

RESEARCH ARTICLE

Body mass affects seasonal variation in sickness intensity in a seasonally breeding rodent

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ABSTRACT

Species that display seasonal variation in sickness intensity show the most intense response in the season during which they have the highest body mass, suggesting that sickness intensity may be limited by an animal's energy stores. Siberian hamsters (*Phodopus sungorus*) display lower body masses and less intense sickness when housed in short, winter-like days as opposed to long, summer-like days. To determine whether reduced sickness intensity displayed by short-day hamsters is a product of seasonal changes in body mass, we food restricted long-day hamsters so that they exhibited body mass loss that mimicked the natural photoperiod-induced loss of body mass in short-day hamsters. We then experimentally induced sickness with lipopolysaccharide (LPS) and compared sickness responses among long-day food-restricted and long- and short-day *ad libitum* fed groups, predicting that long-day food-restricted hamsters would show sickness responses comparable to those of short-day *ad libitum* fed hamsters and attenuated in comparison to long-day *ad libitum* fed hamsters. We found that long-day food-restricted hamsters showed attenuated LPS-induced anorexia, loss of body mass and hypothermia compared with long-day *ad libitum* fed animals; however, anorexia remained elevated in long-day food-restricted animals compared with short-day *ad libitum* fed animals. Additionally, LPS-induced anhedonia and decreases in nest building were not influenced by body mass. Results of hormone assays suggest that cortisol levels could play a role in the attenuation of sickness in long-day food-restricted hamsters, indicating that future research should target the roles of glucocorticoids and natural variation in energy stores in seasonal sickness variation.

KEY WORDS: Acute phase response, Cortisol, Ecoimmunology, Lipopolysaccharide

INTRODUCTION

Mounting an appropriate immune response to infection is a necessity for survival in a pathogen-rich environment. An organism's immune response is sensitive to both its external and internal environments and can be influenced by variables such as environmental temperature, photoperiod, stress, reproductive effort and energy stores (Sheldon and Verhulst, 1996; Demas and Nelson, 1998; Lochmiller and Deerenberg, 2000; Martin et al., 2008a). While each of these variables can fluctuate individually across short time spans, for seasonally breeding animals, these variables often change simultaneously with transitions between seasons. Therefore, one challenge for determining the specific physiological and environmental variables underlying seasonal changes in immunity is disentangling the effects of each of these variables from one another.

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The acute phase response (APR) is one component of the immune system that can vary seasonally (Adelman and Martin, 2009; Ashley and Wingfield, 2012). When the APR is activated at an infection site, neutrophils and macrophages release pro-inflammatory cytokines which not only facilitate the recruitment of other immune cells to this local site of infection but also act on the brain to generate sickness responses. Sickness responses are characterized by fever or hypothermia, anorexia, body mass loss, reductions in social and hedonic behaviors, and hypothalamic-pituitary–adrenal (HPA) endocrine axis activation (Hart, 1988; Tizard, 2008). These sickness symptoms are generated as adaptive mechanisms for fighting off infection (Hart, 1988), and in several seasonally breeding animals, sickness intensity varies across the seasons (Bilbo et al., 2002; Owen-Ashley et al., 2006, 2008; Owen-Ashley and Wingfield, 2006). Because generating a sickness response is critical for eliminating pathogens, variation in the magnitude of this response can profoundly affect an animal's ability to survive an infection (Kluger et al., 1975; Murray and Murray, 1979; Vaughn et al., 1980). Thus, questions remain: why and how are seasonally breeding animals modulating sickness intensity?

When looking across species in which seasonally variable sickness responses have been documented, the common variable that predicts which season an animal will show reduced or enhanced sickness is the animal's body mass, not reproductive state or day length (Ashley and Wingfield, 2012; Carlton et al., 2012). Specifically, seasonally breeding animals show attenuated sickness responses to the bacterial mimetic lipopolysaccharide (LPS) in the season in which they have the lowest body mass (Bilbo et al., 2002; Owen-Ashley et al., 2006, 2008; Owen-Ashley and Wingfield, 2006). Siberian hamsters (*Phodopus sungorus*) are one species that displays seasonal variation in sickness intensity. Hamsters display more intense sickness responses (i.e. greater fever amplitude, longer durations of and greater decreases in food intake and body mass loss and greater decreases in hedonic and nest-building behaviors) to LPS when housed in long, summer-like photoperiods compared with short, winter-like photoperiods (Bilbo et al., 2002; Wen et al., 2007; Wen and Prendergast, 2007). Hamsters housed in long photoperiods remain reproductively active and show higher body masses than hamsters housed in reproductively inhibiting short-day photoperiods. Previous studies have manipulated reproductive state and patterns of endogenous melatonin (an indoleamine hormone whose release acts as a physiological signal of photoperiod) to determine their contributions to seasonal variation in sickness intensity in Siberian hamsters (Freeman et al., 2007; Wen et al., 2007; Prendergast et al., 2008). While these manipulations modulated some sickness symptoms, they also resulted in body mass changes (Bilbo and Nelson, 2002; Wen et al., 2007; Prendergast et al., 2008). Therefore, while there were clearly effects of reproductive state and melatonin on seasonal variation in sickness, it is unclear whether intensity was mediated

directly by the manipulations or indirectly through changes in energetic state.

