EFFECTS OF PINEALECTOMY ON METABOLIC ACTIVITY AND CLOCK GENE EXPRESSION IN *Passer domesticus*

A Senior Scholars Thesis

by

RYAN FRANKLIN MCCORMICK

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2008

Major: Biology

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Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

Effects of Pinealectomy on Metabolic Activity and Clock Gene Expression in *Passer Domesticus* (April 2008)

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The biological clock and the biological rhythms it controls are anticipatory mechanisms found ubiquitously across a wide variety of taxa, ranging from prokaryotes to eukaryotes, from bacteria to animals. Avian species, especially passerine species, rely heavily on melatonin input from the pineal gland, and this experiment further explores the role the pineal gland plays in the organization of the house sparrow clock. The uptake of ^{14}C-labeled 2-deoxyglucose and clock gene transcription were observed in pinealectomized house sparrow brain and peripheral tissue to discern more about the role of the pineal gland in the neuroendocrine loop and its control over peripheral oscillators. Additionally, preliminary *in vitro* studies in chick astrocytes suggest two differentially regulated oscillatory mechanisms at the cellular level: a metabolic clock that rapidly responds to melatonin and a transcriptional clock that likely responds to the entrainment of the metabolic clock. Here we examine the metabolic and transcriptional impacts of pinealectomy in *Passer domesticus* to further explore the mechanics of the neuroendocrine loop in the brain, examine how pinealectomy impacts peripheral

oscillators, and determine if cells are indeed controlled by two coupled oscillatory mechanisms. To this end, we utilized activity-monitoring equipment to measure locomotor activity; 2DG uptake was measured via autoradiography and scintillation counts in the brain and peripheral tissues, respectively; and clock gene transcription was measured via *in situ* hybridization and real time q-PCR in the brain and peripheral tissues, respectively. We hypothesize that, due to the coupled dual cellular oscillators and the lag between the metabolic and transcriptional clock, rhythms of 2DG uptake in peripheral tissue will damp prior to the transcriptional rhythm of the clock genes *Cry1* and *Per2*. Lastly, the SCN's metabolic and transcriptional oscillations will damp, without the pineal gland coupled to it. The results obtained thus far imply that rhythmic 2DG uptake in peripheral tissues of pinealectomized sparrows damps by day 3 in constant darkness, prior to locomotor arrhythmia, and that transcriptional rhythmicity damps in unison with locomotor rhythmicity by day 10. The data to date support the concept of two differentially regulated oscillators at the cellular level, at least within the heart.

DEDICATION

I dedicate this thesis to my mother, my father, and my brother. Without them and their continued support, I would not be where I am today.

ACKNOWLEDGMENTS

I would like to acknowledge all those who put time and effort into helping me pursue this project. I thank Dr. Vincent Cassone for having the patience to put up with a bumbling undergraduate and for entrusting such an important project to my hands. This also could not have been done without the advice of Jiffin Paulose and Vikram Shende, both of which patiently answered any number of questions I had concerning procedures and concepts. Jiffin Paulose also took considerable time out of his work to discuss this project, attend presentations, help with procedures, and generally ensure I was traveling in the right direction. I thank Dr. Paul Bartell for teaching me, albeit indirectly, a valuable lesson about the importance of clear labeling and organization so that those who come after you might continue your work. I would also like to acknowledge Barbera Earnest for never ceasing to be full of energy and always willing to go far out of her way to assist me in my work, and Steve Karagenis for being a voice of experience. The last lab member I would like to thank is Phillip Pippin, a fellow undergraduate whose effort helped further push this project along.

I also give thanks to the Undergraduate Biology and Mathematics program for assistance in funding this work, as well as the National Science Foundation for providing the grant that originally funded the experiment.

Additionally, I would like to thank my family for their continued support and encouragement even when I felt as though I could go no further.

Finally, I would like to thank all those science professors who taught me something practical that I actually used during my work on this project. Perhaps I actually learned something during my four years in college.

NOMENCLATURE

2DG	2-deoxy-[^{14}C]-glucose
CNS	Central Nervous System
Cry1	*Cryptochrome 1*
CT	Circadian Time
CypG	*Cyclophilin G*
DD	Constant Dark Conditions
LD	Light Dark Cycle
LL	Constant Light Conditions
Per2	*Period 2*
PINX	Treated with a Pinealectomy
RHT	Retinohypothalamic Tract
SCN	Suprachiasmatic Nucleus
mSCN	Medial Suprachiasmatic Nucleus
vSCN	Visual Suprachiasmatic Nucleus
SHAM	Treated with a Sham Pinealectomy
q-PCR	Quantitative-Polymerase Chain Reaction

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CHAPTER I

INTRODUCTION

In the slow and laborious march of evolution, biological clocks have been developed and maintained by nearly every organism as anticipatory mechanisms to predict the coming of period events, including the rising and setting of the sun, lunar cycles, and even the tides. Despite the enormous distance between taxa with biological clocks, incredible conservation of its primary mechanisms has been observed. In multi-cellular animals, control of overt rhythmicity is regulated hierarchically by a set of neural and neuroendocrine structures. Many studies have implicated the pineal gland as a primary player in the clock of the passerine species *Passer domesticus*, the house sparrow. Surgical removal shows that the pineal gland is required for self-sustained circadian rhythms of locomotor activity; pinealectomized sparrows display damped rhythms and arrhythmia when subjected to constant darkness (Gaston and Menaker, 1968). Yet rhythmic administration of the hormone melatonin, essentially the messenger produced by the pineal gland for communication with the rest of the body, to pinealectomized sparrows restores the locomotor patterns of activity abolished by pinealectomy (Cassone et al., 1992; Lu and Cassone, 1993b; Heigl and Gwinner, 1995). Additionally, transplantation of the pineal gland into pinealectomized, arrhythmic sparrows confers

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This thesis follows the style of The Journal of Neuroscience.

both the donor's rhythmicity and circadian phase (Zimmerman and Menaker, 1979). Explanted pineal glands contain the oscillators and photoreceptors sufficient to generate circadian rhythms of melatonin biosynthesis and entrain to light:dark regimes (Kasal et al., 1978; Binkley, 1979; Wainwright and Wainwright, 1979; Deguchi, 1979; Takahashi et al., 1980).

