

# Impact of Cage Size and Enrichment (Tube and Shelf) on Heart Rate Variability in Rats

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## Summary

Rats respond physiologically and behaviorally to environmental stressors. As cage conditions can be a stressor, it is important that experimental results acquired from caged rats are not confounded by these responses. This study determined the effects of cage size and cage enrichment (tube and shelf) on heart rate variability (HRV) in rats as a measure of stress. Electrocardiogram data were collected from 5 male Sprague-Dawley rats, each implanted with a radio-telemetric transducer to assess the ratio of the low to high frequency components of the HRV power spectrum (LF/HF). This ratio reflects the degree of sympathetic versus parasympathetic nervous activity and increases with decreasing HRV. Rats were housed for 3 weeks in each of the following cage conditions: small un-enriched, small enriched, large un-enriched and large enriched. Cage enrichment and/or larger cages did not significantly alter LF/HF values compared to the small, un-enriched cage condition, when considered independent of the sleep/wake cycle. However, when results were pooled for all cage conditions, LF/HF significantly increased during the wake cycle compared to the sleep cycle. Further analysis showed that this difference was only statistically significant for the un-enriched cage condition. Thus the presence of a tube and a shelf in a rodent cage can alter the diurnal rhythm of HRV in rats and this should be taken into account when designing experiments in which HRV is an outcome.

## Introduction

According to the U.S. Department of Agriculture (USDA), rodents comprise approximately 90% of all animals used in research today. In order to maintain the validity of data collected from these animals, it is crucial that rodent stressors be minimized as stress adversely affects every physiological system thereby introducing a confounding variable into experimental designs. One potential source of stress is the animal housing environment. Chronic environmental stress due to housing conditions [i.e. barren cage environment (Olsson & Dahlborn, 2002; Sherwin, 2004; Wolfer et al, 2004; Wurbel, 2001) and excessive noise from personnel, machinery, construc-

tion, etc. (Burwell & Baldwin, 2006; Dallman et al, 1999, 2004; Moyaho & Valencia, 2002)] imposes a host of adverse physiological consequences on rodents, including an increase in corticosterone levels (Committee on Recognition and Alleviation of Distress in Laboratory Animals, 2008; Kant et al, 1987), the development of repetitive behaviors (e.g. excessive grooming, digging, rearing, yawning, and fighting/biting) (Dunn et al, 1987; Gonzalez-Burgos & Cuevas-Alvarez, 1992; Moyaho & Valencia, 2002; Olsson & Dahlborn, 2002; Sutton et al, 1982; Veldhuis & De Wied, 1984; Wood et al, 2003; Wurbel, 2001; Wurbel & Garner, 2007; Wurbel & Stauff-

## Abbreviations

HR: heart rate; BP: blood pressure; HRV: heart rate variability; SNA: sympathetic nervous activity; LF: low frequency component; HF: high frequency component; LF/HF: low frequency /high frequency; SU: small un-enriched; SE: small enriched; LU: large un-enriched; LE: large enriched; PVC: Polyvinyl chloride tube; ECG: electrocardiogram; FFT: Fast Fourier Transformation;

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*facher, 1996*.) and a decreased capacity to deal with environmental challenges (e.g. a greater reactivity to stressful stimuli) *de Kloet et al, 1999; Francis et al, 2002; Joseph & Gallagher, 1980; Sutton et al, 1982; Tanke et al, 1996; Wurbel, 2001*).

Environmental stressors can also affect cardiovascular function by increasing sympathetic nervous activity (SNA), which can elevate blood pressure (BP) and heart rate (HR), and can decrease heart rate variability (HRV) *(Costoli et al, 2004; Farah et al, 2001; Inagaki et al, 2004; Nijssen et al, 2000; Sharp et al, 2005; von Borell et al, 2007)*. Heart rate variability is derived from the beat-to-beat changes in HR resulting from a variety of factors including neural input from the parasympathetic and sympathetic nervous systems and input from the mechanical effects of respiration *(Japundzic, 1990; Ning et al, 2006; Perini & Veicsteinas, 2003; Perlini et al, 1995; Rubini et al, 1993; Stein et al, 1994; Stein et al, 1999; Tsai et al, 2002; von Borell et al, 2007)*. An increase in SNA reflects a shift in the rodent's sympathovagal balance *(Kuwahara et al, 1994; Ning et al, 2006; Stein et al, 1994; von Borell et al, 2007)*. Heart rate variability is commonly used as a clinical outcome factor, or a predictor of clinical outcomes in trials involving treatment of heart disease *(Gujjar et al, 2007; Nolan et al, 2008)* in humans. This is relevant to rodents because rats are considered a good model for cardiovascular disease *(Dillman, 2008)*; for example, changes in HRV in cardiac hypertrophy occur in rats similar to humans *(Carre et al, 1994)*. From pharmacokinetic-dynamic reasoning in general, and drug discovery perspective in particular, it is important that potential factors that can confound HRV in rats used for research are identified in order to avoid biased or imprecise results in the drug trial.

