed three times per day: 8:00 am, 5:00 pm, and 10:00 pm (two during light and one during dark), and the ethogram observations were recorded at each time (see Table 2). Tanks 1 and 2 were the tanks of fed fish, and tanks 3 and 4 were the tanks of unfed fish. Tanks 1, 2, and 3 all had marbles, while tank 4 did not. At 8:00 am, the fish in both tanks 1 and 2 were fed the frozen brine shrimp eggs. At 5:00 pm, only the fish in tank 1 were fed. Finally, at 10:00 pm, only the fish in tank 2 were fed. After feeding during the light hours, each tank was observed for 10 minutes, noting behaviors in response to being fed or not. During the dark feeding, each tank was recorded using a digital Panasonic PalmSight video camera and a Maxell mini digital video cassette tape. These tapes were reviewed afterward, and behaviors were noted. After 9 days of study, one fish from each population was removed and killed using quick immersion in ice water. These fishes' heads were removed and stored in formalin fixative for future histological experiments. The remaining fish were maintained in the laboratory for future use.

Statistical analysis was conducted using *StatView*. A one-way ANOVA was used to analyze the weight of fish. To compare response to food of the light fed and dark fed tanks, an unpaired t-test was used. Level of aggression of all four tanks was analyzed using the Kruskal-Wallis nonparametric test (n=9 for each tank). Swimming intensity data was compared for each time of observation using a Kruskal-Wallis nonparametric test (n=9 for each tank).

**Table 2.** Ethogram of observed behaviors and quantification of the behaviors.

| Behavior | Level | Description |
|---|---|---|
| Response to Food | 0 | No Response |
| Response to Food | 1 | Eat if come in contact |
| Response to Food | 2 | Eat when hand removed |
| Response to Food | 3 | Nibble on hand |
| Aggression | 0 | No Aggression |
| Aggression | 1 | Chasing 2-3 fish |
| Aggression | 2 | Chasing 4-6 fish |
| Aggression | 3 | Chasing every fish |
| Swimming Intensity | 0 | Floating/Sleeping Posture |
| Swimming Intensity | 1 | Slow |
| Swimming Intensity | 2 | Fast |
| Swimming Intensity | 3 | Hyperactive |
| Foraging in Marbles/Along Bottom | 0 | No Foraging |
| Foraging in Marbles/Along Bottom | 1 | 1-3 Foragers |
| Foraging in Marbles/Along Bottom | 2 | 4-7 Foragers |
| Foraging in Marbles/Along Bottom | 3 | All Foraging |

# Chapter III

## RESULTS

Table 3 presents results for response to food, level of aggression, swimming intensity, and level of foraging for the four tanks. There was a significant increase in response to food for the light fed (LF) tank compared to the dark fed (DF) tank at 8:00 am ($p<0.0001$). As can be seen in Table 3, on day 1 fish both of the fed tanks were exhibiting the same level of response at all times of observation. However, by day 9, the night fed fish decreased in response to feeding.

For level of aggression, there was a significant decrease in this behavior at 8:00 am from day 1 to day 9 ($p=0.0003$) and at 5:00 pm ($p=0.0015$), but not at 10:00 pm ($p=0.6006$). The no marbles, not fed tank exhibited the lowest level of aggression (usually none) at all times, while the light fed group showed the highest level of aggression during the day. During dark hours, fish in this tank were calm. On day 1, fish in the two non-fed tanks displayed aggression at 10:00 pm, whereas, fish in the fed tanks were not. By day 9, all four tanks showed no aggression at 10:00 pm.

For swimming intensity, there was a significant difference among fish in the four treatments at 8:00 am ($p=0.0062$), at 5:00 pm ($p<0.0001$), and at 10:00 pm ($p=0.0003$). As can be seen in Table 3, all fish were swimming during the light hours, but only the fish of the two fed tanks were swimming during the dark hours on day 1. The marbles,

unfed group exhibited no swimming on day 9. Of the tanks that did exhibit swimming on day 9, they did so at similar levels. Further, the no marbles, not fed group swam the most during the early morning. By day 9, both fed groups had increased the intensity of swimming.