In a previous study, we manipulated a hormonal correlate of energetic state (i.e. leptin) in Siberian hamsters in order to disentangle the effects of seasonal energetic changes from other seasonally modulated variables on sickness intensity variation (Carlton and Demas, 2014). Leptin levels are directly proportional to adipose tissue mass in mammals (Maffei et al., 1995; Johnson et al., 2004), and leptin changes seasonally, in parallel with seasonal changes in body mass, in Siberian hamsters and other seasonally breeding animals (Horton et al., 2000; Concannon et al., 2001; Gaspar-López et al., 2009). In that previous study (Carlton and Demas, 2014), we experimentally elevated circulating leptin levels in short-day Siberian hamsters so that they were comparable to long-day levels. We found that leptin treatment resulted in short-day hamsters showing hypothermic responses to LPS that were more like those of long-day hamsters. Short-day hamsters treated with leptin, however, showed LPS-induced anorexia that was not enhanced to the level of long-day animals, but instead was attenuated even below that of typical short-day animals. These results suggest that leptin modulates some but not all aspects of seasonal sickness variation. As leptin is only one of many energetic hormones, our findings did not eliminate the hypothesis that seasonal variation in sickness intensity is mediated by changes in energy stores. The goals of the present study were to manipulate seasonal energy stores, by using food restriction in long-day Siberian hamsters, *P. sungorus* (Pallas 1773), to mimic the pattern of seasonal body mass loss in short-day hamsters, to determine the effects on sickness intensity and to elucidate a potential hormonal mechanism mediating any changes in intensity. If seasonal variation in sickness intensity is driven by seasonal changes in energy stores, then we expected long-day hamsters displaying body mass loss patterns like those of short-day hamsters to show similar sickness intensity to short-day hamsters and less intensity than long-day hamsters fed *ad libitum*.

RESULTS

An experimental timeline is presented in Fig. 1 in order to clarify at which time points measurements were taken.

Experimental induction of body mass loss

Body mass did not differ among the groups prior to experimental housing ($F_{2,72}=0.281$, $P=0.756$). After 70 days in experimental

photoperiods and food restriction for the long-day food-restricted group (LD-FR), body mass differed among the groups ($F_{2,72}=25.372$, $P<0.001$) (Fig. 2). Long-day *ad libitum* fed (LD-AL) hamsters showed no change in body mass from days 0 to 70 (paired $t=0.230$, $P=0.821$), while short-day *ad libitum* fed (SD-AL) and LD-FR hamsters showed a 22.3% and 22.0% decrease in body mass, respectively. There were no differences in body mass at day 70 between the LD-FR and SD-AL groups ($t_{17}=0.117$, $P=0.907$).

Pre-injection baseline measurements

At the end of those 70 days and until the end of the experiment, LD-FR animals were allocated food at their pre-restriction mean values. Pre-injection baseline food consumption differed among the groups ($F_{2,72}=12.143$, $P<0.001$; Table 1). Specifically, SD-AL hamsters consumed less food than LD-AL and LD-FR hamsters ($t>3.560$, $P<0.001$ for both comparisons), while there was no difference in consumption between the LD-AL and LD-FR hamsters ($t=1.181$, $P=0.241$). LD-FR body mass did increase slightly upon access to pre-restriction mean food levels (paired $t=5.105$, $P<0.001$); however, pre-injection baseline body masses still did not differ between the LD-FR and SD-AL groups ($t=1.495$, $P=0.139$; Table 1). While body mass did not differ between the LD-FR and SD-AL groups, serum leptin levels differed among all three groups ($F_{2,72}=25.412$, $P<0.001$; Table 1). Specifically, LD-AL hamsters showed the highest leptin concentrations, while LD-FR and SD-AL hamsters had leptin concentrations that were 33.5% and 67.1%, respectively, lower than this group.