While the confounding influence of cage conditions on research outcomes has been acknowledged *(Crabbe et al, 1996, 1999; Sherwin, 2004; Wurbel, 2001; 2002)*, few studies have systematically addressed the impact of cage condition on rodent stress in general or on HRV in particular. With regard to cage environment, research rats are typi-

cally housed in small plastic cages that lack items common to their natural environment. These cages provide little beneficial stimuli and limited opportunities for rats to perform natural behaviors *(Clausen et al, 2006; Institute of Laboratory Animal Research, 1996; Jennings et al, 1998)*. Standardized environments have been shown in earlier studies to reduce the extent of inter- and intra-experimental variability that can arise from the addition of superfluous environmental items (i.e. cage enrichment) by decreasing the amount of 'environmental variables' *(Beynen et al, 2001; van der Staay & Steckler, 2001, 2002)*. Reducing inter- and intra-individual variability is essential to decreasing the number of animals required in a particular study, which is the goal of all researchers. However, contrary to expectations, these small cages may actually increase inter-individual variability, necessitating the use of more animals *(Crabbe et al, 1996, 1999; Richter et al, 2009; Wurbel, 2001, 2002; Wurbel & Garner, 2007)*. Thus standardized cages with no enrichment may increase variability through concomitant elevations in rodent stress that, in turn, can adversely affect the validity of experimental outcomes *(Crabbe et al, 1996, 1999; Sherwin, 2004; Wurbel, 2000, 2001; Wurbel & Garner, 2007)*. This problem may be exacerbated in studies of aging because variability in the hypothalamus-adrenal-pituitary response has been shown to be greater in aged rats relative to young or middle-aged rats of the same strain *(Segar et al, 2009)*.

In an attempt to moderate this cage-induced stress and improve rodent welfare, investigators have started using enrichment in their protocols. Enrichment involves providing laboratory animals with objects that stimulate them to explore (i.e. interact with enrichment item(s)) and thereby promote species-specific behaviors, so preventing abnormal or repetitive behaviors *(Balcombe et al, 2004; Clausen et al, 2006; Galef, 1999; Olsson & Dahlborn, 2002; van de Weerd et al, 2002; Wurbel, 2001; Wurbel & Garner, 2007)*. A large body of literature demonstrates how environmental enrichment reduces reactivity to stressful stimuli, and decreases

environmental stress responses (*Balcombe et al, 2004; Chamove, 1989; Diamond, 2001; Joseph & Gallagher, 1980; Olsson & Dahlborn, 2002*). A few studies have shown that enrichment decreases HR and BP (*Sharp et al, 2003, 2005*), while reducing the frequency of behaviors that are indicative of stress (*Olsson & Dahlborn, 2002; van de Weerd, 2002; Wurbel, 2000; Wurbel & Garner, 2007*). Enrichment also reduces corticosterone responses to handling (*Moncek et al, 2004*). Some authors suggest that providing environmental enrichment, such as nesting items, allows rodents to have more control over their environment, which may reduce the rats' response to environmental stressors (*Chamove, 1989; van de Weerd, 1997a, 1997b, 2002, Wiepkema & Koolhaas, 1993*). However, current research on enrichment has yielded inconsistent results due to methodological differences between studies. For example, experimental protocols utilizing cage enrichment often vary in the application and number of enrichment items provided. Due to this lack of standardization, previous studies did not allow the impact of each item to be completely addressed (*Clausing et al, 2006*). Although enrichment has been studied for a half century, the practical application of enrichment items has not been addressed in a systematic way (*Clausing et al, 2006; Tsai et al, 2002; Wurbel, 2002; Wurbel & Garner, 2007*). Thus, while environmental enrichment appears beneficial, the exact type of enrichment item that should be used and its implementation has yet to be determined.

Enrichment is not the only component of the cage that could impact a rodent's stress. The size of the cage relative to the number of animals in that cage could also significantly impact stress. The standard cage size (258 cm<sup>2</sup> (floor area) x 17cm (h) / 350g rat) for group-housed laboratory rodents as dictated by the American Association for Laboratory Animal Science (AALAS) is based on the principle of allowing for the normal physiologic and behavioral needs of rodents, while taking into account the needs of the experimenter (i.e. economics) (*Clausing et al, 2006; Institute of Laboratory Animal Research,*

*1996*). While the latter appears to carry significant weight, it is unclear whether the physiological and behavioral needs of the rodents are adequately addressed in the current system.

Due to the dearth of research on the effects of cage size on rodent stress levels, it is difficult to determine what type of interaction(s), if any, could arise between cage size and animal size/ animal number or enrichment size/enrichment type. Furthermore, it is not known exactly how cage size impacts rodent cardiovascular stress measures and/or behavioral stress measures as experimental outcomes have been largely inconsistent (*Galef & Durlach, 1993, Kitchen & Martin, 1996; Patterson-Kane, 2002; Stayermark & Mueller, 2002; Wurbel & Stauffacher, 1996*). For example, authors in one T maze study showed that rats prefer larger cages over smaller ones, regardless of the presence of a cage mate (*Patterson-Kane, 2002*). Another study showed that there was no observable difference in preference when two cage sizes were compared (*Galef & Durlach 1993*). Moreover, research studies on the effects of cage size are relatively few in number. Alarming, existing cage size standards are not based upon any rigorous physiological-stress response examination (*Galef & Durlach 1993; Institute of Laboratory Animal Research, 1996; Steyermark & Mueller, 2002*). Rather, they are based upon subjective evaluations of physiologic wellbeing (*Galef & Durlach, 1993; Institute of Laboratory Animal Research, 1996; Galef, 1999; Steyermark & Mueller, 2002*). Another possible interaction that has not been systematically addressed is that between cage size and enrichment. Since enrichment decreases the available cage space, one could infer that this could negatively impact the rodent. In contrast, it is possible that the same enrichment in a large cage could be beneficial. Until these types of questions are subject to investigation, it is impossible to predict the impact that cage environment has on the outcome of animal experimentation.

The goal of this study was to evaluate the effects of cage size and cage enrichment on the cardiovascular stress responses of rats that were housed in one

of three standard rodent cages and provided with or without two enrichment items (i.e. tube and shelf). Cages containing a tube and a shelf were referred to as 'enriched cages' in this study, and use of the term 'enrichment' refers only to these particular enrichment items. Experimental measures of stress were HRV, HR and BP. Studies have shown that when rats are stressed by their environment, there is a corresponding increase in corticosterone levels, which increases SNA, HR and BP while decreasing HRV (Dunn *et al*, 1987; Dunn & Swiergiel, 2008; Gonzales-Burgos & Cuevas-Alvarez, 1992; Inagaki *et al*, 2004; Kant *et al*, 1987; Moyaho & Valencia, 2002; Nijssen *et al*, 2000; Olsson & Dahlborn, 2002; Suton *et al*, 1982; Veldhuis & de Wied, 1984; Wood *et al*, 2003; Wurbel, 2001; Wurbel & Stauffacher, 1996; Wurbel & Garner, 2007).