Interestingly, the mammalian pineal gland is neither photoreceptive nor contains circadian oscillators for rhythmic melatonin synthesis, but is considered by some a "slave oscillator" to the master pacemaker, known as the hypothalamic suprachiasmatic nucleus (SCN) (Klein et al., 1997). Analogous to the destruction of the pineal gland in avian species, destruction of the SCN or disruption of SCN neural pathways to the pineal gland in mammalian species abolishes circadian rhythms in melatonin biosynthesis. As such, pineal gland melatonin biosynthesis is a direct output of the mammalian circadian clock. Numerous studies have shown considerable evidence that the mammalian SCN is the master pacemaker and regulates overt circadian rhythms (Moore and Eichler, 1972; Stephan and Zucker, 1972; Warren et al., 1994; Ibuka et al., 1977; Pickard et al., 1987; Earnest et al., 1999; Green and Gillette, 1982; Shibata et al., 1982; Earnest and Sladek, 1987; Newman et al., 1992; Yoo et al., 2004).

It would be interesting if both the mammalian system and the avian system contained the pineal gland yet the avian system had abandoned the SCN structure. This does not seem to be the case as two structures in birds have been labeled the putative homologues of

the mammalian SCN: the medial suprachiasmatic nucleus (mSCN) (Brandstatter and Abraham, 2003) and the visual suprachiasmatic nucleus (vSCN) (Cassone and Moore, 1987). The mSCN has little similarity to the mammalian SCN but does express several clock genes rhythmically on a daily basis (Yasuo et al., 2002; 2003; Abraham et al., 2003), and lesions directed at the mSCN disrupt overt circadian rhythms (Takahashi and Menaker, 1982). Alternatively, the vSCN, unlike the avian mSCN but like the mammalian SCN, receives retinohypothalamic input, has a high concentration of GABA neurons, fibrous astrocytes, and contains neurons that express arginine vasotocin, vasoactive intestinal polypeptide, substance P, and neurotensin. It also receives NPY input from the visual thalamus and dense 5HT input from an unknown source (Cassone and Moore, 1987; Cantwell and Cassone, 2006). Of particular importance to this experiment, the vSCN expresses circadian rhythms in 2-deoxy-[^{14}C]-glucose (2DG) uptake *in vivo* (Cassone, 1988; Lu and Cassone, 1993 a,b; Cantwell and Cassone, 2002) and in sparrow clock gene expression (Abraham et al. 2002).

So it would seem that we have on our hands two conflicting themes; the mammalian SCN master output controls pineal gland output while the avian pineal gland plays a clear role as an independent oscillator and only one part of a complex system of neuroendocrine structures comprising the avian biological clock. For example, most brain structures become arrhythmic in their 2DG uptake following pinealectomy and placement in DD; the exception is the vSCN which persists for at least three days in rhythmicity, losing all 2DG rhythmicity by the 10th day when behavioral rhythmicity is

lost (Lu and Cassone, 1993a). Furthermore, rhythmic administration of melatonin will reestablish both daily activity rhythm and rhythmic 2DG uptake in the vSCN (Lu and Cassone, 1993b). Additionally, even though melatonin rhythms are generated indefinitely *in vivo*, circadian rhythms in melatonin damp after five to seven days *in vitro* (Takahashi et al., 1980; Takahashi and Menaker, 1982; Zatz et al., 1988). Sympathetic denervation of the pineal gland will reproduce this effect *in vivo* (Cassone and Menaker, 1983). Similar to mammals, sympathetic innervations of the avian pineal gland derives from a multi-synaptic pathway arising from the vSCN that releases norepinephrine on a circadian basis such that norepinephrine is released during the day and subjective day, inhibiting melatonin synthesis (Cassone et al., 1986; Cassone and Menaker, 1983; Klein et al., 1997; Zatz, 1991). Lesioning of the vSCN, but not the mSCN, will abolish the rhythm of norepinephrine turnover in the pineal gland (Cassone et al., 1990).

The neuroendocrine loop

In light of this information, a "neuroendocrine loop" model has been proposed for avian circadian organization (Cassone and Menaker, 1984; Cassone and Lu, 1994). Essentially, the model proposes that the system is composed of damped circadian oscillators residing within the vSCN and pineal gland that are not capable of self-sustained oscillation in the absence of photic input and/or neural/endocrine input from the rest of the system. Cassone describes the model sequentially: the vSCN is metabolically and electrically active during the subjective day, stimulating output pathways, including increasing sympathetic tone, which inhibits pineal gland output and

has broad effects throughout the body. Since the vSCN is an oscillator, it spontaneously wanes in its activity as the subjective day progresses, until at subjective dusk it is no longer active. This releases pineal oscillators from their inhibition, allowing pinealocytes to synthesize and release melatonin. Melatonin broadly affects a wide array of processes in tissues that express melatonin receptors, among which is the vSCN whose activity is inhibited by melatonin. The pineal gland too spontaneously wanes in its biosynthesis of melatonin, stopping by subjective dawn and releasing vSCN output from inhibition for the succeeding day. Both of these co-pacemakers are directly affected by light; the pineal gland contains photopigments and photostransduction systems that affect melatonin biosynthesis, and the vSCN receives input from the retinohypothalamic tract (RHT). Additionally, each of these co-pacemakers may independently affect downstream processes. The pineal gland influences central nervous system and peripheral sites via melatonin secretion during the night and tissues expressing melatonin receptors are directly affected by this pacemaker. Conversely, the SCN pacemaker (vSCN and mSCN) affect outputs by several pathways. A humoral output affects local hypothalamic function, at least in mammals (Silver et al., 1996; Allen et al,. 2001), and a neural output via SCN afferents affects CNS and peripheral sites to which they project. Among these is a global regulation of sympathetic tone (Cassone et al., 1990; Warren et al., 1994).

Biological clock organization

To better organize the above information, a brief overview of avian clock organization is displayed in Figure 1 and summed as follows: The pineal gland receives external photic input and synthesizes melatonin which in turn acts both as a messenger to communicate with peripheral oscillators and an inhibitor to the SCN at night. Once melatonin synthesis wanes and releases the SCN from inhibition, SCN activity increases, inhibiting the pineal gland via a norepinephrine output and impacting the central nervous system (CNS). Unlike the pineal gland, the SCN does not receive direct photic input, but rather receives input from the retina via the retinohypothalamic tract (RHT). The retina is also capable of responding to light input, or lack thereof, and synthesizes melatonin which performs the same functions as pineal melatonin. The temporally organized inhibition of the SCN and pineal gland by one another is the basis of the neuroendocrine loop model.