Pre-injection baseline saccharin solution intake varied among the groups ($F_{2,72}=16.917$, $P<0.001$; Table 1). Specifically, LD-FR animals consumed greater volumes of saccharin solution compared with LD-AL ($t=3.428$, $P=0.001$) and SD-AL ($t=5.782$, $P<0.001$) animals. There was no difference in pre-injection baseline saccharin solution intake between the LD-AL and SD-AL groups ($t=1.646$, $P=0.104$). Pre-injection baseline percentage nesting material shredded also differed by group prior to injection ($H=15.105$, $P<0.001$; Table 1). Specifically, LD-FR animals shredded a lower percentage of their cotton nestlet compared with LD-AL ($Z=1.987$, $P=0.047$) and SD-AL ($Z=3.813$, $P<0.001$) animals. There was no difference in percentage nesting material shredded between the LD-AL and SD-AL groups ($Z=1.136$, $P=0.256$).

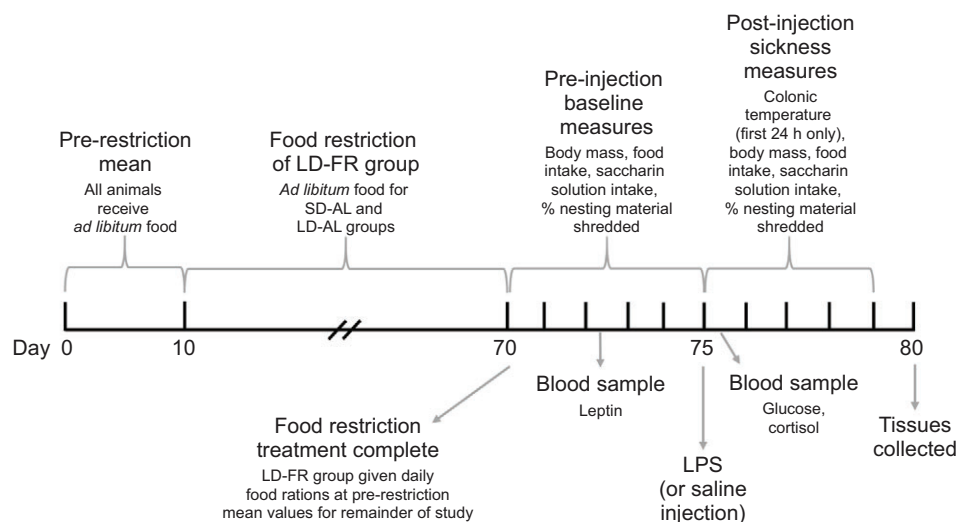


Fig. 1. Experimental timeline indicating when treatments were performed and when measurements were taken. Day 0 refers to the time point when each group was housed in their experimental photoperiods, after 1 week of acclimation to individual housing. LD, long-day photoperiod; SD, short photoperiod; FR, (previously) food-restricted hamsters; AL, *ad libitum* fed hamsters; LPS, lipopolysaccharide.

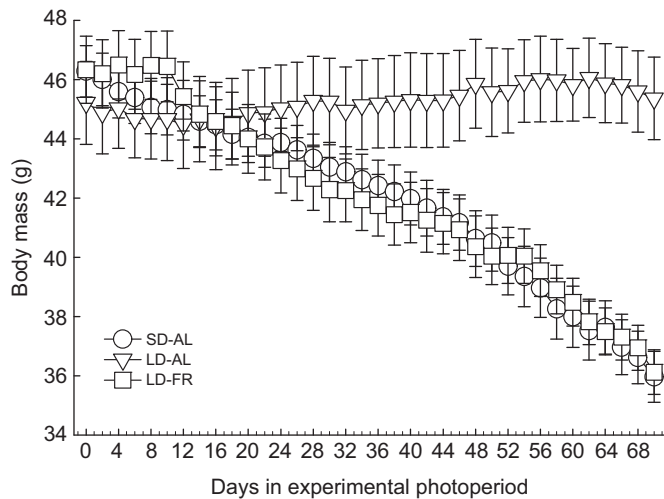


Fig. 2. Mean (\pm s.e.m.) body mass of male Siberian hamsters over the course of the first 70 days of experimental housing. Hamsters were either allowed *ad libitum* (AL) access to food during the experiment and housed in short- (SD) or long-day (LD) photoperiods or had restricted access to food (FR) and were housed in long-day photoperiod.