#### *Hypothesis*

It was anticipated that: rats housed in enriched and/or larger cages would have a lower output of sympathetic nervous activity (i.e. reduced HR, BP; elevated HRV) compared with the rats housed in the smaller cages with no enrichment item present.

#### **Materials and Methods**

**Animals & Housing:** Data were collected from five male Sprague-Dawley rats (1-2 years old) obtained from Charles River Laboratories (Wilmington, MA). Each of the rats was pre-implanted (by Charles River) with a telemetric transducer (C50 PXT, Data Sciences International, St. Paul, MN). Data collection started one month after surgical implantation of telemetric devices. Because rodents are social animals (Institute of Laboratory Animal Research, 1996), each rat was housed, for the entire study, with a non-implanted male cage-mate of equivalent age and size. Three of the implanted rats weighed 350g and two weighed 500g at the start of this study. The small cage was the smallest available, based on weight-age, as dictated by the university animal facility (i.e. IACUC). Likewise, the large cage was the next largest commercially available size offered by the University of Arizona animal facility. Due to the

abovementioned weight difference, three cage sizes were used. The smaller rats (350g) were housed in the 40.8 cm (L) x 21.0 cm (W) x 16.8 cm (H) small cage and the 40.6 cm (L) x 30.5 cm (W) x 30.5 cm (H) large cage, whereas the larger rats (500g) were housed in the 40.6 cm (L) x 30.5 cm (W) x 30.5 cm (H) "small cage" (relative to their weight) and the 58.5cm (L) x 35.2cm (W) x 39.1cm (H) "large cage". In both cases the small cages provided the rats with a floor area of 2.5 cm<sup>2</sup> per gram weight and the large cages provided 3.5 cm<sup>2</sup> / g (350 g rats) or 4.0 cm<sup>2</sup> / g (500 g rats).

Before the experiment the rats were housed in pairs in large, enriched cages because our previous preliminary studies (Baldwin *et al*, 2005) showed that rats housed in large, enriched cages demonstrated less aggressive nocturnal behavior than those housed in small, un-enriched cages.

At the start of the experiment the rats were housed in the small un-enriched cage (SU) and (after the first 3 week assessment) were randomly assigned to each of the other three cage conditions [small enriched (SE), large un-enriched (LU), and large enriched (LE)] until they had experienced (cycled through) each condition once. The fact that there were five pairs of rats and three conditions meant that there would sometimes be two pairs of rats experiencing the same condition during a given period. During the first week, the rats were allowed to acclimate to their new surroundings. Thereafter, data were collected twice a day (8 AM and 8 PM), three days a week, for two weeks. No data were collected from the five cage-mates. All of the cages were provided with a layer of pine shavings as bedding. The enrichment items consisted of a polyvinyl chloride (PVC) tube (19.8cm (L) x 11.2cm (D)) and a wire mesh shelf (40.6cm (L) x 10.2cm (W); or 21.8cm (L) x 36.2cm (W)). PVC tubes and wire mesh shelving units (representative of a nesting environment and an escape route, respectively) were utilized in this study because these items increase the complexity of standardized cages, while stimulating the rodent's natural species-specific behaviors (nesting behaviors and subordinate rat escape behaviors, for

example) (Balcombe *et al*, 1999; Clausing *et al*, 1989; Committee on Recognition and Alleviation of Distress in Laboratory Animals, 2008; Kitchen & Martin, 1996). Our previous observations on seven pairs of rats videotaped for ten 10-minute periods each in the morning and evening (Baldwin *et al*, 2005) showed that on average each rat spent 51%  $\pm$  20% of the observation time interacting with either of the items. In addition, these items were chosen because of their widespread accessibility at most university animal facilities (Institute of Laboratory Animal Research, 1996). Enrichment items were present in the cage for the entire three-week time period. The cages were located in a university animal facility with a 12 hour light-dark cycle (lights on at 6 AM and off at 6 PM) and controlled temperature (20 – 22°C) and humidity (48 – 52%). Rat diet consisted of Harlan Teklad 7001 rat chow (Harlan Teklad, Madison, WI) and de-ionized water, chlorinated to 10 ppm. Cages were changed (i.e. new bedding was provided) once every week. Food and water were provided *ad libitum*. This study adhered to all IACUC and University of Arizona Animal Care Facility regulations. Sentinel animals, tested quarterly for common viruses, are housed in each rodent room and quarterly environmental microbiological surveys are performed. In either case, if problems occur, steps are taken immediately to identify and correct the underlying cause.

*Telemetry System:* The use of radio-telemetric transducers (C50 PXT, Data Sciences International, St. Paul MN) allowed rat BP and electrocardiogram (ECG) to be monitored remotely. The rats' HR, HRV, and BP were determined via analysis of the ECG and BP waveform recordings, respectively using Dataquest A.R.T. 3.0 Analysis software (DSI, St. Paul, MN). To calculate HRV, the inter-beat interval (IBI), or time between beats, was extracted from the ECG waveform. In order to accurately evaluate the rats' HRV from the ECG waveform, variations in IBI of more than 5% from the previous inter-beat-interval were carefully checked against the ECG waveform and removed from the data if the wave-

form was anomalous (this accounted for less than 1% of the data). Such apparent large variations typically reflect a measurement issue rather than a true recording of IBI (Marchant-Forde *et al*, 2004). To ensure adequate fidelity ECG and BP signals were sampled at 1000Hz.

