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Finding a Link Between Circadian Rhythms and the Immune System of Captive Zebra Finches (Taeniopygia Guttata)

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FINDING A LINK BETWEEN CIRCADIAN RHYTHMS AND THE IMMUNE SYSTEM OF CAPTIVE ZEBRA FINCHES(*TAENIOPYGIA GUTTATA*)

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By

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I dedicate this thesis to my parents, Robert and Janice Bishop, who have always encouraged me to do my best and never give up. Also, I dedicate this work to Haley Reed and Matt Duckworth, who helped me through many sleepless nights and frustrating days.

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ABSTRACT

Circadian rhythms are commonplace in organisms and are normally controlled by a master clock. More recent evidence suggests that autonomous clocks operate in various systems in the body, including the immune system. This study looks for such a connection between the circadian rhythm and the immune system. In this study, captive zebra finches (*Taeniopygia guttata)* were exposed to different light and dark cycles and blood samples were taken every six hours. Birds were exposed to 12 hours of light and then 12 hours of darkness (LD), 24 hours of darkness (DD), or 48 hours of DD. After collecting whole blood, RNA was isolated and then RT-PCR was utilized to assess the expression of cytokine genes. It was predicted that cytokine gene expression exhibited rhythmicity in birds exposed to both LD and DD conditions. Alternatively, a lack of a rhythm in DD would suggest that activity of leukocytes was not controlled by a circadian clock. The RT-PCR data analysis of IL-1, IL-6 and IL-10 gene expression showed no significant sustained rhythm over the tested light-dark cycles. This suggests that the expression of these genes was not regulated in a circadian fashion in avian blood. This research has important implications for assessing biological rhythms in immunity and the effectiveness of different drug regimens depending upon the time of day they are administered in birds.

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Contents

Acknowledgements.....iv

Abstract.....v

Vita.....vi

List of Figures.....viii

List of Tables.....ix

Introduction.....1

Material and Methods.....6

Results.....8

Discussion.....11

References.....14

Appendix.....16

List of Figures

Figure 1.....15
Figure 2.....15
Figure 3.....8
Figure 4.....8
Figure 5.....9
Figure 6.....9
Figure 7.....9
Figure 8.....9
Figure 9.....10
Figure 10.....10
Figure 11.....10
Figure 12.....10
Figure 13.....11
Figure 14.....11

List of Tables

Table 1...13

Introduction

Circadian Rhythms

The earth rotates on an axis; this rotation changes which parts of the world are facing the sun, causing a cycle of day and night also known as light and darkness (1). This natural cycle of light has lead to the evolution of systems in all sorts of creatures from single-celled bacteria to complex organisms like humans (1,2). The 24-hour daily rhythm produced in response to these light changes is called a circadian rhythm. The name originates from the Latin phrase *circa diem* meaning about a day (1,2,3,4). This rhythm gives the organism the ability to adapt and predict what might happen, allowing homeostasis to be maintained (3). This cycle not only accounts for light-dark cycles but also predicts when food will be available, when temperature will change, and when risk of exposure to predators or infection will occur throughout the daily cycle(5). These cycles affect the individual by controlling sleep-wake cycles, hormone release, and the expression of certain genes (2,4).

Like other systems and cycles of nature, there are specific criteria for a rhythm to be classified as circadian . The first criteria is that the cycle is self-sustaining. This means that it continues to exist even when external time cues are removed; external cues include factors such as the light-dark cycle in a laboratory setting, or cycles in temperature or food. When theses external cues are experimentally removed the rhythm will begin to free-run. If the light or dark is left on for an extended period, theoretically the rhythm would shift slightly drifting, this is known as free-running. This test is needed so that a circadian rhythm can be distinguished from one that is only present due to the external cyclic changes. The question of whether the cycle is being driven by other external cues or not can be refuted when the cycle persists close to the