Post-injection sickness measurements

Anorexia

Percentage changes in food intake were affected by group ($F_{2,69}=5.325$, $P=0.007$) and by injection ($F_{1,69}=158.030$, $P<0.001$) but not by the group \times injection interaction ($F_{2,69}=0.314$, $P=0.731$; Fig. 3). Percentage changes in food intake varied across the 4 days after injection (within subjects, $F_{3,67}=157.130$, $P<0.001$) and with the time \times group ($F_{6,134}=3.521$, $P=0.003$) and the time \times injection ($F_{3,67}=157.130$, $P<0.001$) interactions. All LPS-treated animals showed greater decreases in food intake compared with their respective saline-treated controls during days 1, 2 and 3 post-injection ($P<0.003$ for all comparisons). By day 4, the LPS-treated LD-FR and SD-AL groups no longer showed reduced food intake compared with controls ($P>0.06$ for both comparisons); however, the LPS-treated LD-AL group still showed reduced food intake on this day compared with controls ($P=0.006$). Additionally, at day 4 post-injection, the LPS-treated LD-AL group showed a greater percentage

Table 1. Mean (\pm s.e.m.) pre-injection baseline food intake, baseline body mass, circulating serum leptin concentration, baseline saccharin intake and baseline percentage nesting material shredded in SD-AL, LD-AL and LD-FR male Siberian hamsters

	SD-AL	LD-AL	LD-FR
Baseline food intake (g day ⁻¹)	4.1 \pm 0.1 ^a	5.0 \pm 0.2 ^b	4.8 \pm 0.1 ^b
Baseline body mass (g)	35.5 \pm 0.8 ^a	45.3 \pm 1.4 ^b	37.3 \pm 0.7 ^a
Leptin concentration (pg ml ⁻¹)	7.07 \pm 0.96 ^a	18.88 \pm 1.41 ^c	12.90 \pm 1.15 ^b
Baseline saccharin solution intake (g 6 h ⁻¹)	1.5 \pm 0.1 ^a	1.8 \pm 0.2 ^a	3.0 \pm 0.3 ^b
Baseline nesting material shredded (%)	99.0 \pm 0.5 ^a	93.8 \pm 3.2 ^a	85.9 \pm 4.1 ^b

SD-AL, short-day *ad libitum* fed hamsters; LD-AL, long-day *ad libitum* fed hamsters; LD-FR, long-day (previously) food restricted hamsters. Pre-injection baseline measurements were calculated by averaging the values collected for the measurements during the 3 days prior to LPS or saline injection. During the 5 days prior to injection, animals in the LD-FR group were allocated food at 100% of their pre-restriction mean values. Different letters indicate statistically significant differences between group means ($P<0.05$); groups sharing the same letter are statistically equivalent.

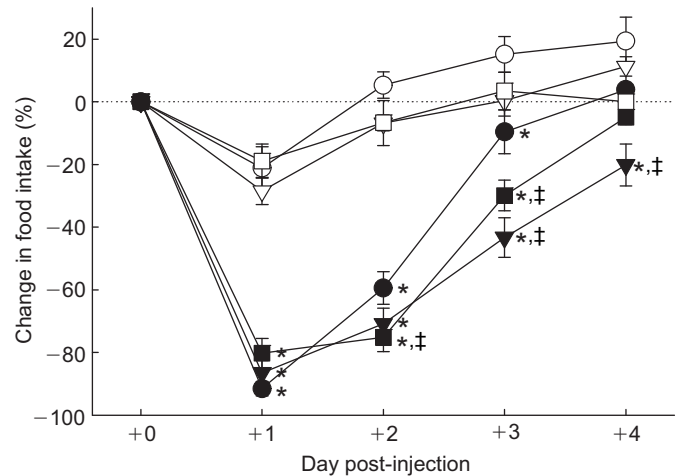


Fig. 3. Mean (\pm s.e.m.) percentage change in daily food intake from baseline following LPS or saline treatment. LPS (black symbols) or saline (white symbols) treatment was delivered on day 0 in SD-AL (circles), LD-AL (triangles) and LD-FR (squares) male Siberian hamsters. Day +1 represents the time period from 0 to 24 h after LPS or saline injection, day +2 represents the time period from 24 to 48 h after LPS or saline injection, and so forth. * $P<0.05$ versus saline-treated group exposed to the same photoperiod and food treatments; † $P<0.05$ versus SD-AL LPS-treated group.

decrease in food intake than the LPS-treated SD-AL group ($P=0.016$) but not the LD-FR group ($P=0.149$).

Body mass loss

Percentage change in body mass was affected by group ($F_{2,69}=11.896$, $P<0.001$) and by injection ($F_{1,69}=174.759$, $P<0.001$) but not by the group \times injection interaction ($F_{2,69}=1.034$, $P=0.361$; Fig. 4). Percentage change in body mass differed across the 4 days post-injection (within subjects, $F_{2,2,149,4}=7.545$, $P<0.001$) and with the time \times group ($F_{4,3,149,4}=2.809$, $P=0.024$), time \times injection ($F_{2,2,149,4}=12.564$, $P<0.001$) and time \times group \times injection