*ECG Data Acquisition & Processing:* ECG and BP were recorded at 8 AM and at 8 PM for 25 minutes; cycling two at a time between the five rats for 2.5 minutes, giving a total of 10 minutes per rat. Blood pressure readings were averaged over the AM and over the PM periods. During the 25-minute data collection period, video recordings of the rats were acquired for 10 minutes (2 minutes per rat) for behavioral analysis. Analysis of the ECG waveforms was performed to quantify the sympathetic and parasympathetic neural inputs that govern the variability in time between heart beats (i.e. HRV) (Stein *et al*, 1994; von Borell *et al*, 2007). A Fast Fourier Transformation (FFT) was used to transform the data from the time domain to the frequency domain, in terms of a power spectral density (Balcombe *et al*, 2004; Baldwin *et al*, 2005; Kleiger *et al*, 2005; Kuwahara *et al*, 1994; Ning *et al*, 2006; Stein & Kleiger, 1999; von Borell *et al*, 2007). Data were pooled for each rat over the 6 measurement periods (3 times a week for 2 weeks AM and similarly for PM).

The power spectral density captures the frequency content of random processes and helps identify periodicities. Specifically, the frequency-domain analysis yields data in two distinct frequency ranges that allows for the assessment of the relative contributions of the sympathetic and parasympathetic autonomic nervous systems (Ning *et al*, 2006; Stein *et al*, 1994; Tsai *et al*, 2002). The low frequency (LF) range (0.25 – 1.0 HZ) encompasses the sympathovagal balance of the autonomic nervous system inputs (i.e. sympathetic and parasympathetic) (Aubert *et al*, 1999; Japundzic *et al*, 1990; Kuwahara *et al*, 1994; Malik & Camm, 1993; Ning *et al*, 2006; Stein *et al*, 1994; Stein & Kleiger, 1999; von Borell *et al*, 2007). The high frequency (HF)

range (1.0 – 3.0 HZ) reflects the parasympathetic and mechanical-respiratory components (Japundzic et al, 1990; Kuwahara et al, 1994; Ning et al, 2006; Perlini et al, 1995; Rubini et al, 1993; Stein & Kleiger, 1999; Tsai et al, 2003; von Borell et al, 2007). Thus the ratio of the LF power component to the HF power component (LF/HF) reflects the contribution of SNA and PNA to the beat-to-beat changes in HR (Aubert et al, 1999; Kuwahara et al, 1994; Malik & Camm, 1993; Ning et al, 2006; Rubini et al, 1993; Stein & Kleiger, 1999; Tsai et al, 2003; von Borell et al, 2007). An increase in LF/HF (i.e., decreased HRV) is illustrative of a change in the sympathovagal balance between SNA and PNA (Inagaki et al, 2004; Japundzic et al, 1990; Kuwahara et al, 1994; Ning et al, 2006; Rubini et al, 1993; Stein & Kleiger, 1999; Tsai et al, 2003; von Borell et al, 2007). Studies have shown that a significant increase in LF/HF ratio is representative of an increased stress (analogous to a significant increase in corticosterone levels) (Inagaki et al, 2004; von Borell et al, 2007). Specifically, a significant increase in a rodent's stress response(s) (cardiovascular and behavioral) elicits a corresponding increase in the rodent's LF/HF ratio, which is inversely proportional to HRV (i.e. there is a corresponding reduction in HRV). In summary, increased stress responses cause a reduction in HRV.

**Behavioral Observations:** Activity data were collected for ten minutes (2 minutes per rat) in tandem with the cardiovascular data. The video recordings were performed using a video recorder (Sony Digital Camcorder, Model #SCD 23) attached to a tripod that was manually focused on each cage for two minutes in turn. The order of recording of each cage was randomized in case the order made a difference to the results. In the dark phase the camera was operated in the infrared mode. The tripod was kept in the same position on the floor so as not to disturb the animals. Rat behaviors involving activity were classified from video recordings by means of an established Rat Ethogram (Table 1) (Dunn et al, 1987; Gonzales-Burgos & Cuevas-Alvarez,

1992; Moyaho & Valencia, 2002; Wood et al, 2003). Behaviors were enumerated by noting each time a specified behavior was performed and by recording the duration of that behavior. Behaviors were then evaluated to quantify the amount of time, during the observation period, the rats spent performing each of the specified behaviors. These data were then converted into the percentage (%) of total time (AM and PM) each rat spent performing active behaviors. All measurements on a given rat for AM or for PM were averaged over a given cage condition (i.e. 3 measurements per week for 2 weeks AM and similarly for PM). During the first three weeks of the experiment the videotapes were examined to determine which animal of each pair was dominant. The two animals of each pair could be distinguished from each other because the implanted animal had an ear tag. In each case the number of times each one of the pair initiated an encounter with the other within a given time period was recorded. For all five pairs of rats there was very little difference in these numbers, indicating that there were no dominance issues at least at the start of the experiment.

**Statistical Analysis:** The data were analyzed with a 3-way repeated measures ANOVA with time (AM vs PM) as the first within-subject factor, condition (enriched vs un-enriched) as the second and cage size (small vs large) as the third. Post hoc analysis was performed using paired Student t-test adjusted

**Table 1.**

<b>Active (Locomotive)</b>
Moving on cage floor
Moving on shelf
Moving on/off of tunnel
Moving in/out of tunnel
Climbing up/down from shelf
Eating
Drinking

for multiple comparisons. A probability of  $p < 0.05$  was deemed significant.

## Results

### *Effects of Age and Treatment Order on the Study*

Even though age and treatment order were not explicitly included in the ANOVA, they are present as Between-Subject difference (ie, if there was an impact of age or order, there would have been a significant Between-Subjects effect). As Between-Subjects differences were not observed in any of the ANOVAs, we can conclude that age and treatment-order do not appear to affect the outcome of this study.