original 24 hour cycle but shows a slight shift (1). This imprecision in the free-run is evidence that no other factors are acting on the cycle, because if other factors were present then, the shift would not exist. More concisely, the shift shows that the cycle is self-sustaining. The second criteria for a rhythm to be classified as circadian is that the rhythm can be entrained. This means that if exposed to an external cue the rhythm would accommodate the cue and shift as shown in Figure 1 in the appendix. A third characteristic is that the rhythms are ubiquitous in nature, meaning that the same rhythms with similar peaks appear in many different species (1). The most recent discovery is that circadian rhythms have a cellular origin, even in complex organisms that have a central time-keeping structure in their brain (1,3,5). This means that while there is a central control in higher organisms, different systems such as the immune and endocrine systems can produce their own cycles.

Immune System

The immune system can be broken down into two branches: the innate and adaptive systems. The innate immune system is an organism's first line of defense, which includes the skin and mucosal membranes. These act as physical barriers. It also includes cellular components such as natural killer-cells, macrophages, neutrophils, eosinophils, basophils, and mast cells that use pattern recognition receptors to detect and rid the body of foreign substances on a non-specific basis (5). The adaptive immune system is made up of T and B lymphocytes that recognize specific parts of foreign substances. These cells are capable of recognizing specific antigens as well as producing memory cells which provide longer lasting immunity that can respond to future invasions by the same foreign substances (5,6). Since the immune system passes through the entire body, it is necessary for the different parts and cells to communicate

with one another (6). One way in which the immune system does this is through the use of cytokines (3).

Cytokines

As mentioned previously cytokines are used by the immune system to communicate with itself as well as with other parts of the body (3). Cytokines are small proteins that are secreted and are classified based on what type of cell produces them, their activity, or where they act (7). The different classifications include: lymphokines which are produced by lymphocytes, monokines produced by monocytes, chemokines which have chemotactic activities, and interleukins which are made my leukocytes and act on other leukocytes (7). Cytokines function for communication as well; when one cell begins secreting a cytokine and other cells detect it, they begin producing their own cytokines, creating a cascade effect. While some cytokines increase activity, others have the ability to suppress activity. Since cytokines are produced by many different cells and have various influences on receptors, different types of cytokines have different functions which can range from pro-inflammatory properties to anti-viral properties (7,8).

Avian Circadian Rhythm

As previously mentioned many organisms follow a 24 hour cycle of light and darkness. This includes the avian species who like humans are diurnal, meaning that they are awake and active during the day and sleep at night. The exception to the pattern is during migration when birds will sleep during the day and travel at night to avoid predators (9). The most obvious cue for this daily cycle is the rising and falling of the sun since it can be observed. The external cues however are not alone in producing this pattern, internal systems and molecular changes account

for the observed behavior. The external light cues begin to work within the avian circadian organization through photoreceptors that register the changing light cues. Unlike mammals where the majority of the photopigments are found in the eyes, birds also have functional photopigments in the brain that are critical to the circadian rhythm. These photopigments lie within the pineal gland, preoptic area, lateral septum, and tuberal hypothalamus (9). The suprachiasmatic nuclei (SCN) in the brain and pineal gland work to create the observed circadian rhythm (10). There are two structures to the avian SCN the medial (mSCN) and visual (vSCN) structures each having its own function in the expression of circadian associated genes (11,12,13,14). Similar to mammals, birds express certain circadian genes that are collectively known as the clock genes because their expression is known to directly cause rhythmic patterns in the expression of other genes, biochemical and physiological processes, and behavioral expressions (15,16) These genes include the positive elements, *clock* and *baml1*, the negative elements, *Period 1* (*per 1*, not expressed in birds), *Period 2* (*per 2*), *Period 3* (*per 3*), and the cryptochromes, *cryptochrome 1* (*cry 1*) and *cryptochrome 2* (*cry 2*) (17,18,19). *Clock* and *baml1* are positive elements because they work together and promote the expression of the negative elements. The negative elements are grouped together because together they cause a repression in expression of the positive elements.

