MATERIALS AND METHODS

Phase-shift experiments

Aged mice (C57bl/6 male) were purchased from the National Institute on Aging's Aged Rodent Colonies. Young control mice were retired breeders (8-12 months old estimated) from Charles River Laboratories. Once the mice arrived at our facility, they were immediately transferred to individual cages held in light-tight boxes. Initially all mice were housed in a 12:12 light dark cycle. Light intensity in the boxes ranged from 7 to 23 $\mu W/cm^2$ depending on the position in the box (Philips 40W fluorescent tubes). Ad-libitum food and water were provided throughout all 4 experiments. Animals were allowed one week of habituation before any adjustments to the lighting cycles were begun. The advance was accomplished by advancing the time of lights-on. The delay was accomplished by delaying the time of lights-off on the shift day. Cages were changed every week on the day before the shift. All experiments used naïve mice.

Glucocorticoid measurements

To determine the level of HPA axis activity in mice we have developed and implemented a non-invasive method for the measurement of fecal glucocorticoids by radioimmunoassay (*1-3*). The protocol was adapted from a method developed by T. Good and colleagues ((*4*) with minor modifications by T.E. Ziegler (personal communication). Briefly, aged animals (>28 m.o.; n=3 per group) were housed in metabolic cages (Nalgene) to separate feces and urine within a light-controlled dark box and provided with food and water ad-libitum. Sample collection tubes passed below the cages and through small holes in the bottom of the light-controlled box. Fecal samples were collected every six hours (Supplemental Fig 1a) or daily at 08:00h (Supplemental Fig 1b) through the attached dark box below the animals and immediately frozen at -80C until extraction of corticosterone.

To verify that fecal corticosterone determination is comparable to serum measurements we collected at 6h intervals in order to plot the 24h profile for three mice housed in a 12:12 L/D cycle. Similar to serum measurements, fecal corticosterone in these mice peaked between midnight and 0600.

Initially animals were allowed to habituate to the wire-bottom cages for a minimum of 2 weeks. Following this period, fecal samples were collected every 4 hours for 48 hours (n=3 each group). Additional aged mice were again habituated to the metabolic chambers and daily samples were collected for 6 days. On day 7 the 12:12 L:D cycle was either advanced (n=6) or delayed (n=3) and fecal samples were collected once daily for 7 days. On day 13 the L:D cycle was again advanced or delayed six hours, followed by 7 more days of fecal collection.

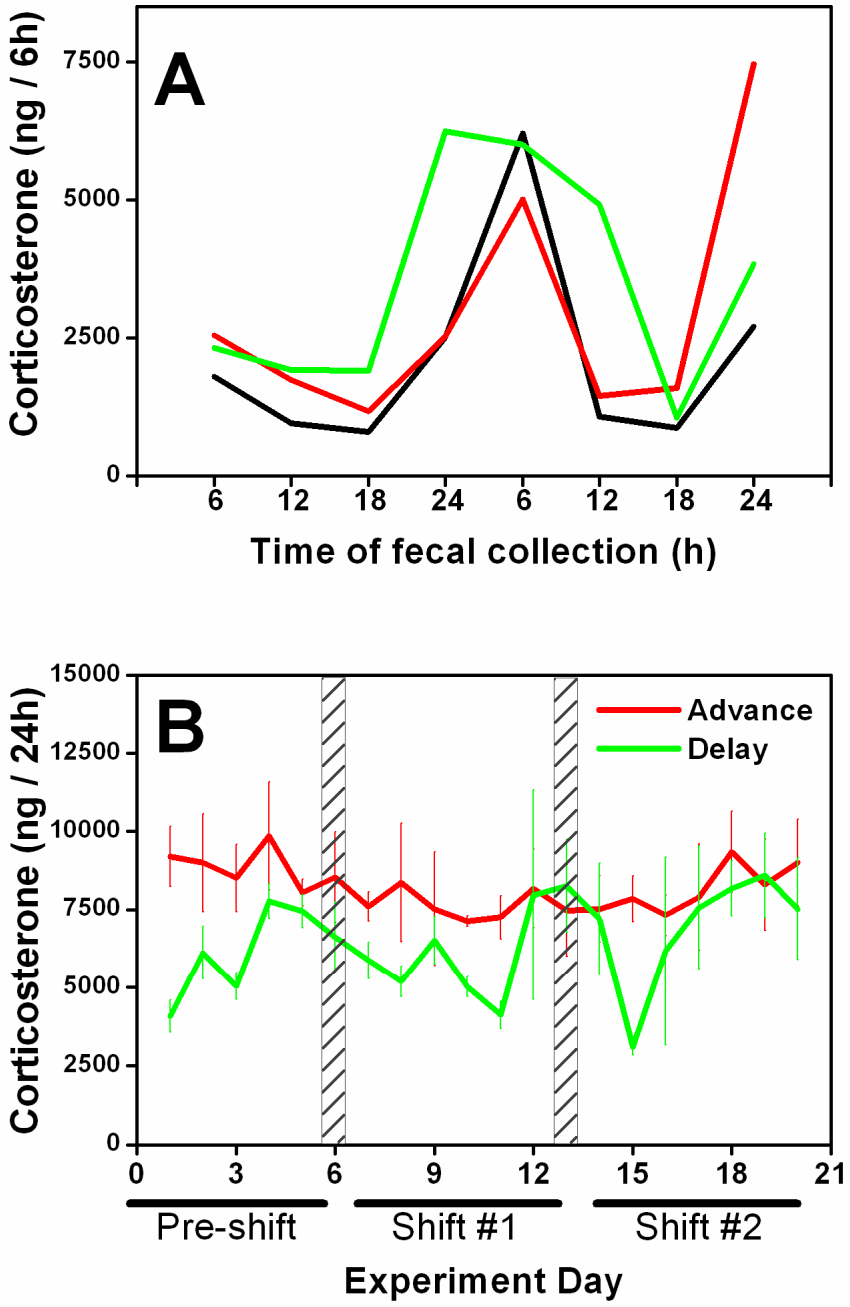
Fecal samples were thawed, separated from food particulate and weighed. A fraction of the total fecal mass (250mg) was isolated and saturated with 100ul of sterile ddH_20. Samples were then crushed with a glass rod (Kontes) in 1 ml of 90% MeOH and separated by centrifugation at 2500g for 15 mins. Supernatants were removed, dried in a vacuum evaporator and reconstituted in 300µls of 30% MeOH. Samples were poured onto equilibrated 3cc HLB hydrophobic solid-phase extraction columns (HLB 60mg, Waters Inc., Milford, MA). Columns were rinsed twice with 2 mls 20% MeOH and samples were eluted with 2 mls 100% MeOH by gravity. Samples were then dried in a vacuum evaporator and brought to a final volume of 300µls with 90% MeOH. For CORT RIA, 50 µls of fecal extracts was diluted 1:5 with buffer and 100uls was assayed for CORT by RIA according to the manufacturers protocol