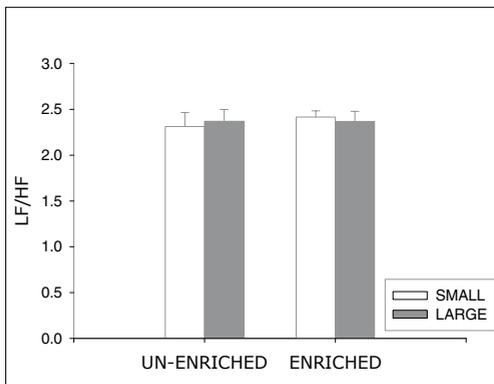
### *Effect of 'Sleep/Wake Cycle' on Rodent Stress as Measured by LF/HF*

There was no discernible difference in LF/HF between the four cage conditions, when considered independent of sleep/wake cycle (Figure 1). This was also true for HR, BP and activity levels (data not shown). To determine the extent to which the rodents' sleep/wake cycle affected SNA under the

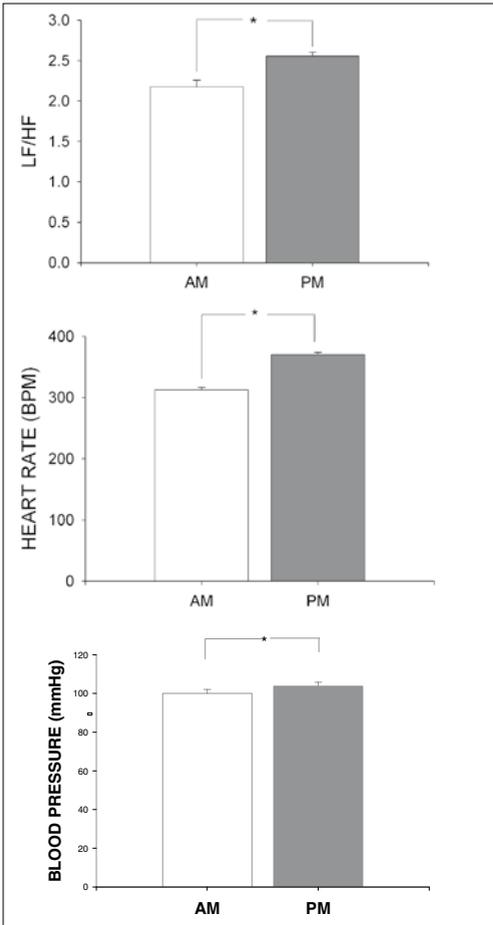
different cage conditions, LF/HF was examined in the morning (sleep) relative to the evening (wake). First, data are presented pooled over all four of the cage conditions. Analysis of the ECG recordings revealed an increase in LF/HF (i.e. SNA), when the rats were awake and active ( $p < 0.05$ ,  $F = 32.3$ ) (Figure 2a). Since the HF component (primarily parasympathetic nervous activity) was not different, regardless of cage condition or time of day (data not shown), the increase in LF/HF ratio reflects an increase in SNA. This elevation in LF/HF is consistent with the observed increases in HR ( $p < 0.05$ ,  $F = 169.21$ ) and BP ( $p < 0.05$ ,  $F = 6.76$ ) during the evening (Figure 2a). As expected, based on the rats' nocturnal behavior, the amount of time spent in the active state increased during the evening ( $p < 0.05$ ,  $F = 80.47$ ) (Figure 2b). In summary, the data suggest that rats experience an increase in LF/HF, HR, BP and active behaviors during the evening, when they are awake.

### *Effects of 'Cage Enrichment' on Rodent Stress as Measured by LF/HF*

To determine if the presence of cage enrichment, regardless of cage size, induced a change in rodent SNA, the effect of cage enrichment on LF/HF, independent of time of day, was investigated. There was no discernible difference in LF/HF, BP or in activity levels between the un-enriched and enriched cages, when considered independent of sleep/wake cycle (data not shown). However, HR was significantly higher in rats housed in large enriched cages compared to the other conditions ( $353 \text{ bpm} \pm 10$  (standard error) for LE vs  $333 \pm 11$  for SE,  $337 \pm 12$  for LU and  $341 \pm 13$  for SU;  $p < 0.05$ ,  $F = 7.22$ ). This difference in HR was not accompanied by any differences in activity levels. When the sleep/wake cycle was taken into account, no significant difference in LF/HF between the un-enriched and the enriched cages was observed in the AM or the PM. However, as mentioned previously, when AM and PM values of LF/HF were compared there was an increase in LF/HF (i.e. increase in SNA) when the rats were awake compared to asleep (Figure 2a).

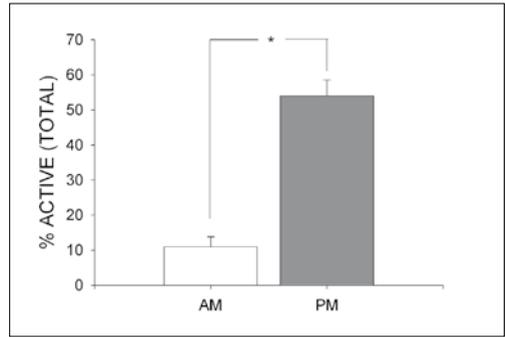


**Figure 1.** Mean LF/HF ratio for each of the four cage environments: small un-enriched, small enriched, large un-enriched, and large enriched. There was no significant difference in LF/HF across each of the four cage conditions when considered independent of time of day. \* $P \leq 0.05$  was considered significant. Error bars represent standard errors.