While the eyes and other photoreceptive parts of the brain are important to circadian control the pineal gland is the master pacemaker of the system. Gaston and Menaker showed this best when they removed the pineal gland from house sparrow that then lost the ability to sustain a rhythm in constant dark conditions(10). This showed that the pineal gland is necessary to self-sustain the circadian rhythmicity. Another study done by Zimmerman and Menaker showed that pineal glands transplantation from house sparrows with entrained circadian rhythms to those

whom had their pineal gland removed restored the circadian rhythm (20). This experiment also showed that a secretion of the pineal gland affected behavior since re-innervation does not occur after only one day. This secretion was the hormone idoleamine melatonin that had been studied by Lerner, Axelrod, Klein in their experiments to find the biochemical basis for melatonin biosynthesis in the pineal gland (21). It has also been found that chick, *Gallus gallus*, pineal glands show rhythmic expression of clock genes consistent with the circadian patterns of clock gene expression in *Drosophila* and mice (22,23,24). Studies have even been conducted that how that the pineal gland of chicks expresses transcripts associated with cytokine biosynthesis, immune function, and lymphopoesis rhythmically.

Experiment

This study examines whether the immune systems of captive zebra finches vary on a circadian basis by specifically measuring cytokine gene expression. Zebra finches were chosen for this study because they are easy to keep and breed in captivity, as shown by their status as a common bird sold at pet stores. Birds were chosen for the study since unlike mammals, birds' red blood cells are nucleated. This means that it is possible that cytokines could not only be secreted by circulating white blood cells but also by circulation red blood cells. The collection of serial blood samples within individuals allows the rhythm of individual birds to be tracked. This experiment examines the expression of three different cytokines of the immune system: interleukin-1 (IL-1) and interleukin-6 (IL-6) which are pro-inflammatory and interleukin-10 (IL-10) which is an anti-inflammatory cytokine. If the expression of these cytokines follows a circadian rhythm then it should follow a cycle of peaks and valleys during light-dark and constant dark conditions. The constant dark conditions allows for the system to free-run since no external environmental cues are present. Given the previous research that has documented

circadian rhythms in cytokine variation in murine models and humans, it was predicted that birds would exhibit similar circadian rhythms in cytokine gene expression.

Materials and Methods

Animals and Experimental Design

Zebra finches were maintained in an indoor flight aviary at 22±1C^{o} with seed and water constantly available. The colony is normally maintained on a 12L:12D light cycle with lights on at 7am. Four adult male and four adult female birds were separated from the flock and placed in individual cages in the same room to minimize their stress when blood was being sampled (Figure 2). Light exposure was controlled at three different settings: 12 hours of light and 12 hours of dark(12L:12D), 24 hours of dark(DD), and 48 hours of DD. During the dark hours, a dim blue light was present. This blue light does not affect the brain waves and/or circadian rhythms of birds, including zebra finches, since the wavelengths cannot penetrate the skull. A timing system was set in place to automatically turn the lights on at 7a.m. and off at 7p.m. This system was manually turned off for the constant dark conditions. Birds were taken from the flock 24 hours before the experimental period for acclimation to the new environment and a week was given between experimental cycles with the exception of the DD time period where they were in the individual cages for an additional 24 hours of constant dark conditions before samples were taken. Blood samples of one capillary tube were taken from the wing vein of each bird and stored in 1.0mL of Tri-reagent at -20C^{o}. The samples were taken every six hours beginning at 1a.m. until 7p.m. over a 24 hour period.