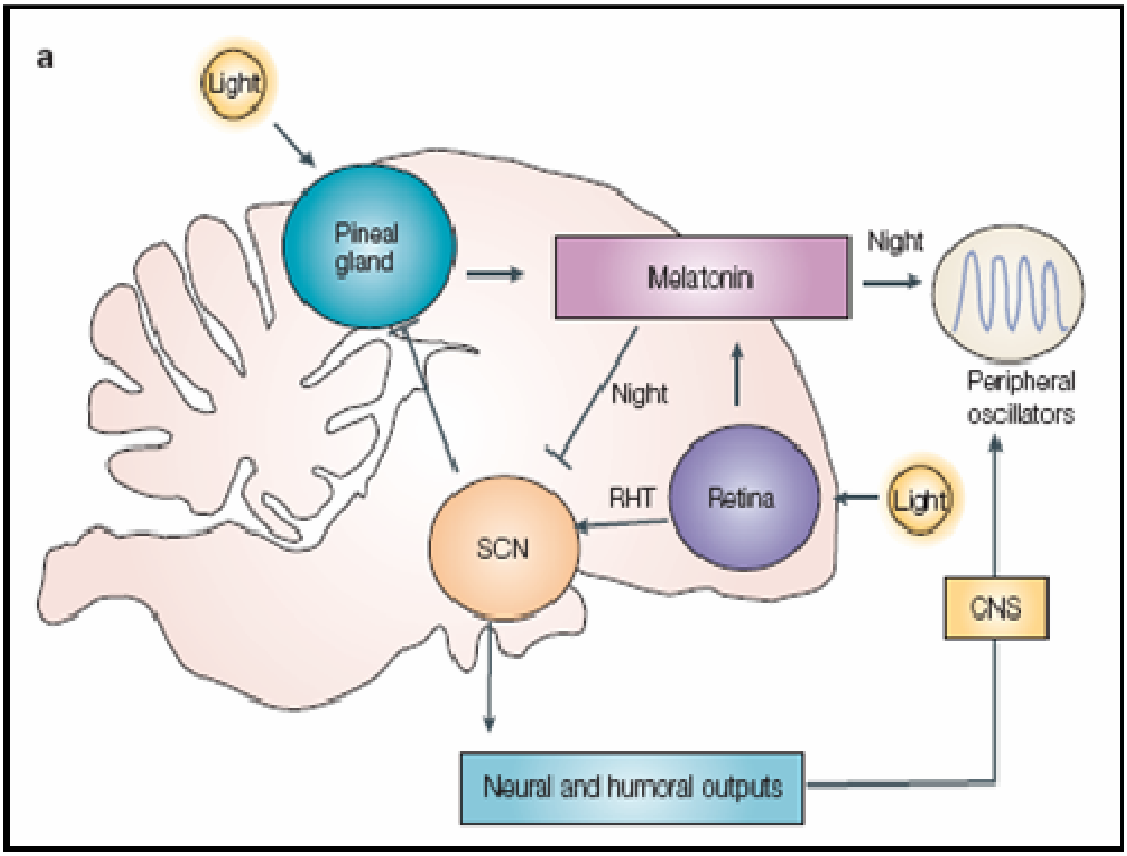

Figure 1. Avian clock organization. One current model for avian (and mammalian) clock organization is the neuroendocrine loop. In short, the SCN and pineal gland are coupled oscillators that both receive external input (light) and inhibit one another (Figure from Bel-Pedersen et al. 2005).

Metabolic and transcriptional oscillators

Little work has been done to show the metabolic and transcriptional effects of pinealectomy at the cellular level in peripheral tissue. However, when *in vitro* preparations of astrocytes derived from the diencephalon of E17 *Gallus domesticus* chicks are subjected to melatonin cycles, melatonin imposes rhythmic 2DG uptake in two rhythms in 180° anti-phase with melatonin administration, yielding 2 damped cycles of 2DG uptake after removal of melatonin. Since the cells expressed both

cryptochromes and opsins, the effects of LD cycles and anti-phase DL cycles were also examined. LD or DL had no effect on 2DG uptake, but they imposed a distinct rhythm of clock genes (Paulose et al., unpublished, 2008). As such, it would appear as though, at the cellular level, the clock is controlled via two different mechanisms. At the organismal level, the pineal gland and the SCN appear to coordinate to control peripheral clocks, but at the cellular level it would appear the metabolic clock is sensitive to melatonin while clock gene expression is coordinated by another method with input from the metabolic clock (Fig. 2). Although most cells within the body are probably not photoresponsive, the fact that astrocytes are organized via two different inputs supports the model that two coupled oscillators exist in the cell.

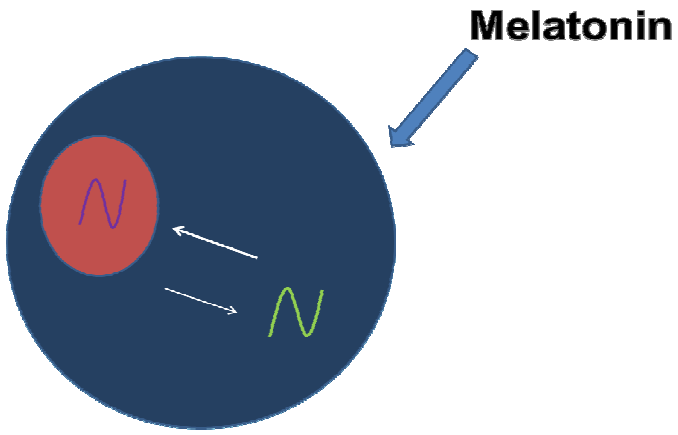

Figure 2. The dual cellular oscillators hypothesis. At the cellular level, the biological clock appears to be under the control of two coupled oscillators: a metabolic oscillator and a transcriptional oscillator. If this is true, the metabolic oscillator can react more rapidly to melatonin input due to its location to melatonin receptors. The transcriptional clock's response lags behind the metabolic clock response. Interestingly, photoresponsive chick astrocytes yield metabolic rhythms in response to melatonin, yet when subjected to light cycle conditions, a transcriptional rhythm is seen, but no metabolic rhythm was induced.