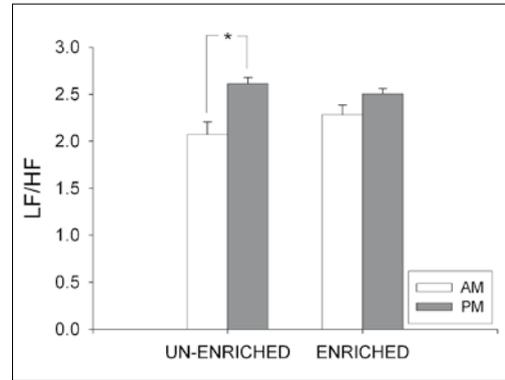


**Figure 2a.** Effect of diurnal cycling on mean values of LF/HF, HR and BP, regardless of cage condition. \* $P \leq 0.05$  was considered significant. Error bars represent standard errors.

This increase in LF/HF (PM vs. AM) was driven by the un-enriched cage condition ( $p < 0.05$ ,  $F = 5.63$ ) as no significant change in LF/HF (PM vs. AM) was observed in the enriched environment (Figure 3a). On the other hand, the differences in activity levels observed between AM and PM were seen in both enriched and un-enriched conditions ( $p < 0.05$ , Figure 3b). This was true for HR and BP (see summary data in Figure 5). In summary, the data suggest that enrichment significantly reduces the difference in



**Figure 2b.** Effect of diurnal cycling on percentage activity, regardless of cage condition. \* $P \leq 0.05$  was considered significant.

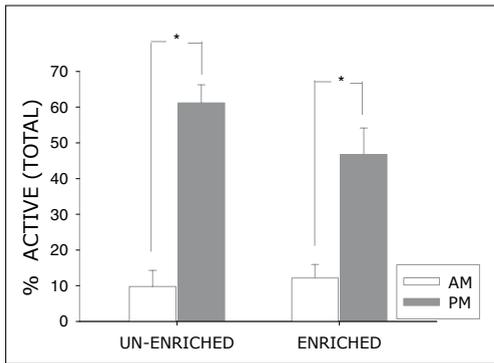


**Figure 3a.** Mean LF/HF ratios for the un-enriched and enriched cage conditions in the PM relative to the AM. LF/HF was significantly greater for the un-enriched cage condition only. \* $P \leq 0.05$  was considered significant. Error bars represent standard errors.

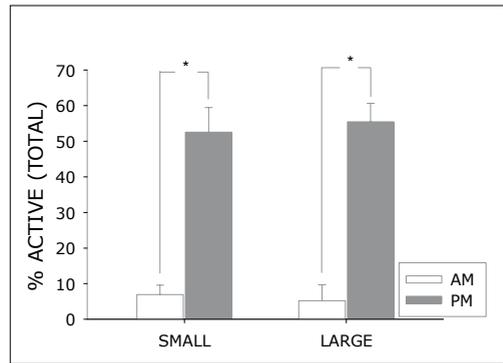
sympathovagal balance (LF/HF) experienced by the rats throughout the sleep/wake cycle in the un-enriched cage condition and that this effect is not explained by a significantly reduced variation in activity levels.

*Effects of 'Cage Size' on Rodent Stress as Measured by LF/HF*

To determine if different cage sizes elicit a specific rodent SNA response, regardless of the presence of

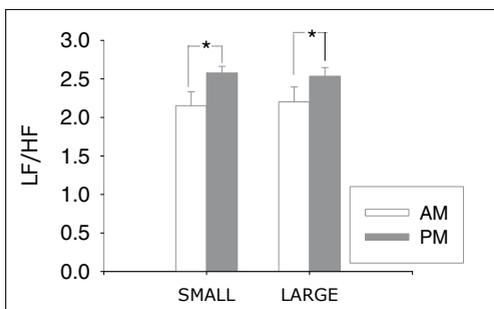


**Figure 3b.** Average percentage locomotive activity for the un-enriched and enriched cage conditions in the PM relative to the AM. \* $P \leq 0.05$  was considered significant. Error bars represent standard errors.



**Figure 4b.** Average percentage locomotive activity for the small and large cage conditions in the PM relative to the AM. \* $P \leq 0.05$  was considered significant. Error bars represent standard errors.

cage enrichment, the effect of cage size on LF/HF, independent of time of day, was investigated. There was no discernible difference in LF/HF, HR, BP or in activity levels between the small and large cages, when considered independent of sleep/wake cycle (data not shown). However, as mentioned previously, there was an increase in LF/HF (i.e. increase in SNA) when the rats were awake (Figure 2a). This increase in LF/HF (PM vs. AM) occurred for both the small cage condition ( $p < 0.0003$ ) and for the large cage environment ( $p < 0.01$ ) (Figure 4a). In ad-



**Figure 4a.** Mean LF/HF ratios for the small and large cage conditions in the PM relative to the AM. LF/HF was significantly greater in the PM for both small and large cages. \* $P \leq 0.05$  was considered significant. Error bars represent standard errors.

