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Alcohol Usage and Abrupt Cessation Modulate Diurnal Activity

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Abstract

Alcohol has many effects throughout the body. The effect on circadian rhythms and the correlation of these effects to withdrawal effects of alcohol present interesting findings. By measuring 3 planes of activity of female Sprague-Dawley rats during alcohol usage and continuing study through the first two days following withdrawal of alcohol allow for the observation of a drastic modulation of the circadian pattern of activity.

Introduction

The Greek poet Hesiod wrote in 700 B.C., “Of themselves, diseases come among men, some by day and some by night. Whoever wishes to pursue the science of medicine in a direct manner, must first investigate the seasons of the year and what occurs in them.” (41). Patterns of health and homeostasis have been noted throughout history. The observable daily pattern in humans is that of circadian rhythmicity. Human circadian rhythms have been noted in a wide range of aspects of homeostasis, including physiology, behavioral, endocrinology, neurology, and metabolism (48). It is argued that “the concept of homeostasis should be extended to include the precisely timed mechanisms of the circadian timing system which enables organisms to predict when environmental challenges are most likely to occur...It must be concluded that the day-night cycle of the natural environment has played a fundamental role in shaping the evolutionary development of homeostatic mechanisms because of the dominating predictability of diurnal changes in illumination, temperature, food availability, and predator activity.” (28). The circadian rhythm is controlled by an internal “clock” in mammals, which is located in the suprachiasmatic nucleus (SCN), found in the anterior hypothalamus (36,40,47,48). The SCN receives information about levels of light via the retinohypothalamic tract (29,36).

Disruption of circadian rhythms can have many effects. For instance, there are short term conditions such as jet lag and its associated symptoms of fatigue, disorientation and insomnia (3,41). Disruption of circadian rhythms can also be seen in numerous long term

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diseases. It has been noted in everything from arthritis, asthma, cancer and diabetes to heartburn, heart disease, hypertension, multiple sclerosis, and stroke (41). Although there is mounting evidence for chronotherapy, the process of synchronizing treatment with body rhythms, for being successful in the administration and effectiveness of drug therapies (41), there is little knowledge of how drugs themselves impact circadian rhythms.

Alcoholic beverages have been consumed for centuries for a variety of purposes, including hygienic, anesthetic, religious, and recreational. Alcohol, specifically ethanol, is hydrophobic in nature, which allows it to cross cell membranes easily. So once alcohol is in the bloodstream it can diffuse into nearly every tissue in the body, including the brain. The actual site of action, however, remains controversial (43). Initially, alcohol crosses the blood brain barrier and produces feelings of relaxation and cheerfulness by causing the release of dopamine and serotonin in the nucleus accumbens, the brain's pleasure center (50). Further exposure results in alcohol's dose-dependant CNS depression, leading to ataxia, slurred speech, blurred vision, slowed reaction times, and uninhibited behavior (15,18,19,20). In addition to alcohol's depressive actions, repetitious alcohol consumption elicits tolerance and withdrawal effects. Tolerance occurs when a subject's reaction to a drug decreases after repeated exposure so that larger doses are required to achieve the same effect (13,15,18,19,20,34). The body makes adaptations to the drug which requires that more of the drug be applied in order to reach the same bodily changes produced by the drug. Once these adaptations are set, the body then becomes dependent on the drug, needing it to function physiologically in the same manner (15,18,19,20). These adaptations remain after use of the drug is discontinued and subsequently appear as "withdrawal" signs and symptoms which are commonly the opposite effects that the drug produces. Alcohol withdrawal symptoms classically consist of hyperexcitability and insomnia (6,8,15,18,19,20).

Nearly all of the published studies on the effects of alcohol analyze the effects of the drug during usage and immediately following cessation of the treatment. No studies to date have looked at how the drug modulates the behavioral circadian rhythm, not only during intoxication and immediately following cessation, but also for the subsequent hours and days following the termination of treatment. We hypothesize that chronic alcohol administration will alter the behavioral circadian rhythm activity and that subsequent withdrawal of the alcohol will cause changes in the circadian rhythm that will produce the symptoms of alcohol withdrawal.

Methods

Animals

Pregnant, female Sprague-Dawley rats (n=38) weighing from 160–190 g were housed in the experimental room in groups of four at an ambient temperature of 21 ± 2 °C and a relative humidity of 37–42%. The rats were acclimated to the test environment for seven days prior to experimentation on a 12:12 h light (07:00–19:00)/dark (19:00–07:00) cycle. At the beginning of the experiment all rats were weighed and individually housed in Omnitech Digiscan RXYZM DVA animal activity computerized monitoring system test cages. After 12 hours of accommodation to the test cages, rats were continuously monitored (sample time: 10 min) throughout the 24 hour cycle for 16 days (1,7,16).