RNA Extraction and cDNA Synthesis

RNA was extracted from each of the blood samples using a modified Qiagen RNeasy mini kit quick-start protocol. The procedure had to be modified since normally the kit is used for homogenized tissue samples. The modification included the addition of 0.1mL of 1-bromo-3-cloropropane per 1.0mL of Tri-reagent used, shaking the sample for 15 seconds then a 15 minute rest period, centrifugation for 15 minutes at 12xg between 2Cº and 8Cº. The colorless layer was then collected. The remaining protocol followed the manufacturer's instructions starting with the addition of 350µL of RLT buffer until the end. The samples were then analyzed spretrophotometrically to check purity of the RNA sample as well as concentration(NanoDrop 2000). Each sample of RNA was diluted to the same concentration and then converted to cDNA using a Thermo Scientific High Capacity cDNA synthesis kit. The 10µL master mix was made of 2µL 10x RT buffer, .8µL 25x DNTP, 2µL 10x random RT primers, 1µL reverse transcriptase, and 4.2µL of nuclease free water. The 10µL of master mix was then combined with 10µL of RNA to make a 20µL solution. The solution was then run in an Eppendorf Master cycle pro set to a protocol of 25ºC for 10 minutes, 37ºC for 120 minutes, and 85ºC for 5 minutes. The samples were then collected and stored at -20ºC.

RT-PCR and analysis

RT-PCR was run using a custom primer/probes specifically designed for zebra finch: (1) the endogenous control PPIA (peptidylprolyl isomerase A), and IL-10, IL-6 and IL-1, Taqman Gene Expression RT-PCR master mix using an Applied Biosystem 7300 Machine was used. The gene sequences and identifying information are provided in Table 1 of the appendix. The amplification protocol was as follows: 50ºC for 2 minutes, 95ºC for 10 minutes, 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. The reactions were carried out using the manufacturer's protocol with the exception that the sample volume was cut in half from 50µL to

25µL. A standard curve was created using cDNA of a liver from a Zebra Finch injected with bacterial lipopolysaccharide (LPS) acute injection of 2mg/kg of body weight and euthanized 2 hours later. The standard curve was a dilution serially (1:1, 1:10, 1:100, 1:1,000, 1:10,000) and run with each plate. The samples were run in duplicate and the C_t scores were averaged. The relative expression rates at each collection time were averaged and then used to create a plot of gene expression over the complete 72 hour testing cycle. Error was assessed using the standard error associated with the 8 samples for a specific gene and collection time. Statistical analysis using one-way repeated-measure ANOVA was used to detect a significant cycle. Significance was designated as a P value less than 0.05.

Results

IL-1

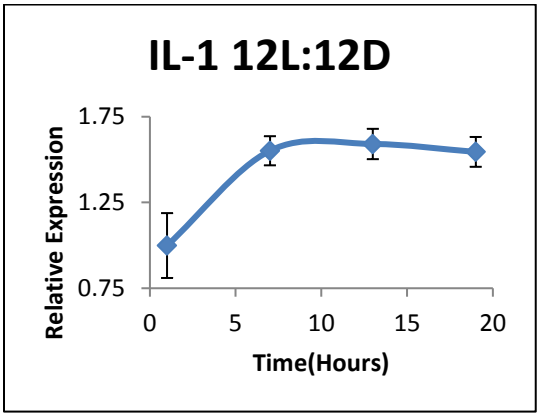

Figure 3. Graphical representation of relative expression of IL-1 at sample collection times during 12L:12D cycle.

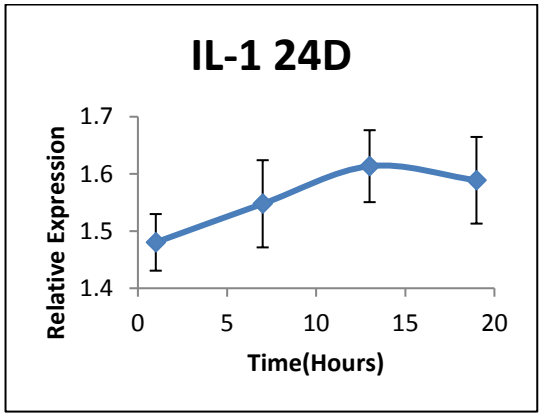

Figure 4. Graphical representation of relative expression of IL-1 at sample collection times during 24D cycle.