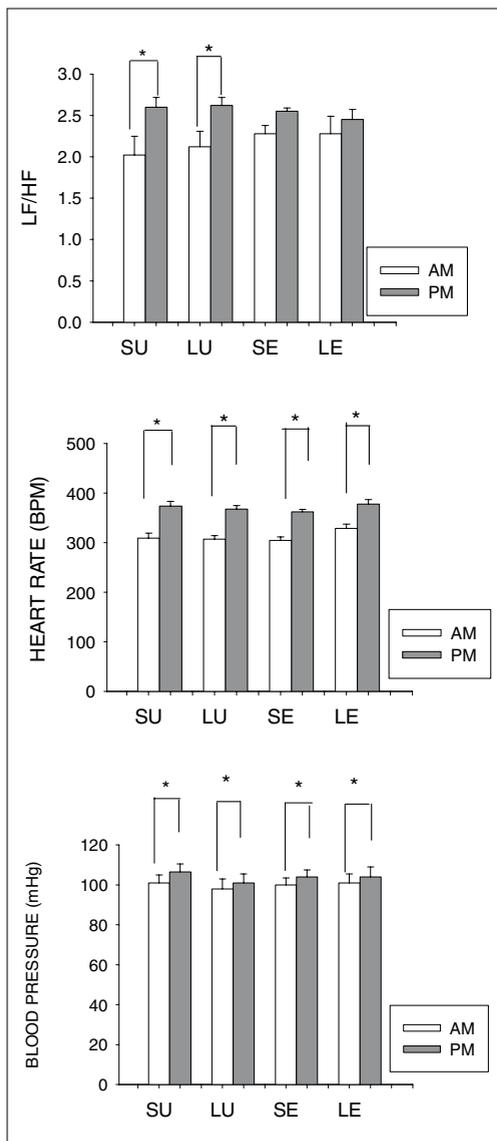
dition, the difference in activity levels observed between AM and PM was seen in both small cage and large cage conditions ( $p < 0.05$ , Figure 4b). This was true for HR and BP (see summary data in Figure 5). In summary, the data show that an increase in cage size above the recommended minimum, regardless of the presence or absence of enrichment, was not sufficient to reduce the difference in LF/HF experienced by the rats throughout the sleep/wake cycle.

#### *Effects of 'Cage Enrichment' and 'Cage Size' on Rodent Stress as Measured by LF/HF*

This study showed that the presence of enrichment in the cage reduced the difference in LF/HF experienced by the rats throughout the sleep/wake cycle. However, increasing cage size had no significant effect except to cause an increase in HR in the enriched condition. There was no measurable interaction between cage size and presence of enrichment regarding LF/HF. The data are summarized in Figure 5. The results for HR and BP are also included.

#### **Discussion**

The goal of this study was to assess the effects of cage enrichment and/or cage size on rat cardiovascular stress responses by measuring the rats' LF/HF, HR, and BP in four different housing conditions: SU,



**Figure 5.** Mean LF/HF ratio HF and BP for each of the four cage environments: small un-enriched (SU), small enriched (SE), large un-enriched (LU), and large enriched (LE) (AM vs. PM). LF/HF significantly increased in the PM relative to the AM in the un-enriched cage condition for both the small and large cage sizes. \*P<0.05 was considered significant. Error bars represent standard errors.

SE, LU and LE. In accordance with previous studies of normal rodent (nocturnal) cardiovascular function, it was established that LF/HF (i.e. SNA), HR, BP and activity levels increased in tandem during the evening when the rats were awake. Furthermore, addition of enrichment, regardless of cage size, significantly reduced the apparent diurnal rhythm in LF/HF observed in the un-enriched cages.

*Confirmatory Results and Novel Findings:*

As expected, rat SNA and overall activity increased concomitantly during the evening. Previous studies have established that when rats are awake and alert there is a corresponding increase in HR, BP (Miki & Yoshimoto, 2005; Smith et al, 1987) and LF/HF (Hashimoto et al 1999). The increase in these cardiovascular parameters at night when the animals are active compared to during the day when they are mostly sleeping has been termed a diurnal rhythm. It should be noted that all of these previous studies were conducted on rodents housed in standardized small un-enriched cages.

Although the present study does confirm results from these previous experiments, the data further establish that the increase in LF/HF, but not the increase in HR and BP, from day to night includes a cage enrichment-dependent component. For instance, LF/HF (i.e. SNA) increased in un-enriched cages, when the rats were awake and active, whereas, there was no significant change in LF/HF when rodents were housed in enriched cages. It is important to separate out possible effects of physical activity from effects of the stress response on LF/HF. The more active a rodent becomes, the greater the increase in SNA (Miki & Yoshimoto, 2005; Sherwin, 2004). However, in this study it was found that the percentage of time that the rats spent in locomotive activities, or eating or drinking (activities that also involve movement) while being videotaped, was independent of the cage conditions. In addition, in humans at least, mild physical activity has been found to mainly affect the very low frequency component of HRV (< 0.03 Hz) (Bernardi et al, 1996) that was not considered in this study. Thus it is highly unlikely

ly that the disappearance of the diurnal rhythm of LF/HF when the rats were housed in enriched cages was caused by differences in activity levels. Interestingly, one study (Inagaki *et al*, 2004) reported that experimentally produced anxiety states in rats resulted in a significant increase in HR and LF/HF with no change in HF compared to control conditions, similar to the PM versus AM responses we observed in rats housed in un-enriched cages. The study by Inagaki *et al* was performed when the rats were in the dark phase. Another study showed that when miniature swine were housed together in pairs instead of in isolation, the diurnal rhythm of LF/HF also disappeared (Kuwahara *et al*, 2004).

The present study provides further evidence that changes in sympathovagal balance of caged animals throughout the day are dependent on the housing environment. When the rats were in the enriched cages they showed slightly more sympathetic activation during the day and less at night compared to the un-enriched condition. During the day the pairs of rats were often sleeping in close contact with each other in the tunnel and this close proximity may have stimulated the locus coeruleus, a part of the brain that mediates arousal and exploratory activity and is involved in stress reactions, leading to release of norepinephrine from the resident noradrenergic neurons (Rosenzweig *et al*, 1999). On the other hand, at night when the rats were awake, the tunnel and shelf may have provided a refuge for one of the pair, should the other become aggressive, thus circumventing stimulation of SNA. Since the rats showed a distinct preference for interacting with the tunnel and shelf, this suggests that the enriched cage condition is beneficial to animal welfare.

#### *Advantages and Limitations of Experimental Design*

The experimental design used in this study had several major benefits. Firstly, well established measures of stress (i.e. LF/HF, HR, and BP) were used to evaluate the rats' response to the different cage conditions and also measures could be taken remotely by means of the radiotelemetric technology.