Testing Apparatus

The test chambers for constant activity monitoring consisted of clear acrylic open field boxes (40.5×40.5×31.5) with 2 levels of infrared motion sensors consisting of 16 infrared beams spaced 2.5 cm apart. The first and second levels of sensors were 6 cm and 12.5 cm above the cage floor, respectively. The activity monitoring system checked each of the

beams at a frequency of 100 Hz to determine whether the beams were interrupted. The interruption of any beam was recorded as an activity score. Interruptions of two or more beams separated by at least a second was recorded as a movement score. The total distance traveled in centimeters was calculated from the total number of beams interrupted in consecutive sequence. Cumulative counts were compiled and downloaded every 10 minutes into OASIS data collection program, and organized into 24 motor indices (1,7,16).

Horizontal activity measures the amount of movement along the floor of the cage. Vertical 1 measures any sort of activity above the floor, as in standing or rearing of the head. Vertical 2 requires that the rat be fully extended along the wall of the cage, as in reaching or jumping.

Procedure

The first 3 days of recording served as a baseline measurement of activity for each individual rat so they could serve as their own control on the days of alcohol consumption (1,16). During this time, food pellets and water were continuously supplied. During the experiment, ethanol was administered in the following manner: free access to a nutritionally balanced liquid diet containing ethanol (5%, w/v, 36% ethanol-derived calories) was available. The average daily intake of ethanol was 19.9 ± 0.8 g/kg, which was previously found to produce blood ethanol levels of 198 ± 6 mg/dl (9).

Data Analysis

Twenty-four hour activity patterns were evaluated for the three levels of activity indicated above and plotted displaying the hourly means and standard deviations. The original histograms were evaluated and paired data points reflecting the appropriate hourly means and standard deviations. These paired data points were characterized by fitting a cosine curve with a twenty-four hour period. Such curves are typically parameterized by estimating the mesor (baseline, average), amplitude (distance from mesor to highest point), and acrophase (time at which the maximum occurs) using the cosinor analysis procedures and f-tests for statistical significance as described by et al., (2). Table 1 displays the days on which activity was measured and the corresponding experimental description. Parameter estimates of the cosine curves with the appropriate standard errors and p-values are presented in Table 2. Here the standard errors of the mesor estimate are presented; however for presentation of the results below the mesor estimate and amplitude of the cosine are represented as the mesor value $\pm$ a second amplitude estimate. Table 3 displays the tests for significance of the parameter estimate differences. Activity for the control days were evaluated to determine whether the days could be pooled as an overall control group. Representative treatment days were compared to the control group and to other treatment days using appropriate F-tests to qualify the effects of the treatment changes on the activity patterns. Representative plots contrasting specific treatment days with control days were constructed to visually present the effects of various stages in the treatment cycle. F-tests contrasting cosine parameter estimates were computed to test for significant differences between parameter estimates.

Results

Control

Fig. 1 summarizes the total locomotor activity for 24 hour periods for the 3 control days. The data demonstrates that the control group exhibits similar activity with the minor fluctuations during the days of control. The 24 hour total activity level in the control rats remains consistent during the control portion of the experiment for all three locomotor indices: horizontal, vertical 1, and vertical 2.

Fig. 2 summarizes the hourly horizontal, vertical 1, and vertical 2 locomotor activity, also during the control portion of the experiment. Days 1, 5, and 9 were arbitrarily chosen as representative of the daily pattern of activity in the rats. They display activity consistent with nocturnal behavior. These three days of control were also determined to be statistically similar using the cosine curve analysis (2). Therefore, the control days 1, 5, and 9 are grouped together for purposes of simplicity in demonstrating the data gathered. The horizontal activity of the control group shows an average (mesor) of 2158.54 beam crossings per hour during the 24 hour period, with an amplitude above and below that value of 2,308.51 and maximal activity approximately 3:15 AM. The mesor for the vertical activity level 1 (sitting posture) was 279.03 ± 262.61 , with maximal activity close to 1:40 AM, and for the vertical activity level 2 (reaching posture) the mesor was 30.66 ± 29.92 beam crossings, with maximal activity close to 2:05 AM.