Thus, we propose the hypothesis that there must be at least two oscillatory mechanisms; one mechanism involves the rhythmic transcription of clock genes and their outputs and the other mechanism, independent of clock gene transcription, is sensitive to the hormone melatonin. The data clearly show that metabolic and clock gene rhythms are regulated differentially in cultured astrocytes.

This report will examine the effects of pinealectomy on the metabolic and transcriptional activity of brain structure and peripheral tissues, providing more information on how the two primary structures of the neuroendocrine loop, the SCN and the pineal gland, communicate, how oscillators in peripheral tissues respond to the melatonin provided by the pineal gland, and determining whether or not biological clocks at the cellular level are indeed controlled by a coupled dual cellular oscillators. Brain, heart, lung, liver, kidney, and skeletal muscle will be examined by measuring 2DG uptake and expression of Cry1 and Per2 at mid-subjective day (CT 6) and mid-subjective night (CT 18). Should two oscillatory mechanisms exist, the metabolic and transcriptional rhythms will not damp in unison.

CHAPTER II

MATERIALS AND METHODS

House sparrows were collected locally and maintained in an indoor quarantine aviary for two weeks. Following quarantine, sparrows were placed in cages equipped with infrared movement detectors that input into QA4A activity modules of the Minimitter Dataport 24 interface. The cages were placed in light-tight environmental chambers with continuous ventilation and clock-controlled lighting. All birds will be provided food and water *ad libitum*. Following a week of acclimation to the cages, 30 birds were anesthetized and surgically pinealectomized (PINX) as in Lu and Cassone (1993a,b), while 30 birds received a sham surgery (SHAM). Following recovery from surgery, the birds were returned to their cages, and locomotor activity was recorded for one week. After one week, all birds were placed in DD.

On day 1 (termed day 0 for all results) of DD, all birds were rhythmic, as has been shown repeatedly (Gaston and Menaker, 1968; Lu and Cassone, 1993a,b). At circadian time (CT) 6, the mid-point in the bird's activity, 5 PINX birds and 5 SHAM birds received intramuscular injections of 100μCi/kg 2-deoxy-[^{14}C]-glucose (2DG), and, after 60 minutes, they were sacrificed by CO_2 asphyxiation in the dark. Brains, retinae, liver, heart, kindey, lung and the pectoral muscle contralateral to the 2DG injection were removed and rapidly frozen in -40° isopentane. This procedure was repeated at CT 18, the middle of the inactivity phase.

On day 3 of DD, all SHAM birds were rhythmic, but CT 6 in the PINX birds had expanded. At CT6 and CT18, 5 SHAM and 5 PINX birds were injected with 2DG, sacrificed by CO_2 asphyxiation as above, and their tissues were processed as above. This procedure was repeated on day 10 of DD. By day 10 of DD, all SHAM birds were still robustly rhythmic, but the PINX birds were completely arrhythmic. At CT 6 and CT 18, 5 SHAM birds were injected with 2DG, sacrificed as above, and their tissues processed. The PINX birds were arrhythmic, so ascribing a phase of their activity was difficult. Fortunately, since sparrows damp gradually to arrythmicity, we were able to project at what time CT 6 and CT 18 might be. We injected 2DG and then sacrificed the remaining 20 birds at these times and processed their tissues accordingly.

Brains were transversely sectioned on a cryostat at 20µm through the preoptic and hypothalamic region in 3 bins, such that adjacent sections could be probed and analyzed for different signals, 2DG autoradiography, *pbmal1 in situ* hybridization and *pper2 in situ* hybridization. Although the sparrow vSCN is 800µm long, the mSCN is only 300µm long (Cassone and Moore, 1987). Therefore, it would have been difficult to also analyze adjacent sections for sense controls. We therefore sectioned through the pineal gland, which expresses these genes, and cut 5 bins: 2DG, antisense *pclock*, sense *pclock*, antisense *pper2* and sense *pper2*. The remaining forebrain (anterior of the preoptic area) and hindbrain (posterior of the pineal gland), the liver, heart, kidney, lung and pectoral muscle were homogenized (FastRNA Pro Green Kit, MP Biomedicals). An aliquot was removed to determine 2DG uptake in all tissues by processing the tissue homogenate in

scintillant and taking scintillation counts. This latter analysis is a measure of glycogen biosynthesis. The remaining homogenates were processed for clock gene expression via RNA isolation (RNeasy Kit, Qiagen), a reverse transcriptase reaction (Superscript First Strand Synthesis, Invitrogen), and real time q-PCR for mRNA transcripts of the genes *Per2* and *Cry1* using *cyclophilin G* (*CypG),* a constitutively expressed housekeeping gene, and *beta-actin*, a constitutively expressed structural gene, as endogenous control genes. At the time of the writing of this report, only the peripheral tissues had been analyzed in any depth. Only preliminary observations had been made on brain sections and their quality is suspect; they will not be reported here.

CHAPTER III

RESULTS

Behavioral

Considerable time was spent on the development of an effective data acquisition system for house sparrow locomotor activity. Previously, a perch-switch system had been utilized to monitor locomotor activity, but the resolution, accuracy, and utility of this system was suspect. As such, a new system was installed: new cages were built, infrared detectors were installed to monitor activity, new exhaust systems were installed, the entire system was re-wired and labeled, a back-up system was initiated, and data acquisition was also programmed to be duplicated and saved on a network server. As a result, we can show that this system is superior to a perch-switch system (Fig. 3).