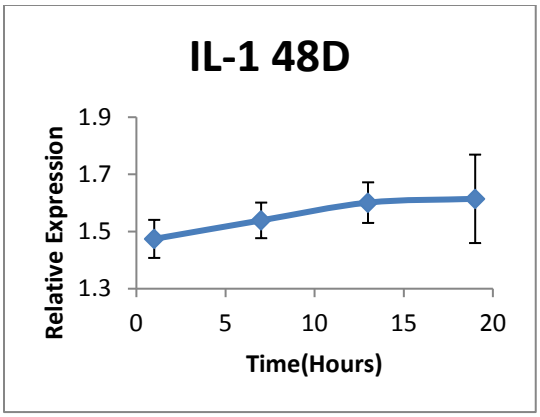

Figure 5. Graphical representation of relative expression of IL-1 at sample collection times during 48D cycle.

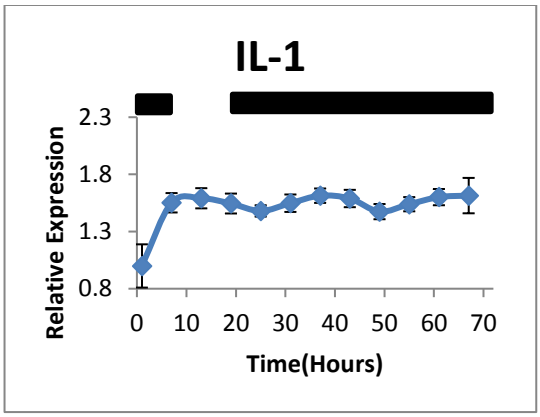

Figure 6. Graphical representation of relative expression of IL-1 at sample collection times combined over 72 hour period.

IL-1 shows a significant cycle over the 12L:12D but no significant cycle is shown over the other time periods(12L:12D $F_{3,21}$=5.223, P=0.0075, 24D $F_{3,21}$=2.098, P=0.131, and 48D $F_{3,21}$=1.367, P=0.260).

IL-6

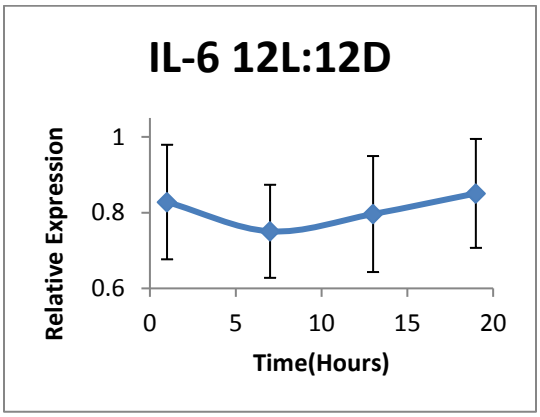

Figure 7. Graphical representation of relative expression of IL-6 at sample collection times in the 12L:12D cycle.

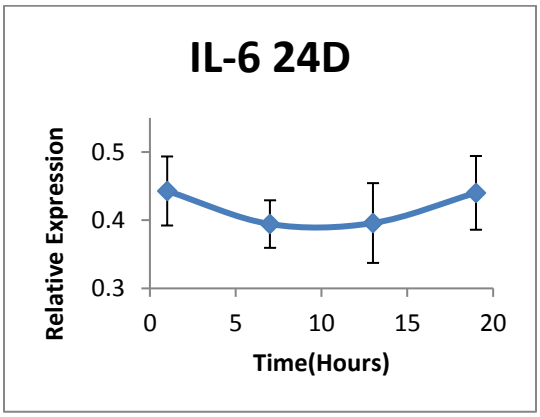

Figure 8. Graphical representation of relative expression of IL-6 at sample collection times during 24D cycle.