Experiment

Fig. 3 shows the average counts for 24 hour periods during all phases of experimentation. Days 1–3 account for a control baseline. This control was previously established and is the control used for statistical comparison. The average counts for the days of alcohol treatment and those for the days of alcohol withdraw display statistical differences, not only one from the other, but also each from the control.

Alcohol Consumption, Experimental Day 26—Fig. 4(a) shows the hourly activity recorded for Day 26, again arbitrarily chosen to represent the effects of alcohol usage. All levels of activity, horizontal, vertical 1, and vertical 2 display statistical difference during peak activity hours compared to that of the control as shown in Fig. 2(c). The activity recorded during the 12 hours of light, when the rats sleep, however, is similar to that of the control. The most notable difference is that the activity during the 12 hours of dark, when the rats are awake and is usually more depressed. More specifically, the horizontal beam crossings during this alcohol consumption phase showed a mesor of $1,068.33 \pm 998.2$ ($p < 0.001$), for vertical 1 was 164.38 ± 167.76 ($p < 0.001$), and for vertical 2 were 14.96 ± 16.65 ($p < 0.001$). Also, the times of the maximum activity (acrophase) for each level of measurement did not significantly change.

Alcohol Withdrawal, Experimental Days 29 and 30—Figs. 5 and 6 show the hourly activity recorded for Day 29 (Fig. 5(a)) and Day 30 (Fig. 6(a)), the first 2 days after alcohol consumption was discontinued. Day 29 shows a mesor for beam crossings for the horizontal and vertical 1 activity is increased from Day 26 by almost 70%, but remained below normal ($p < 0.001$); and the vertical 2 activity increased by 170%, but also remaining below normal ($p < 0.001$). Again, the times of maximal activity (acrophase) for the horizontal and vertical 1 levels of activity were not significantly different between alcohol usage, Day 26 and alcohol withdrawal, Day 29. However upon inspection of Figure 5(a) a dramatic, although non-significant ($p = 0.390$) change in the timing of maximal vertical 2 level activity is indicated. The maximum activity for the alcohol usage (Day 26) is approximately 3 AM, while the withdrawal activity (Day 29) increases during daylight hours, reaching a maximum at close to 9 PM, thus demonstrating an altered circadian rhythm.

The second day of withdrawal, Day 30, indicates a tendency to return to normal activity during the period of darkness, but with persistent differences. For Day 30, there was an elevated horizontal activity mesor of 2559.58 beam crossings, significantly different from normal ($p < 0.001$) with a significantly lower ($p < 0.001$) amplitude of ± 386.65 . The maximal activity time, 5:30 AM, returns to approximately normal ($p = 0.087$). The mesor of vertical 1 activity, 385.88, on Day 30, was also elevated ($p < 0.001$) while the amplitude remains significantly ($p < 0.001$) reduced at 88.74 beam crossings, with maximal activity at 2:00 AM,

again returning to normal ($p=0.062$). Vertical 2 activity mesor, 28.63, returns to normal ($p=0.400$), while the amplitude, 15.92 remains reduced ($p<0.001$), and the acrophase, 5:40 AM indicates a shift ($p<0.001$) toward daylight hours.

Discussion

The data displays a marked difference in the levels of activity between the control, the application of alcohol, and the withdrawal thereof. A decrease in the total activity of the rats was observed after 26 days of alcohol consumption, most of the decrease occurring during the 12 hours of dark phase, the time when the rats are awake. This depression of motor activity can be explained by alcohol's depressive actions on the CNS (15,18,19,20,46). This alteration in the awake locomotor activity creates an alteration in the circadian activity pattern of the animal. In every axis, horizontal, vertical 1, and vertical 2, there were changes in the level of activity. Once the alcohol was removed, and blood alcohol concentrations began to drop, the rats increased their locomotor activity which can be interpreted as withdrawal behavior. This alteration in the circadian rhythm is apparent due to the significant change in the acrophase from 3 AM to 9 PM. The characteristic signs of withdrawal manifested in the data collected during the hourly recordings of activity are hyperactivity, hyperexcitability, and insomnia similar to other reports (6,13,18,19,20). These symptoms are notably the exact contraries to the effects during alcohol consumption, and agree with previous observations (6,8,15,18,19,20,40).