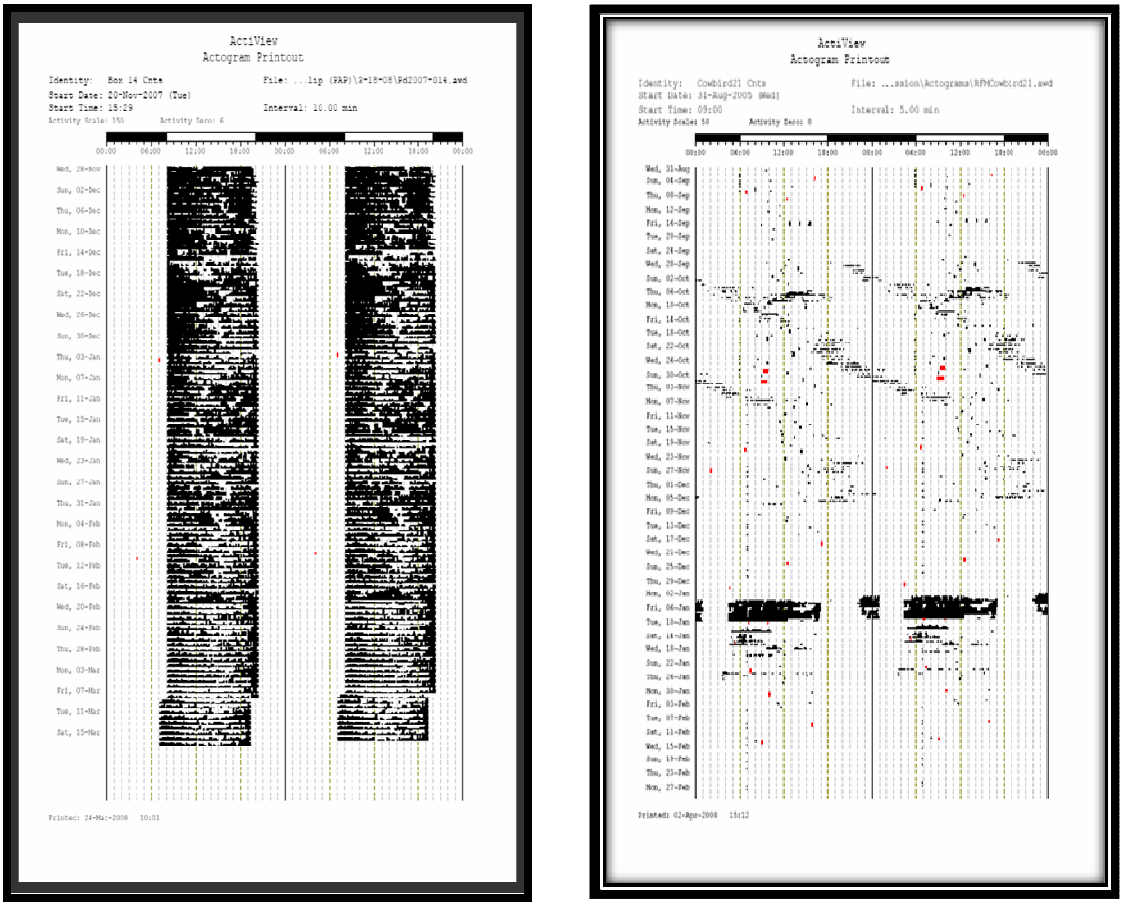

Figure 3. Comparison of locomotor activity data acquisition systems. The new infrared locomotor detection system is superior to the previous "perch-switch" locomotor detection system. As observed in the left actogram, less "noise" is produced and activity is monitored with greater resolution. The shift left is due to daylight savings time and is cleanly monitored by the infrared system. However, the actogram on the right is an example of an actogram generated by the "perch-switch" system before it was torn replaced. The right actogram shows some uncharacteristic patterns for a diurnal bird in a 12:12 LD cycle, and, although it is an extreme example of poor actograms generated by the old system, is one of the primary motivators for spending a considerable amount of time generating a new locomotor activity monitoring system.

In this experiment, locomotor behavior was used primarily to determine timing of arrhythmia and length of free running period. The free running period, tau, was used to

calculate CT 6 and CT 18, or middle of subjective day and subjective night respectively. Although calculating length of free running period in arrhythmic sparrows is considerably more difficult, sparrows damp gradually and the locomotor activity prior to complete damping gives a useful estimation. Figure 4 displays an actogram of a SHAM sparrow for 19 days: 13 days of a 12:12 LD cycle and the final 6 are under DD conditions.

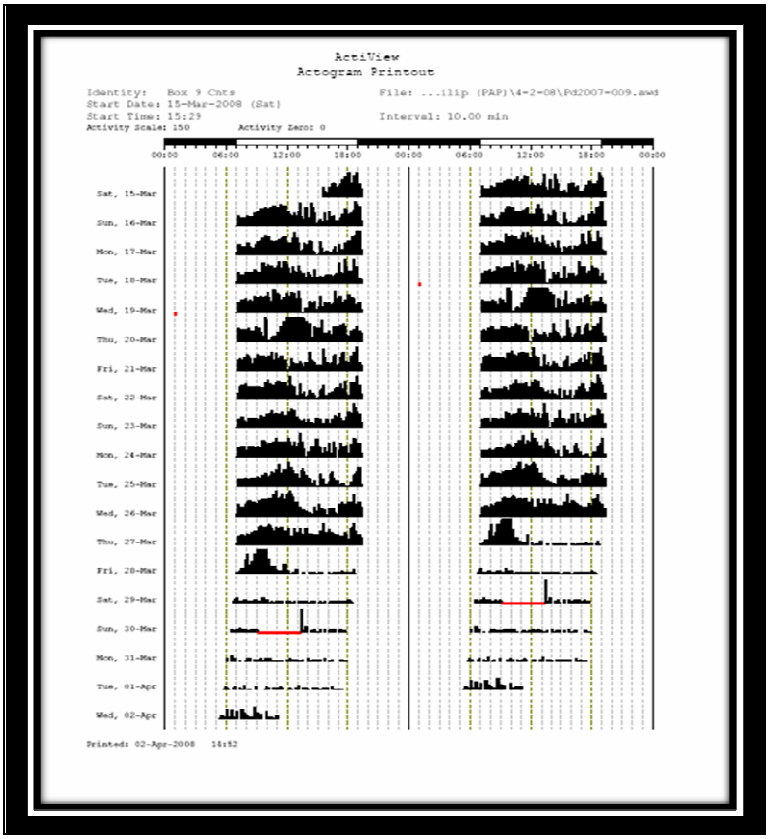

Figure 4. Free running periods observed on actograms were used to calculate tau. Both SHAM and PIXN sparrows display free-running locomotor behavior after exposure to constant darkness and display behavior like that seen here. The middle of their activity phase (CT 6) and inactivity phase (CT 18) were caclulated by determining the length of their free running period (tau) and using tau to predict CT 6 and CT 18 on the day of tissue harvesting.