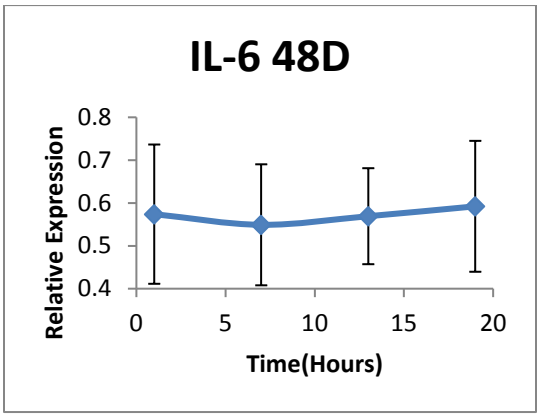

Figure 9. Graphical representation of relative expression of IL-6 at sample collection times during 48D cycle.

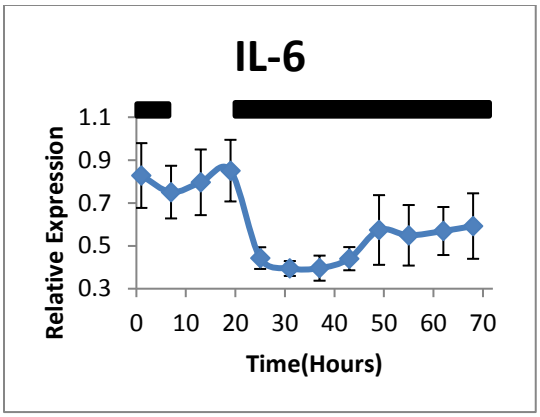

Figure 10. Graphical representation of relative expression of IL-6 at sample collection times combined over the 72 hour period.

IL-6 expression was detected to have no significant cycle in whole blood (12L:12D $F_{3,21}$=0.307, P=0.82, 24D $F_{3,21}$=0.638, P=0.599, and 48D $F_{3,21}$=0.142, P=0.934).

IL-10

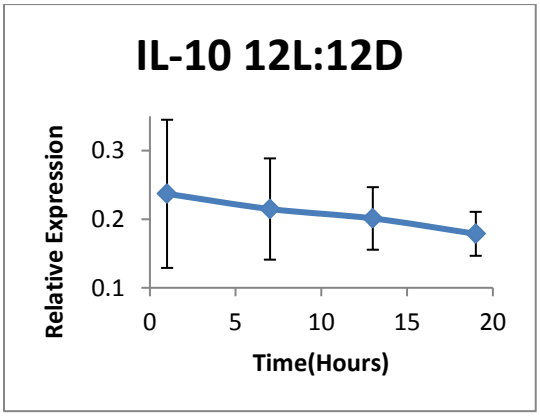

Figure 11. Graphical representation of relative expression of IL-10 at sample collection times during 12L:12D cycle.

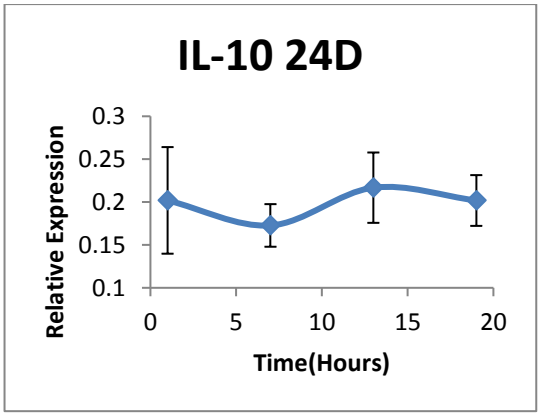

Figure 12. Graphical representation of relative expression of IL-10 at sample collection times during 24D cycle.