This, therein, begs the question: Does withdrawal from alcohol create the disturbance in circadian rhythms or does a disturbance in the circadian rhythm produce withdrawal-like effects? The data suggests that ethanol creates changes throughout the brain that have many effects that lead to dependence and subsequent withdrawal effects. These changes involve changes which alter the circadian rhythm. Furthermore, the data suggests that this modulation in circadian rhythm activity is then responsible for perpetuating the symptoms of withdrawal and that once the circadian rhythm activity is corrected, the signs of withdrawal can be reversed, similar to the idea that once the circadian rhythm is restored in a jet lagged traveler, their symptoms of jet lag fade away (41). This warrants further investigation.

Although no specific receptor for ethanol has been found, ethanol has been found to produce effects on numerous receptors throughout the body. Such receptors include serotonin (5-HT) (11,35) voltage gated calcium channels (17); receptors for the excitatory amino acid, glutamate (12,35); GABA neurotransmission (23,44); catecholamines such as norepinephrine and dopamine (39). Changes in each of these receptors are suspected in not only producing dependence on alcohol, but also in claiming responsibility for the withdrawal syndrome.

The relationship of 5-HT and ethanol self-administration has long been realized. It is agreed that increasing 5-HT neurotransmission will produce reductions in alcohol self-administration (32). It was reported that depletion of 5-HT in the brain slowed the rate of development of tolerance. Data has shown that ethanol withdrawal alters the function of 5-HT in the brain, suggesting that reduced 5-HT activity in the brain may be a secondary consequence of ethanol withdrawal. 5-HT, however, does not appear to play a role in the development of dependence on ethanol (11,32).

Serotonin seems to display a particular connection between circadian rhythms and withdrawal (35). In the rat, 5-HT levels are highest during the hours of light, and lowest during hours of darkness. The amplitude of the rhythm in 5-HT tissue concentration has been shown to be the largest in the suprachiasmatic nucleus (SCN) (20). Both in vivo (5,21,45) and in vitro (24,33) experiments have shown that pharmacological intervention

with selective ligands for 5-HT receptors produces phase shifts and/or changes in amplitude of the measured rhythm. On the basis of these findings, it is clear that serotonergic innervation of the SCN plays an important part in modulating the activity and output of the circadian oscillator (22,25,35).

Furthermore, ethanol has been shown to upregulate long-lasting (L-type) calcium channels (14). L-type calcium channels are primarily found on dendrites and neuronal cell bodies in the brain (17). Activation of these channels mediate long lasting increases in cytosolic calcium that may be linked with second messenger functions of calcium in brain, which includes involvement in neurotransmitter release. Evidence further indicates that chronic ethanol administration may result in upregulation of these L-type calcium channels with increasing calcium influx and that the temporary maintenance of these changes are involved in the development of functional tolerance, dependence, and in the withdrawal syndrome (14,17,32).

Glutamate is the major excitatory neurotransmitter in the CNS (15). One of the primary receptors for glutamate is the NMDA receptor (12,15). Ethanol is a potent and selective inhibitor of the function of the NMDA receptor (32). Chronic ethanol usage results in an upregulation of the NMDA receptor function. This function of ethanol is thought to underlie the mechanism of ethanol tolerance and the symptoms of ethanol withdrawal, especially withdrawal seizures (12,32,35).

Ethanol also produces some of its effects by modulating the GABA receptor in the brain (23). GABA is an inhibitory neurotransmitter of which ethanol is believed to be an agonist. Ethanol has the effect of downregulating GABA receptors. Alcohol binding of the ligand produce a conformational change in the receptor and the control ion pore opens to allow the flow of ions. In some receptors the binding of the ligand produces a closed conformational state in spite of the continuing presence of the ligand. This state is called “desensitization” (tolerance). The delta subunit-containing GABA_AR_S and tonic inhibition mediated by them appears to cause the sobriety impairing concentration of ethanol (27). The delta subunit-containing GABA_AR_S considered by some as the “ethanol receptor” of the brain (10). The tonic inhibition mediated by these GABA_AR_S has an equally high sensitivity to ethanol (49), suggesting that the delta subunit-containing GABA_AR_S of the brain are the target for sobriety impairing concentration of ethanol (27). Specifically, some studies suggest that chronic ethanol application alters the GABA_A receptor gene expression, which suggests a method of ethanol tolerance and withdrawal (32,34,44) as well as modulation of the circadian clock gene (23).

Chronic ethanol ingestion increases the activity of NE and decreases the activity of DA in the brain (39). Both of these systems are thought to be involved in the development of physical dependence on ethanol and in ethanol withdrawal; however data suggests that the ethanol induced alterations in DA activity and sensitizes the DA receptors, which may contribute more highly to certain signs of alcohol withdrawal (32). Acutely, dopaminergic neurons are resistant to the effects of alcohol, possibly mediating a reinforcing effect of ethanol in the short term (32,39).