Transcriptional

The transcriptional data presented here are among the first produced for the house sparrow. To investigate clock gene expression in peripheral tissues, it was first necessary to ensure that the endogenous control genes used for real time q-PCR analysis were not rhythmic in their expression. Three samples each with four tissues at CT 6 and four tissues at CT 18 with *Per2* primers were processed via real time q-PCR in parallel with one of two known housekeeping genes: *beta-actin* or *cyclophilin G*. Lack of rhythmicity in these endogenous control genes would be indicated by rhythmic *Per2* transcription, a clock gene known to be rhythmic in other organisms. If either *beta-actin* or *cyclophilin G* were rhythmic, then *Per2* expression would appear differentially rhythmic or arrhythmic upon comparison of relative transcript quantity for both control genes. Simultaneously, *Per2* could be identified as rhythmic in the house sparrow circadian system. Figures 5 and 6 below display *Per2* expression in SHAM birds at CT 6 and CT 18 on day 0.

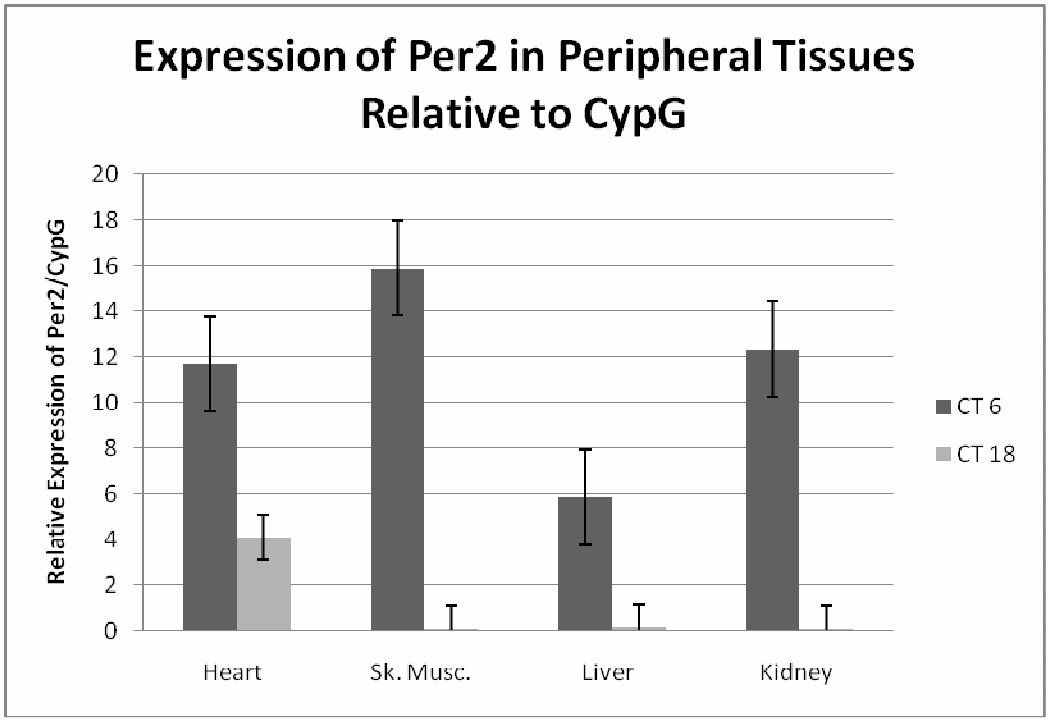

Figure 5. *Per2* expression on day 0 was examined in SHAM heart, skeletal muscle, liver, and kidney relative to *CypG*. *Per2* mRNA transcript was present at considerably higher levels than *CypG* transcripts during the mid-subjective day in all tissues. During mid-subjective night, all tissues displayed a significant lowering of *Per2* expression relative to *CypG*. These results are the average of two identical replicates; at first, a 16 fold increase between night and day such as in skeletal muscle seemed out of place, but a second run showed this result was consistent.

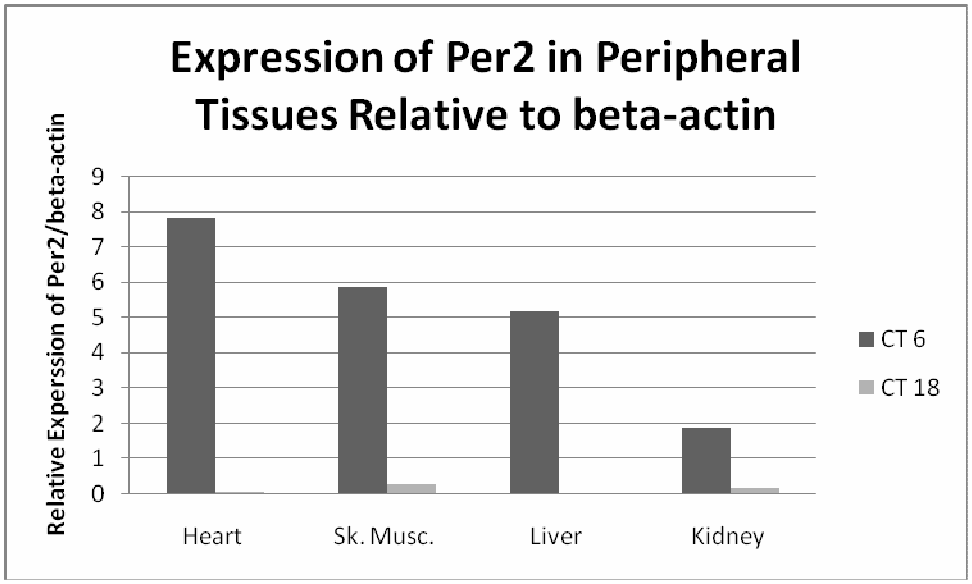

Figure 6. Per2 expression on day 0 was examined in SHAM heart, skeletal muscle, liver and kidney relative to *beta-actin*. *Per2* mRNA transcript was present at considerably higher levels than *beta-actin* transcripts during mid-subjective day in all tissues. During mid-subjective night, all tissues displayed a significant lowering of *Per2* expression relative to beta-actin. These results display one replicate.