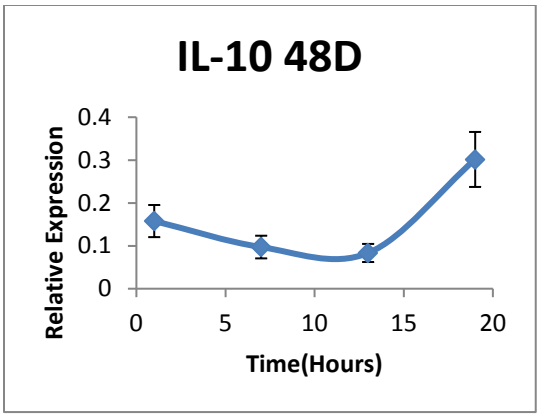

Figure 13. Graphical representation of relative expression of IL-10 at sample collection times during 48D cycle.

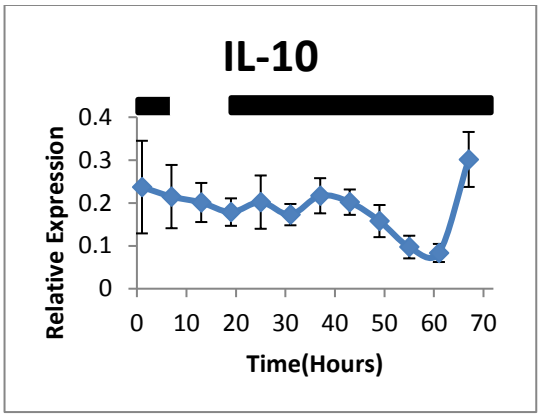

Figure 14. Graphical representation of relative expression of IL-10 at sample collection times combined over 72 hour period.

Relative expression of IL-10 showed no significant cycle until the 48D period(12L:12D $F_{3,21}$=0.311, P=0.934, 24D $F_{3,21}$=0.222, P=0.880, and 48D $F_{3,21}$= 6.739, P=0.0023).

Discussion

My hypothesis was that pro-inflammatory IL-1 and IL-6 and anti-inflammatory IL-10 cytokine gene expression would exhibit a circadian rhythm in whole blood samples. This would be supported by each of the genes being expressed in a rhythm in the 12L:12D cycle as well as in the 24D and 48D cycles. The results failed to support my original hypothesis.

The RT-PCR data collected from whole blood samples and examined for IL-1 showed a significant rhythm in the 12L:12D cycle but the rhythm did not continue when in constant dark conditions. Figure 6 shows the graphical representation of the data collected and it can be observed on the graph that the error associated with each data point does not overlap with other data points along with the ANOVA analysis giving a P value of 0.0075 shows the present rhythm. The overlap of the error bars in Figures 3-6 and the P values of 0.131 and 0.260

respectively show no significant rhythm. Although a rhythm is present in the 12L:12D cycle expression of IL-1 cannot be said to have a circadian rhythm because it is not self-sustaining(1,3). Alternatively, it is possible that the actual light and darkness of the room is having a masking effect upon IL-1 levels such that variation occurs in the 12L:12D, but not in constant darkness.

RT-PCR data showed that under all the conditions tested IL-6 showed no significant rhythm. This means that the expression of IL-6 in not controlled by a circadian rhythm. If the data in Figures 7-10 were to be examined without taking into account the error bars then a rhythm would appear in all three time cycles with a significant decrease in expression after the first 24 hours. The error bars and ANOVA statistical analysis allow the variances between the birds to be considered rather than just looking at the averages. The standard error shown in Figures 2-5 allows the overlap of the points to be seen that combined with a P value of 0.82, 0.599, and 0.934 respectively leads to the conclusion that the differences between data points are not significant enough for a true rhythm to be present.