Ethanol creates these neuromodulatory adaptations involving 5-HT, L-type calcium channels, glutamate, GABA, NE, and DA (23,32,35). These adaptations are proposed to account for not only the acute effects of alcohol intoxication, but also withdrawal. The effects involve an alteration of a gene, which then produces either an upregulation or downregulation of a gene product (3,31,32).

All of the above interpretations consider synaptic interaction. Recently, it was reported that neurons are able to communicate without synapses. They are able to send chemical

messages by means of diffusion to target cells via the extracellular space (52). These observations led to the suggestion that in addition to synaptic chemical communication, there is another form of interaction among neurons (51). Moreover, in the CNS most of the drugs are modulators and their targets are non-synaptic receptors, transporters, enzymes, and ion channels. These non-synaptic receptors are more affected because they outnumber synaptic, the synaptic ones and they are more sensitive to fine modulation of ambient in the extracellular space compared with receptors in the synapses (52), the non-synaptic function are thought to be responsible for the modulation, tuning and alteration of many physiological processes such as the level of arousal and may be also circadian activity patterns shown in this study following chronic alcohol consumption. Furthermore, studies have shown (4,25,37,38) that both chronic ethanol intake and ethanol withdrawal may be associated with changes in a fundamental parameter of the circadian pacemaker (3,31). The results of this study further supports the evidence that ethanol alters the circadian rhythm.

The mammalian clock system contains a master clock located in the suprachiasmatic nucleus (SCN) (23,34). In mice, the system includes three *Period* (*Per*) genes (*Per 1*, *Per 2*, *Per 3*), two *Cryptochrome* genes, the *Clock* gene, and the gene encoding brain-muscle Arnt-like protein 1 (30,42). Recent studies have shown that ethanol consumption does alter the circadian expression patterns of *Per* genes in the suprachiasmatic nucleus (42). Evidence suggests that it is this ability of ethanol to alter genetic expression involved in the circadian rhythm that is paramount in discovering the causal relationship to withdrawal. The disruption in circadian rhythms by alteration of the genetic controllers, leads to the behavioral effects of withdrawal.

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Figure 1.

The figure summarizes (N=8) the total 24 hour activity of three locomotor indices (A,B, and C) recorded from three randomized control days. The recordings were obtained from 31 consecutive non-stop recording days. The data shows that the control group exhibited similar levels of activities with minor non significant fluctuation.

Figure 2.

The figure summarizes the hourly sum (N=8) of the rat described in figure 1 of the three locomotor indices (A,B, and C) evaluated. The hour's histogram show the hourly activity pattern typical of nocturnal animals. The number under the column indicates the recording time. The hourly activity pattern on experimental day days 1,15, and 29 exhibit similar levels and patterns with minor non-significant fluctuation.

Figure 3.

The figure summarizes the total 24 hours of the three locomotor activity indices obtained on experimental days 1 to 3 (control), 26 to 28 the last three days of the alcohol consumption and the last three experimental day after abrupt alcohol cessation (experimental days 29 to 31)

Figure 4.

The figure shows the 24 hourly activity of the three locomotor indices (Fig. 4A) and the superimposed cosine curve analysis of the control day with experimental day 26 (Fig. 4B). Similar observations were obtained from experimental days 27 and 28.

Figure 5.

The figure shows the 24 hourly activity of the three locomotor indices (Fig. 5A) and the superimposed cosine curve analysis of the control day with experimental day 29 the first day of abrupt alcohol cessation (Fig. 5B).

Figure 6.

The figure shows the 24 hourly activity of the three locomotor indices (Fig. 6A) and the superimposed cosine curve analysis of the control day with experimental day 30 the second day of abrupt alcohol cessation (Fig. 6B).

Table 1

Summary of the days, general treatment categories, and treatment phase.