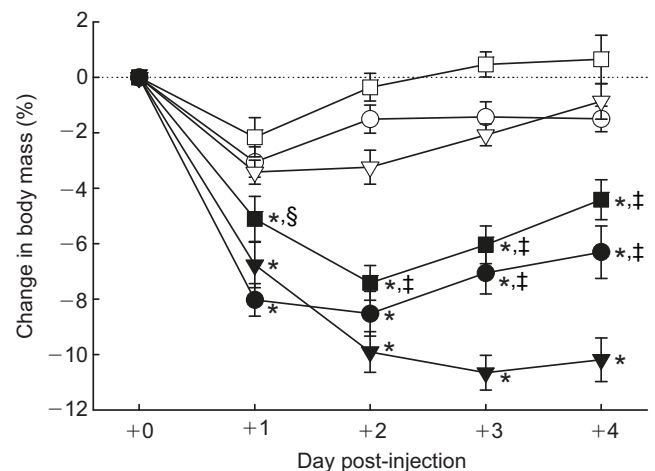


Fig. 4. Mean (\pm s.e.m.) percentage change in body mass from baseline following LPS or saline treatment. LPS (black symbols) or saline (white symbols) treatment was delivered on day 0 in SD-AL (circles), LD-AL (triangles) and LD-FR (squares) male Siberian hamsters. Day +1 represents the time period from 0 to 24 h after LPS or saline injection, day +2 represents the time period from 24 to 48 h after LPS or saline injection, and so forth. * $P<0.05$ versus saline-treated group exposed to the same photoperiod and food treatments; † $P<0.05$ versus LD-AL LPS-treated group; § $P<0.05$ versus SD-AL LPS-treated group.

($F_{4,3,149.4}=4.096$, $P=0.003$) interactions. During all 4 days after injection, LPS-treated animals showed a greater percentage decrease in body mass compared with their respective saline-treated controls ($P<0.001$ for all comparisons); however, the LPS-treated LD-FR and SD-AL groups showed attenuated percentage decreases in body mass relative to the LPS-treated LD-AL group. Specifically, the LD-FR group showed a smaller percentage body mass decrease compared with the LD-AL group on days 2, 3 and 4 post-LPS injection ($P<0.02$ for all comparisons), while the SD-AL group showed a smaller percentage body mass decrease on days 3 and 4 post-LPS injection ($P<0.002$ for both comparisons).

In LPS-treated animals, the percentage change in body mass was negatively correlated with pre-injection baseline body mass, such that animals with a greater initial body mass showed a greater percentage decrease in body mass on day 3 (body mass: $F_{1,34}=9.011$, $P=0.005$; group: $F_{2,34}=2.644$, $P=0.086$) and day 4 (body mass: $F_{1,34}=6.037$, $P=0.019$; group: $F_{2,34}=3.771$, $P=0.033$) but not days 1 or 2 ($P>0.05$ for body mass on both days). In addition, the maximum percentage change in body mass that each LPS-treated animal displayed after injection was not correlated with pre-injection baseline body mass (body mass: $F_{1,34}=1.890$, $P=0.178$; group: $F_{2,34}=2.852$, $P=0.0716$).

Body temperature

Colonic temperature differed by group ($F_{2,69}=7.368$, $P=0.001$) and injection ($F_{1,69}=44.515$, $P<0.001$) but not by the group×injection interaction ($F_{2,69}=1.302$, $P=0.279$; Fig. 5). Colonic temperature changed across time (within subjects, $F_{6,0,415.4}=180.880$, $P<0.001$) and with the time×injection ($F_{6,0,415.4}=34.795$, $P<0.001$) and time×group×injection ($F_{2,3,12.0}=2.329$, $P=0.007$) interactions. Among saline-injected animals, the SD-AL group showed higher temperatures than the LD-AL and LD-FR groups at 8, 10 and 16 h post-injection ($P<0.05$). LPS injection resulted in a hypothermic response across all groups at most time points after injection. However, at 2 h post-injection, LPS-treated SD-AL and LD-FR hamsters showed greater temperatures compared with their respective saline controls ($P<0.003$); the temperature of LPS-treated LD-AL hamsters did not differ from that of the saline-treated controls at this time point ($P=0.369$). LPS-treated animals in all groups exhibited hypothermic responses compared with their respective saline controls starting at either 4 h post-injection (LD-AL) or 6 h post-injection (SD-AL and LD-FR) through to the end of the 24 h measurement period ($P<0.05$ for all comparisons except SD-AL at 20 h post-injection). The temperature of the LPS-treated LD-AL group was lower than the temperature of the LPS-treated SD-AL group at 4, 8, 10 and 16 h post-injection ($P<0.05$ for these comparisons), while the temperature of the LD-FR group was only lower than that of the SD-AL group at 8 h post-injection ($P=0.016$).