(Corticosterone I^{125} RIA kit, MP Biomedicals, Orangeburg, NY). The total ng/mg fecal content was calculated and we have presented our data as ng/mg total feces produced per collection period. Inter- and intra-assay coefficients of variation were both <5%. Data were analyzed with one-way ANOVA for time, followed by student-neuman keul's post-hoc tests when appropriate. Differences were considered significant at $p<0.05$. All graphs were produced with Origin Pro 7 (Origin Labs Corp, Northampton MA) and statistics were conducted with Graphpad Prism 4 (GraphPad Prism Inc).

1. R. Palme, S. Rettenbacher, C. Touma, S. M. El-Bahr, E. Mostl, *Ann N Y Acad Sci* **1040**, 162 (Apr, 2005).
2. C. Touma, R. Palme, *Ann N Y Acad Sci* **1046**, 54 (Jun, 2005).
3. C. Touma, R. Palme, N. Sachser, *Horm Behav* **45**, 10 (Jan, 2004).
4. T. Good, M. Z. Khan, J. W. Lynch, *Physiol Behav* **80**, 405 (Nov, 2003).
5. S. Yamazaki *et al.*, *Science* **288**, 682 (2000).

Supplementary Figure 1. Fecal corticosterone in aged mice.

Phase-shifting results in temporary desynchrony among oscillators in mammalian organ systems (*5*) . Therefore we sought to determine if chronic stress is induced in mice undergoing repeated advance or delay phase shifts. Mice were housed in metabolic cages; repeated fecal collection was performed in a manner that did not disturb the mice. (A) To verify that fecal corticosterone determination is comparable to serum measurements, feces from three aged mice in a stationary 12:12 L/D cycle was sampled every 6h for two days. These mice displayed consistent 24h profiles of fecal corticosterone characterized by highest levels measured between 0000 and 0600h ($p<0.01$ $F=4.125$). (B) Aged mice (n=4 advancers; n=3 delayers) were housed in metabolic cages for 3 weeks while total daily (24h) fecal corticosterone levels were assayed. Days 1-6 represent baseline 24h corticosterone levels in naïve (never shifted) aged mice. After the first or second shift of the light cycle (hatched vertical bars), data from neither group indicated an increase in glucocorticoid release above baseline levels. Data are presented as mean $\pm$ SEM. These data suggest that chronic stress does not underlie the increase in mortality observed in phase-shifted mice


A
Corticosterone (ng / 6h)
7500
5000
2500
0
6
12
18
24
6
12
18
24
Time of fecal collection (h)
B
Advance
Delay
Corticosterone (ng / 24h)
15000
12500
10000
7500
5000
2500
0
0
3
6
9
12
15
18
21
Pre-shift
Shift #1
Shift #2
Experiment Day