Secondly, by cycling the rats through all four of the conditions, each rat acted as its own control. By this design the sensitivity of each test was optimized, thereby decreasing the number of animals needed for the experiment. Thirdly, in this study the impact of two commonly available enrichment items on rats was methodically and critically assessed. By using only two enrichment items, this study reduced the confounding influence of multiple items on experimental outcome.

The occurrence of fewer confounding variables leads to a reduction in the number of animals needed for an experiment, while increasing the reproducibility of each experiment. The practical application of enrichment items in previous animal studies has yielded experimental results that have been largely inconsistent due to the lack of scientific scrutiny of these items prior to their application (Clausing *et al*, 2006). Enrichment items need to be critically assessed to determine if they meet the criteria for enrichment as set forth by The Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources (ILAR), 1996, which states that: "Animals should be housed with the goal of maximizing species-specific behaviors, increasing animal-to-habitat interactions, and minimizing stress-induced behaviors."

Fourthly, very few studies have systematically addressed the impact of increased cage size alone or in combination with cage enrichment on experimental outcomes. Since cage enrichment obviously takes up available space that rodents normally would use to perform their natural behaviors, the ratio of space taken by cage enrichment to total cage volume becomes an important issue that should be addressed. While there are currently criteria available for the minimum cage space (258 cm<sup>2</sup> (floor area) x 17cm (h) / 350g rat) allowed, these AALAS guidelines have not been updated in almost fifteen years (Institute of Laboratory Animal Research, 1996). The minimal rodent requirements needed for animal welfare and procurement of valid data have evolved over time, thereby necessitating the need for housing conditions to be re-assessed. Research needs to

be performed that systematically assesses the impact of 'enrichment' in a variety of cage sizes to determine if an enrichment item elicits a specific response based on an available cage volume. By using two different cage sizes (small and large) this study had the potential to identify any size-dependent effects that may have influenced the cage enrichment effect.

There were, however, some limitations with this study, the most prominent of which was small sample size used in this study. The low 'n' in this study is a consequence of using telemetric transducers. Wireless transducers restrict the number of animals that can be used in any given study because of their high cost and the limited data processing capacity of their accompanying receivers. Previous experiments performed on rats implanted with telemetric devices have all involved low numbers of animals. With respect to statistical power (the probability that the null hypothesis will be rejected when the alternative hypothesis is true), the fact that significant differences in heart rate variability (stress) in the AM vs PM were observed with the small number of animals argues that our power was adequate for that variable. What it does not address is whether other variables might become significant if we increased the number of rats in the study. Another important issue for future consideration is that any enrichment item identified here as 'beneficial' to rodents is not necessarily 'beneficial' to all animal species. Rodents inhabit specific environments that are ideally suited to their own species-specific needs and as such require particular items that may or may not fulfill the same purpose to another animal species. Another limitation of this study is that both increased cage size and/or the addition of an enrichment item raises the initial cost of experimentation. However, when the small standardized cages are used, there is a subsequent increase in the cost of the experiment due to an increase in variability between animals and hence the need to use more animals. Therefore, when choosing a cage environment the investigator must evaluate the cost-benefit ratio to determine the appropriate cage environment for their specific ani-

mal model and to determine whether or not enrichment will be 'beneficial' to their study.

An additional limitation of this study was that rodent cardiovascular data were only analyzed in the "implanted" rats. While rats are social animals they have been shown to develop a social hierarchy where one rodent may become dominant over its cage mate. Since physiologic patterns can change based on the established caste system it is possible that the rodent's cardiovascular response was a consequence of social stress rather than environmental stress.

### *Significance*

On the surface these results appear to show that cage environment does not affect rat BP, HRV or locomotive activity levels and this may give investigators reason to economize on rat accommodation, only needing to provide small un-enriched cages. However, this study has shown that the cage enrichment does affect the diurnal rhythm of HRV in rats. This means that different studies involving measurement of HRV in rats, for example rat models for human heart disease, can only be directly compared with each other if the cage environments are identical. More importantly, the items of enrichment selected for this study, the tunnel and the shelf, seemed to improve animal welfare, as demonstrated by our previous observation of the "increasing animal-to-habitat interactions" (*Baldwin et al, 2005*) defined by ILAR as a sign of enhanced animal welfare. Therefore it appears from this experiment that rats used for research purposes should all be provided with at least these types of enrichment.

The rodent is the most commonly used animal (~90%) in research today (including pharmacologic studies). Since it is critical that experimental outcomes are accurate and reproducible, it is important that the data obtained from rodents correctly represent the actual (average) response(s) to whatever perturbation is under investigation. Even though most investigators design their experimental protocols with this principle in mind, they often ignore the behavioral needs of the animal under in-

vestigation and how this will affect their data. It is therefore imperative that experimenters consider all of the pertinent behavioral and physiological responses that result from diurnal variations and from the rodent cage-environments when interpreting experimental data. Overall, the fact that the circadian rhythms for heart rate and blood pressure were conserved regardless of cage condition, but the circadian rhythm for HRV was not, implies that the latter rhythm is much weaker and only manifests in the absence of outside environmental stimuli. This is not surprising because HRV is a very sensitive physiological measure that is affected by emotions. As stated by Borell et al, 2007, "HRV is a promising approach for evaluating stress and emotional states in animals". From these results, it appears that HRV is an excellent parameter for monitoring the more subtle effects of the environment on caged animals; effects that might be missed by only measuring heart rate and blood pressure. Investigators who discount the more subtle consequences of housing rodents in un-enriched cages are in danger of denying their research animals a whole dimension of their normal life experience, that of emotion, and this will adversely affect not only animal welfare but will also oversimplify the interpretation of experimental data and its relevance to the human condition.

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ALB conceived and designed the experiments. AEB and AI performed the experiments. AEB, DTK, AI, and ALB analyzed the data and contributed reagents/materials/analysis tools. AEB, DTK, and ALB wrote the paper.

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