Metabolic

Currently, only heart tissues have been processed for 2DG uptake and are the only peripheral tissue presented here. Figures 7-9 below represent the rate of 2DG uptake; a greater concentration of ^{14}C in the tissue indicates a greater uptake of the glucose analogue, and, as such, a greater uptake of glucose.

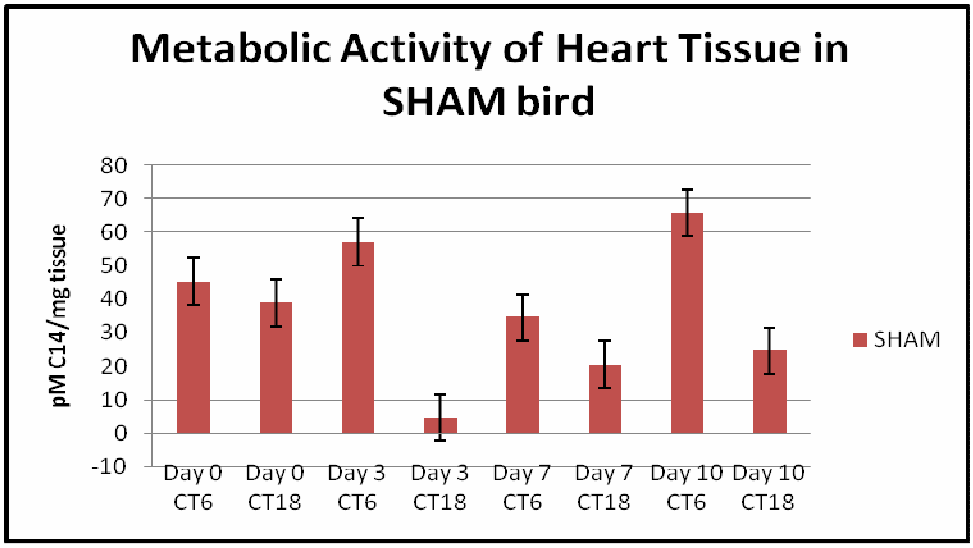

Figure 7. The metabolic activity of heart tissue in the SHAM bird across the time series. Greater concentrations of C14 indicate greater 2DG uptake. Day 0 has only slight variation between CT 6 and CT 18, but all days show increased 2DG uptake during mid-subjective day and decreased 2DG uptake at mid-subjective night.

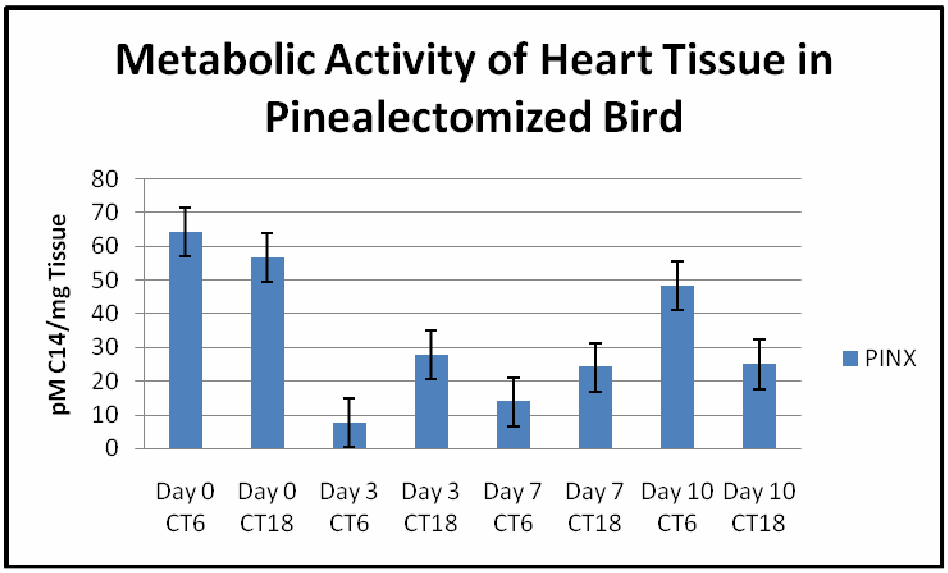

Figure 8. The metabolic activity of heart tissue in the PINX bird across the time series. Greater concentrations of C14 indicate greater glucose uptake. Day 0 has only slight variation between CT 6 and CT 18, as seen in Figure 7. The rest of the days have differences between mid-subjective day and mid-subjective night activity, but it does not follow the same explicit pattern as the SHAM bird.

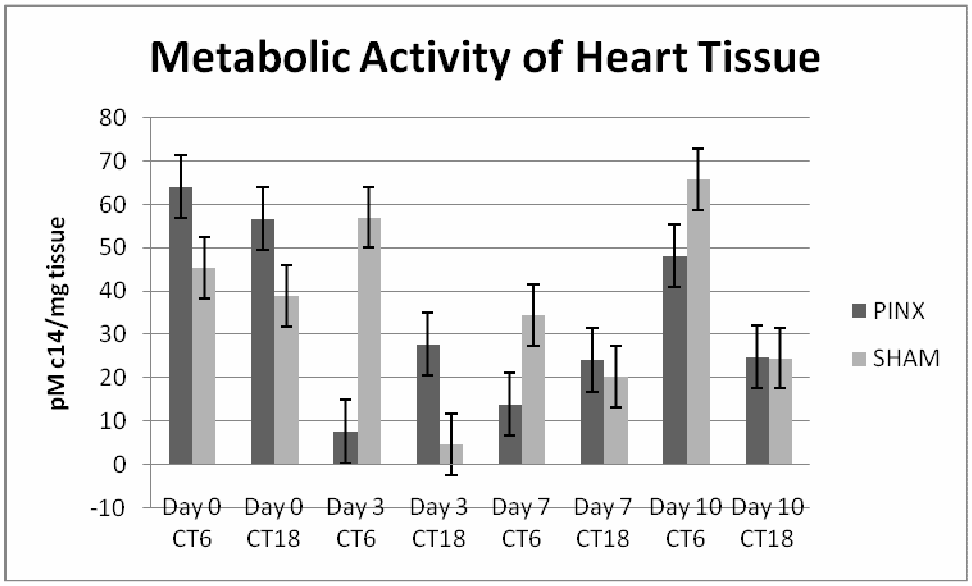

Figure 9. Metabolic activity for both SHAM and PINX birds. Figure 7 and Figure 8 were paired together for comparison. Although a rhythm is seen in the PINX sparrow, it is not as significant as the SHAM sparrow rhythm and it phase shifts twice.