Day	Treatment Group	Description
1	Controls	Normal Activity
5	Controls	Normal Activity
9	Controls	Normal Activity
26	Study	During Alcohol Use/Drug Effect
30	Study	Removal of Alcohol/Withdrawal
31	Study	Withdrawal

Table 2
Cosine curve parameter estimates and amplitude significance tests. (Period=24 Hours)

Tau	Grp	N	Activity Reading	Mesor	Mesor (se)	Amp.	Amp. p-value	Acro. (deg.)	Acro (hrs)	R-square
24	1,2,3	144	Hz	2158.54	49.06	2308.5	<0.001	-33.28	3.22	0.8870
	4	48	Hz	1068.33	59.14	0998.2	<0.001	-30.49	3.03	0.7599
	5	48	Hz	1777.50	67.86	537.52	<0.001	-346.43	24.1	0.4108
	6	48	Hz	2559.58	80.50	386.65	0.0059	-65.32	5.35	0.2040
24	1,2,3	144	Valz	279.03	10.26	262.61	<0.001	-9.21	1.61	0.6992
	4	48	Valz	164.38	9.43	167.76	<0.001	-8.64	1.58	0.7785
	5	48	Valz	272.88	9.72	146.71	<	-16.01	2.07	0.7166
	6	48	Valz	385.88	11.78	88.74	<	-39.34	3.62	0.3873
24	1,2,3	144	Va2z	30.66	1.32	29.92	<	-16.98	2.13	0.6468
	4	48	Va2z	14.96	1.26	16.65	<	-27.16	2.81	0.6608
	5	48	Va2z	41.83	1.35	09.05	<	-304.33	21.29	0.3315
	6	48	Va2z	28.63	01.22	15.92	<	-70.39	05.69	0.6528

Table 3

F-tests of significance for difference between cosine curve parameter estimates.

Animal Group 1	Animal Group 2	H ₀ : Equal Parameter	F-stat	Degree of Freedom	p-value
Day 26	Control	Mesor	141.00	(1,186)	<0.001
		Amplitude	101.9	(1,186)	<0.001
		Acrophase	0.176	(1,186)	0.676
Day 30	Control	Mesor	16.53	(1,186)	<0.001
		Amplitude	178.5	(1,186)	<0.001
		Acrophase	2.45	(1,186)	0.119
Day 31	Control	Mesor	17.13	(1,186)	<0.001
		Amplitude	196.70	(1,186)	<0.001
		Acrophase	2.97	(1,186)	0.087
Day 30	Day 31	Mesor	55.18	(1,90)	<0.001
		Amplitude	1.03	(1,90)	0.314
		Acrophase	6.57	(1,90)	0.012
Day 26	Day 30	Mesor	62.07	(1,90)	<0.001
		Amplitude	13.10	(1,90)	<0.001
		Acrophase	2.38	(1,90)	0.127
Day 26	Day 31	Mesor	222.9	(1,90)	<0.001
		Amplitude	18.74	(1,90)	<0.001
		Acrophase	4.42	(1,90)	0.038
Day 26	Control	Mesor	37.81	(1,186)	<0.001
		Amplitude	12.95	"	<0.001
		Acrophase	0.01		0.945
Day 30	Control	Mesor	0.11	(1,186)	0.743
		Amplitude	19.22	"	<
		Acrophase	0.52	"	0.471
Day 31	Control	Mesor	31.40	(1,186)	<
		Amplitude	41.57	"	

Animal Group 1	Animal Group 2	Ho: Equal Parameter	F-stat	Degree of Freedom	p-value
		Acrophase	3.54	"	0.062
Day 30	Day 31	Mesor	54.82	(1,90)	<
		Amplitude	7.21	"	0.009
		Acrophase	4.01	"	0.048
Day 26	Day 30	Mesor	64.17	(1,90)	
		Amplitude	1.20	"	0.276
		Acrophase	1.10	"	0.298
Day 26	Day 31	Mesor	215.8	(1,90)	
		Amplitude	13.72	"	
		Acrophase	7.04	"	0.008
Day 26	Control	Mesor	42.78	(1,186)	<
		Amplitude	15.26	"	<
		Acrophase	0.911	"	0.241
Day 30	Control	Mesor	21.34	(1,186)	<
		Amplitude	37.26	"	<
		Acrophase	3.58	"	0.060
Day 31	Control	Mesor	0.72	(1,186)	0.400
		Amplitude	17.07	"	<
		Acrophase	18.33	"	<
Day 30	Day 31	Mesor	52.36	(1,90)	<
		Amplitude	7.09	"	0.009
		Acrophase	01.21	"	0.274
Day 26	Day 30	Mesor	211.5	(1,90)	
		Amplitude	8.47	"	
		Acrophase	4.40	"	0.39
Day 26	Day 31	Mesor	60.64	(1,90)	<
		Amplitude	0.09	"	0.767
		Acrophase	23.32	"	<