Saccharin solution intake

Percentage change in saccharin solution intake was affected by injection treatment only ($F_{1,69}=7.600$, $P=0.008$) and not by group ($F_{2,69}=1.089$, $P=0.342$) or the group×injection interaction ($F_{2,69}=0.885$, $P=0.418$; Fig. 6A). Percentage change in saccharin solution intake varied across time (within subjects, $F_{2,5,173.8}=3.674$, $P=0.019$) and with the time×group ($F_{5,0,173.8}=4.966$, $P<0.001$) and time×injection ($F_{2,5,173.8}=9.081$, $P<0.001$) interactions. While there was considerable variance in this measure, *post hoc* analyses revealed that saccharin solution intake was reduced in the LPS-treated LD-AL and LD-FR groups during the 0–6 h time point compared with their respective saline-treated controls ($P<0.009$ for both comparisons).

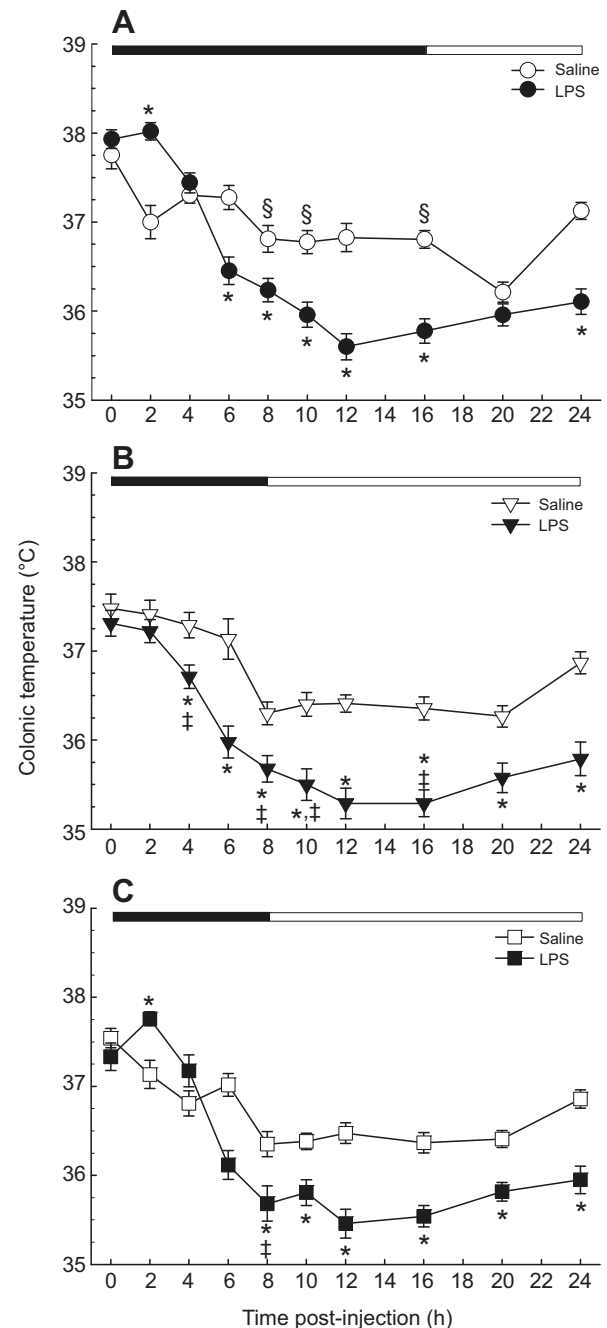


Fig. 5. Mean (\pm s.e.m.) colonic temperature following LPS or saline treatment. Treatment was delivered at the 0 h time point in SD-AL (A), LD-AL (B) and LD-FR (C) male Siberian hamsters. Black and white bars at the top of the graphs indicate the active, dark (black) and inactive, light (white) phases of the light–dark cycle for each photoperiodic morph. Within each panel: * $P<0.05$ versus saline-treated group exposed to the same photoperiod and food treatments. Across panels: † $P<0.05$ versus LPS-treated SD-AL group; § $P<0.05$ versus saline-treated LD-AL and LD-FR groups.

Nest-building behavior

Percentage change in nesting material shredded differed among the groups at the 0–6 h ($H=43.543$, $P<0.001$), 24–30 h ($H=59.684$, $P<0.001$) and 48–54 h ($H=50.964$, $P<0.001$) time points but not at the 72–78 h time point ($H=4.356$, $P=0.499$; Fig. 6B). Specifically, at the 0–6 h, 24–30 h and 48–54 h time points, all three LPS-treated groups showed decreases in nesting material shredded compared with their respective saline-treated controls ($Z>3.003$,