These metabolic results, while interesting on their own, require a complete picture of the transcriptional activity of peripheral tissues before any conclusions may be drawn concerning the presence of the dual cellular oscillators. At the time of composition, most tissues and samples had been harvested but only a fraction had been processed. Thus, these results represent only a portion of the work to come.

CHAPTER IV

DISCUSSION

Behavioral

The behavioral data presented are expected but significant in that they show the superiority of an infrared data acquisition system over the traditional perch switch system. For future behavioral studies of the house sparrow in which locomotor activity is an output, infrared detection systems should be used. As expected, SHAM birds displayed free running rhythmicity while PINX birds had become arrhythmic by day 10. The infrared system allowed easy measurement of tau and accurate calculation of CT 6 and CT 18 for each sparrow. Although not shown in an actogram, we also observed a common issue when measuring sparrow locomotor activity: sparrows put in constant darkness after a 12:12 LD light cycle occasionally become totally inactive and activity cannot be measured until a few days after beginning of constant conditions. This problem will probably never be resolved as the birds cannot be forced to be active, but it fortunately did not pose any issues in this experiment.

Once the rest of the molecular and metabolic results are obtained for a given sparrow, they will be compared its locomotor activity. This will provide insight as to whether locomotor activity becomes arrhythmic in at the same time 2DG uptake and clock gene transcription become arrythmic. Initially, our data indicates that locomotor behavior and

2DG uptake in the heart become arrhythmic at different times. Locomotor rhythms are still succumbing to arrhythmia by day 3 when 2DG uptake is already arrhythmic.

Transcriptional

The molecular data here are among the first presented for *Passer domesticus* and the results appeared as expected. *CypG* and *beta-actin* are known to be constitutively expressed at stable levels in other organisms, and it is no different in the house sparrow. In the SHAM tissues analyzed, *Per2* transcripts were present in greater amounts relative to *CypG* than to *beta-actin* at all time points in heart, liver, kidney and skeletal muscle, a good indication that there is more *beta-actin* transcript present in sparrow cells and that both *CypG* and *beta-actin* are constitutively and stably expressed in the house sparrow. These observations are necessary to continue any real time q-PCR analysis of house sparrow genes. Additionally, *Per2* was shown to be rhythmic in the heart, skeletal muscle, liver, and kidney with high expression during mid-subjective day and low expression during mid-subjective night on day 0.

As of yet, samples have not been processed for the remaining tissues and time points for real time q-PCR analysis. However, we expect that *Per2* and *Cry1* expression will remain rhythmic through day 10 in SHAM birds. Conversely we should see a damping of this transcriptional rhythm over the course of the time series into complete arrhythmia by day 10 in pinealectomized birds. If the dual oscillator model is indeed plausible, the transcriptional rhythm will damp sometime after day 3, the day by which metabolic data

becomes arrhythmic. Whether transcriptional rhythmicity will damp in unison with locomotor rhythmicity is unknown, but quite possibly could be the case.

Metabolic

Lastly, as predicted, the metabolic data show robust rhythms of 2DG uptake in the SHAM sparrow heart under conditions of constant darkness. Even by day 10, the heart is maintaining a robust rhythm of high 2DG uptake in the subjective day and low 2DG uptake in the subjective night. However, the PINX sparrow heart under constant conditions succumbs to what is presumably metabolic arrhythmia by day 3. It is presumed to be arrhythmia because a phase shift occurring between day 0 and day 3 to yield low 2DG uptake in mid-subjective day and high 2DG uptake in subjective night and then again between day 7 and day 10 would be unlikely in a PINX sparrow. However, it is worth noting that the 2DG uptake had a fairly pronounced difference between CT 6 and CT 18 for each of the days, excluding perhaps day 0. This could be a result of chance and simply catching high and low points of the arrhythmia, or it could be indicative of something even more incredible, such as self-sustained peripheral oscillators that function independent of the pineal gland. As interesting as it would be, the latter is unlikely as the said oscillator would have to phase shift twice in the ten day period. Future work will look into this and show more clearly the damping of 2DG uptake. Another interesting observation is that it would appear both SHAM and PINX sparrows have only a weak difference between day and night metabolic activity in the heart at day 0, but this different becomes and remains pronounced by day 3. This could

again be simply due to small sample sizes not providing enough resolution, but perhaps another cause could be contributing. Again, future work will look into this observation as well. Based upon the data presented here, it is likely that PINX sparrow hearts are arrhythmic in 2DG uptake by day 3. Coupled with the observation that locomotor activity is still rhythmic in the PINX bird at day 3, this supports a dual cellular oscillator model; assuming transcriptional rhythms are responsible for locomotor rhythms, transcriptional rhythms will damp after metabolic rhythms. However, if all three rhythms (behavioral, metabolic, and transcriptional) damp at varying times between day 0 and day 10, this would indicate that all three outputs are under different controls. Either this, or, at the very least, responding differentially to the same control.

CHAPTER V

SUMMARY AND CONCLUSIONS

At the composition of this paper, only a fraction of the whole picture has been developed, but that bit of the picture is important and interesting indeed. While the results shown here focus on the effect of pinealectomy on peripheral tissues, the brain samples obtained during the course of this experiment will also yield valuable data concerning the transcriptional and metabolic activities of the brain. While there is still much work to be done, this first portion of the experiment is a valuable cornerstone and lays important groundwork on which this wide reaching experiment may be continued.

The next step, upon completion of this first experiment, is to administer melatonin cycles to arrhythmic pinealectomized sparrows under constant darkness and examine the same outputs observed here. This will provide insight into how melatonin re-entrains the oscillators in question and will determine if these oscillators are sensitive to melatonin directly or rather another input that is dependent upon melatonin.

Although the experiment is an ambitious one, it will be a significant publication upon completion, answering many questions concerning biological clock organization throughout the entire body.

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