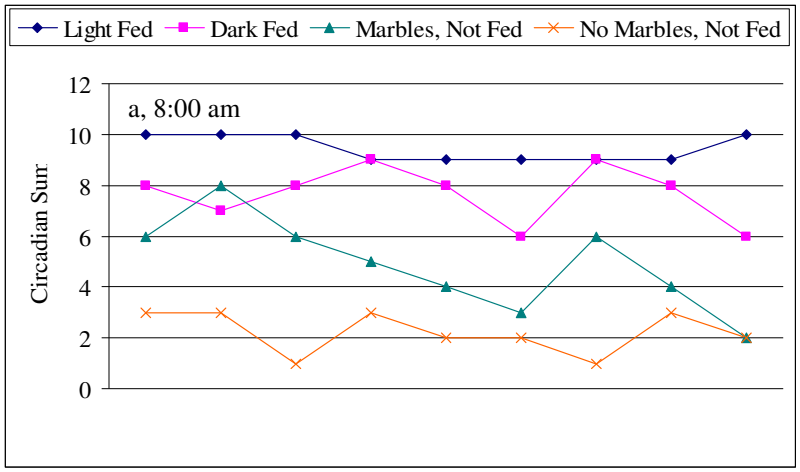
For level of foraging, there was a significant difference among the four treatments at 8:00 am ($p$=0.0195) and at 5:00 pm ($p$=0.0066), but not at 10:00 pm ($p$=0.2290). The light fed fish foraged in the marbles the most on day 1. The no marbles, not fed fish only foraged during the 8:00 am hour both days. On day 9, all the fish that foraged did so at equal levels. The dark fed fish foraged both days at 10:00 pm.

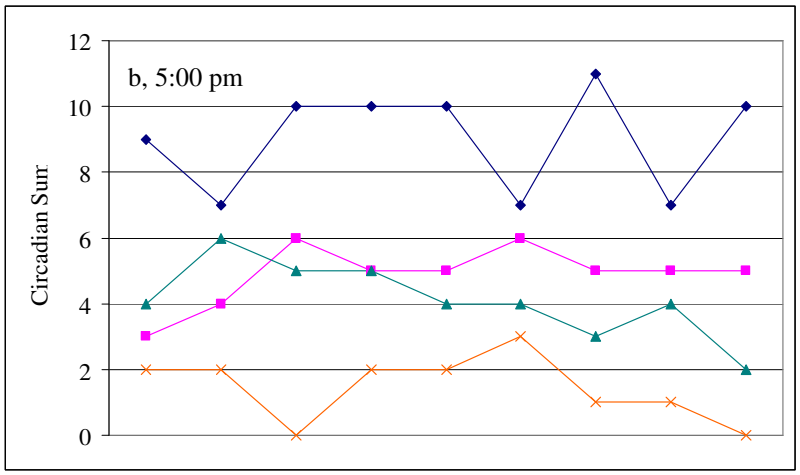
**Table 3.** Results for response to food, level of aggression, swimming intensity, and level of foraging for the four tanks (LF = light fed; DF = dark fed; M, NF= marbles, not fed; NM, NF = no marbles, not fed) for days 1 and 9. Bold signifies significance.