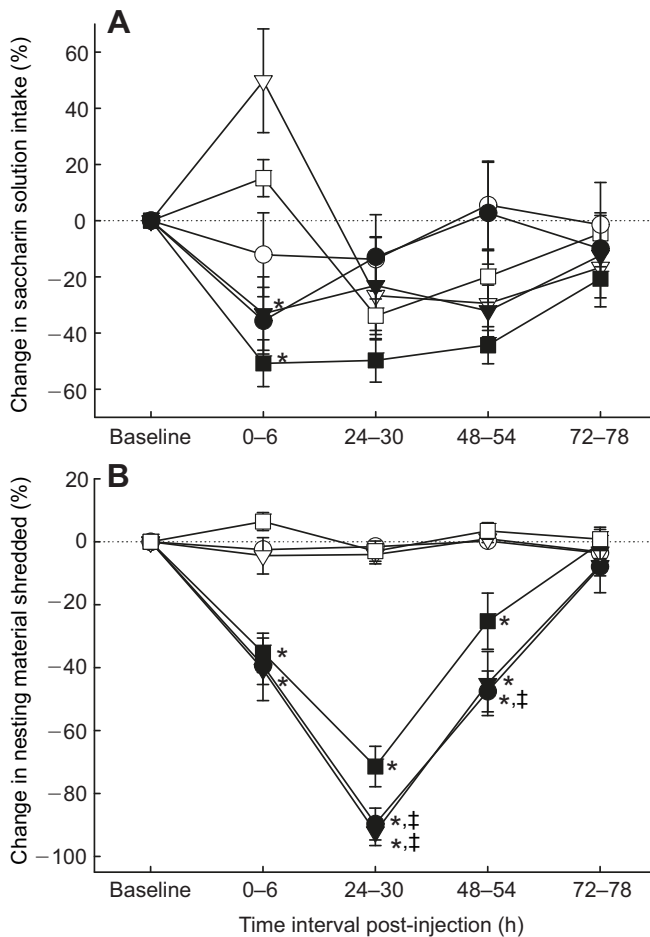


Fig. 6. Mean (\pm s.e.m.) percentage change from baseline in saccharin solution intake and nesting material shredded following LPS or saline treatment. (A) Saccharin solution intake; (B) nesting material shredded. LPS (black symbols) or saline (white symbols) treatment was delivered at the 0 h time point in SD-AL (circles), LD-AL (triangles) and LD-FR (squares) male Siberian hamsters. * P <0.05 versus saline-treated group exposed to the same photoperiod and food treatments; † P <0.05 versus LD-FR LPS-treated group.

P <0.002 for all comparisons); however, LPS-treated LD-FR animals showed a smaller percentage decrease in nesting material shredded than the LPS-treated SD-AL group at the 24–30 h ($Z=2.483$, $P=0.013$) and 48–54 h ($Z=2.037$, $P=0.042$) time points and the LPS-treated LD-AL group at the 24–30 h time point ($Z=2.111$, $P=0.035$).

Blood glucose

Blood glucose levels were affected by group ($F_{2,69}=3.77$, $P=0.028$) and injection ($F_{1,69}=114.23$, $P<0.001$) but not the group \times injection interaction ($F_{2,69}=0.24$, $P=0.784$; Fig. 7A). All LPS-treated hamsters showed lower blood glucose levels than their respective saline-treated controls ($t>5.75$, $P<0.001$ for all comparisons). Additionally, saline-treated LD-FR hamsters had lower blood glucose concentrations than saline-treated LD-AL hamsters ($t=2.42$, $P=0.018$).

Cortisol

Cortisol was affected by group ($F_{2,69}=18.082$, $P<0.001$) and injection ($F_{1,69}=65.005$, $P<0.001$) but not by the group \times injection interaction ($F_{2,69}=0.402$, $P=0.671$; Fig. 7B). LPS-treated animals from all groups showed elevated cortisol levels compared with their