IL-10 shows strange results in that the RT-PCR data showed that no significant rhythm was present in the 12L:12D and 24D cycles but showed a significant rhythm in the 48D cycle. It can be seen from Figures 11 and 12 that the error bars for points overlap with other points causing no rhythm to be present. This holds true with the ANOVA analysis that gave a P value of 0.934 and 0.880 respectively. The 48D cycle has a P value of 0.0023 and the error bars do not overlap in Figure 13. This cannot be a circadian rhythm though because the rhythm does not appear during the 12L:12D cycle and is not sustained meaning it does not comply with the rules given to define a circadian rhythm(1,3). The appearance of a rhythm at the 48D cycle means that the expression on IL-10 is most likely controlled by a factor other than light-dark cues, this

factor could be food or behavior related but further studies would need to be conducted to determine this.

I predicted that the cytokine gene expression in avian whole blood would follow a circadian rhythm. This was not shown however since IL-6, Il-10 and IL-1 did not sustain a rhythm throughout the 12L:12D, 24D and 48D cycles. IL-1 showed light dependent cues since a significant rhythm was detected during the 12L:12D cycle, then lost when the rhythm was left to free run in the constant dark conditions. Expression of IL-10 showed no rhythm until the 48D period leading to the conclusion that it is synchronized with some other factor. One such factor is food since during the 48D period the bird had been in the cage a total of 72 hours instead of 48 hours with the previous cycle. This extended period could have allowed the bird to become more comfortable in its new environment and resume regular eating habits. An experiment could be done where there is a longer acclimation period and food intake is monitored both in the original aviary as well as in the individual cage to test for the food depend expression of IL-10.

The study of avian immune systems and their regulation factors is important to humans since many avian species are used as a food source and source of income (25). Many Americans consume chicken and turkey products leading to a demand on chicken and turkey farms. The health of these birds is important for those raising them so that demand can be met but also to the consumer. Had this study shown significant results its implications could have been used towards the administration of an anti-biotic in the case of infection. The timing of administration could have been adjusted so that it coincided with the natural rhythm of the immune system to allow for boosts when the immune system was at its weakest point. This would presumably help the bird overcome the infection sooner and lead to a healthier product for consumers since the use would be minimalized.

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Appendix

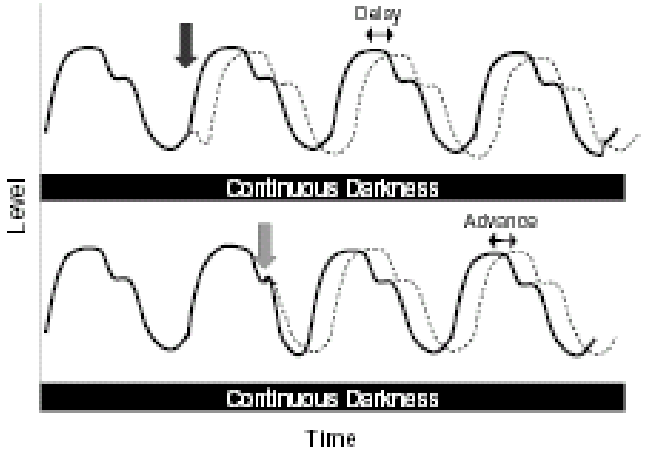

Figure 1. The dark line in the above figure shows the normal circadian rhythm. The dotted line of the first graph shows the shift caused by constant dark conditions. The dotted line in the second graph shows the entrained rhythm caused by a pulse of light during the constant darkness

Figure2. Picture of a Zebra Finch in a separate cage from flock

Table 1

Gene	Name	ID	Sequence
PPIA	NM_001245462.1	AIRSBD1	ACGGTTCNCAGTTCTTCATCTGCACTGCCAAGACTGAGTGGCTGGATGGC
IL-10	XM_002194605.1	ASI09J9	CTGCTGGAGGAAATCAAGGGCAGGCTCGGCTGCCAGTCGGTGTCGGAGT
IL-6	XM_012571854.1	AIT97QH	GTACCATAAGACAGATGGTGATCAATCCCGAAGAAGTGATCATTCCAGAT
IL-1	XM_002195564.3	AIVI5WP	TTCTTGGATGATATTTTCGAGCCCGTCTCCTTCCGGTGCATCAGAGGCAG