| | | 8:00 am | | 5:00 pm | | 10:00 pm | |
|---|---|---|---|---|---|---|---|
| | | Day 1 | Day 9 | Day 1 | Day 9 | Day 1 | Day 9 |
| Response to Food | LF | **3** | **3** | 3 | 3 | n/a | n/a |
| | DF | **3** | **1** | n/a | n/a | 3 | 2 |
| | M, NF | n/a | n/a | n/a | n/a | n/a | n/a |
| | NM, NF | n/a | n/a | n/a | n/a | n/a | n/a |
| Level of Aggression | LF | **2** | **3** | **3** | **3** | 0 | 0 |
| | DF | **2** | **2** | **1** | **2** | 0 | 0 |
| | M, NF | **3** | **1** | **2** | **1** | 1 | 0 |
| | NM, NF | **1** | **0** | **1** | **0** | 1 | 0 |
| Swimming Intensity | LF | **2** | **3** | **2** | **3** | **1** | **2** |
| | DF | **2** | **2** | **1** | **2** | **1** | **2** |
| | M, NF | **2** | **0** | **1** | **0** | **0** | **0** |
| | NM, NF | **1** | **1** | **1** | **0** | **0** | **0** |
| Level of Foraging | LF | **3** | **1** | **1** | **1** | 1 | 0 |
| | DF | **1** | **1** | **1** | **1** | 1 | 1 |
| | M, NF | **1** | **1** | **1** | **1** | 0 | 1 |
| | NM, NF | **1** | **1** | **0** | **0** | 0 | 0 |

Figure 5 shows the circadian sum of all four tanks on each day for the three times of observation. At 8:00 am, fish in the light fed group were the most active (see Figure 5a, mean =9.44), and the no marbles, not fed fish were the least active (mean =2.22). Figure 5b shows that the light fed group was still the most active (mean =9.00), and fish

in all three of the other treatments had decreased their activity at 5:00 pm (mean dark fed =4.89, mean marbles, not fed =4.11, and mean no marbles, not fed =1.44). Finally, Figure 5c shows that the dark fed tank was the most active at 10:00 pm (mean=3.33), and the other groups were not as active, if at all (mean light fed =2.22, mean marbles, not fed =0.889, and mean no marbles, not fed =0.222).



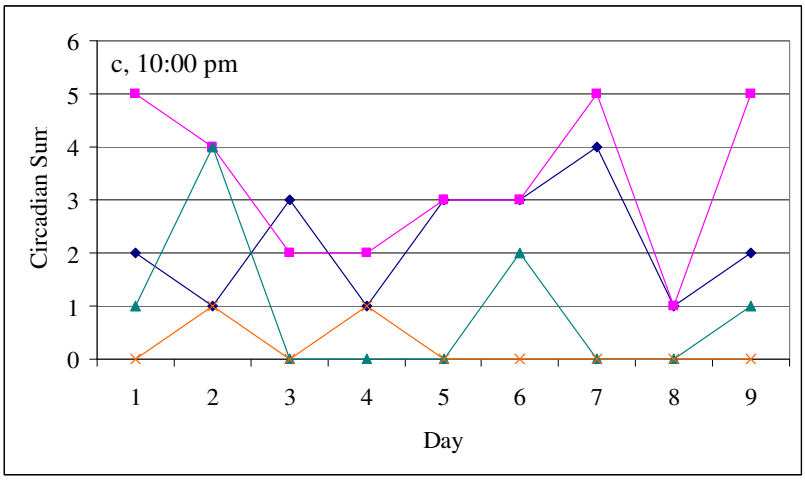




**Figure 5.** (a) Circadian Sum of the four tanks at 8:00 am. (b) Circadian Sum of the four tanks at 5:00 pm. (c) Circadian Sum of the four tanks at 10:00 pm.

# Chapter IV

## DISCUSSION

The hypothesis that fish fed during daylight hours would exhibit greater activity compared to fish fed at night was supported. However, the hypothesis that those fed at night would have unaltered behaviors was not supported, as dark fed fish actually had significantly increased circadian-dependent behaviors. This unexpected result is shown in Figure 6, and a hypothetical melatonin response is proposed. The response of the dark fed group is anticipated to be due to a decreased melatonin concentration, as a result of feeding during dark hours. The unfed group exhibited expected behaviors: low activity during the dark hours and high, normal activity during the light hours. Finally, the hypothesis that fish not fed would exhibit different behaviors from those that were fed was supported. Thus, researchers using *Danio rerio* are justified in the practice of not feeding during experiments.

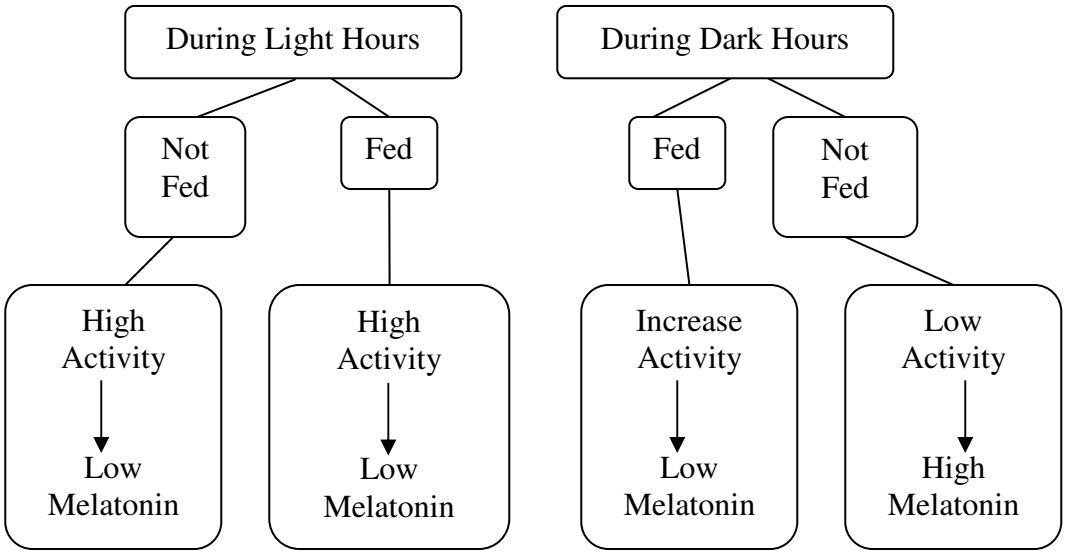



**Figure 6.** Adjusted, new hypothesized response of zebrafish to feeding during both the light and dark hours.

The unfed group with no marbles had access to all the eggs produced by the reproductively active females; therefore, they were able to consume and recycle their eggs. This group was the most successful, with the least number of deaths and the highest circadian sum at 8:00 am and 5:00 pm. The unfed group with marbles had no access to their eggs, and the level of foraging with this group was remarkably low. In each behavior studied, the group with the highest values was the group that was fed two times during the day. All groups exhibited lower activity during the dark hours than during the light hours. This is probably a result of melatonin production, which agrees with results found by Kazimi and Cahill in that with darkness, melatonin concentration increases (1999). The group that was fed during the dark hours showed a decrease in activity during the dark hours, but this group had the highest circadian sum at 10:00 pm. This indicates that this group was the most active group at 10:00 pm.

The two unfed groups showed low levels of aggression throughout the experiment. This can be attributed to energy preservation, as food was scarce. In relation to swimming intensity, the no marbles, unfed group swam the most during the morning hours because that is when the unfertilized, oviposited eggs were eaten to recycle nutrients (and preserve energy).

The circadian sum of fish in the dark fed tank was the highest at 10:00 pm. Melatonin production is stimulated by darkness and causes activity to decrease. In fact, sleeping posture could be observed in all four tanks a certain points. Zhdanova *et al.* found that the sleeping posture and resting state of zebrafish are much like the sleeping state seen in mammals (2001). However, feeding the dark fed fish, which normally would have similar behavior patterns to the light fed fish, increased in the observed behaviors during the dark hours. To explain this, it is further hypothesized that the response to feeding during the dark hours increased the activity and decreased the melatonin level. In a study by Tavartkiladze *et al.*, it was found that high calorie food decreased melatonin synthesis and secretion in white rats (2006). The zebrafish were fed frozen brine shrimp eggs, a high calorie food.

In future studies, the hypothesis that increased activity patterns in dark-fed fish is due to depressed melatonin concentrations should be tested by measuring melatonin in fish fed at different times of the day. Doing this would further elucidate the effects of feeding on zebrafish circadian-dependent behaviors.

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