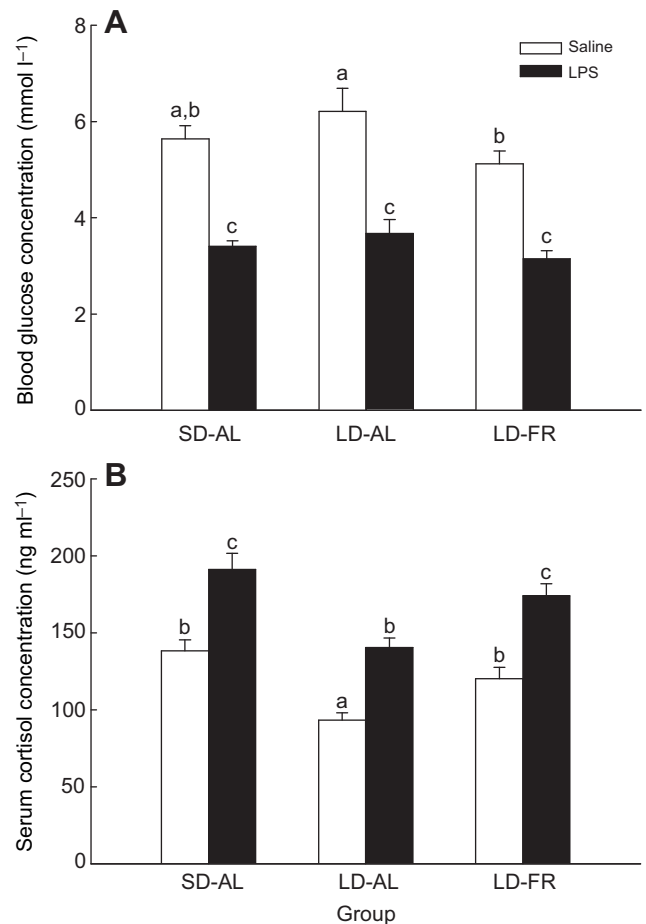


Fig. 7. Mean (\pm s.e.m.) blood glucose and circulating serum cortisol concentrations 4 h following LPS and saline treatment. (A) Blood glucose; (B) circulating serum cortisol. Treatment was delivered to SD-AL, LD-AL and LD-FR male Siberian hamsters. Within each panel, groups with different letters indicate statistically significant differences between group means (P <0.05); groups sharing the same letter are statistically equivalent.

respective saline-treated controls (P <0.001 for all comparisons). LPS-treated LD-FR and SD-AL animals had higher cortisol levels than LPS-treated LD-AL animals (P <0.02 for both comparisons), while saline-treated LD-FR and SD-AL animals had higher cortisol levels than saline-treated LD-AL animals (P <0.006 for both comparisons). Cortisol levels of the LPS-treated LD-AL animals were similar to those of saline-treated LD-FR and SD-AL animals (P >0.05 for both comparisons).

Tissue mass

Paired testes mass was affected by group ($F_{2,69}=1425.837$, $P<0.001$) but not by injection ($F_{1,69}=2.454$, $P=0.122$) or the group \times injection interaction ($F_{2,69}=0.373$, $P=0.690$; Table 2). Specifically, SD-AL hamsters had paired testes masses that were regressed in comparison to LD-AL and LD-FR hamsters, regardless of injection treatment (P <0.001 for all comparisons). Inguinal white adipose tissue (IWAT), epididymal white adipose tissue (EWAT), retroperitoneal white adipose tissue (RWAT) and composite fat masses were affected by group ($F_{2,69}>15.69$, $P<0.001$ for all models), but not injection ($F_{1,69}<0.07$, $P>0.806$ for all models) or the group \times injection interaction ($F_{2,69}<0.23$, $P>0.799$ for all models; Table 2). All groups showed significantly different composite fat masses from each other, with the LD-AL groups having the greatest

Table 2. Mean (\pm s.e.m.) paired testes, IWAT, EWAT, RWAT and composite adipose tissue masses from SD-AL, LD-AL and LD-FR male Siberian hamsters

	SD-AL	LD-AL	LD-FR
Paired testes mass (g)			
Saline	0.049 \pm 0.004 ^a	0.778 \pm 0.042 ^c	0.623 \pm 0.025 ^b
LPS	0.046 \pm 0.003 ^a	0.671 \pm 0.033 ^{b,c}	0.606 \pm 0.028 ^b
IWAT mass (g)			
Saline	0.488 \pm 0.084 ^a	1.062 \pm 0.141 ^b	0.627 \pm 0.085 ^a
LPS	0.465 \pm 0.077 ^a	1.083 \pm 0.107 ^b	0.513 \pm 0.054 ^a
EWAT mass (g)			
Saline	0.357 \pm 0.044 ^a	0.935 \pm 0.109 ^{b,c}	0.624 \pm 0.067 ^b
LPS	0.340 \pm 0.049 ^a	0.999 \pm 0.119 ^c	0.610 \pm 0.055 ^b
RWAT mass (g)			
Saline	0.057 \pm 0.009 ^a	0.164 \pm 0.024 ^e	0.106 \pm 0.019 ^{c,d}
LPS	0.064 \pm 0.011 ^{a,b}	0.155 \pm 0.018 ^{d,e}	0.087 \pm 0.012 ^{b,c}
Composite adipose tissue mass (g)			
Saline	0.902 \pm 0.133 ^a	2.160 \pm 0.264 ^c	1.357 \pm 0.166 ^b
LPS	0.869 \pm 0.134 ^a	2.237 \pm 0.237 ^c	1.210 \pm 0.112 ^b

IWAT, inguinal white adipose tissue; EWAT, epididymal white adipose tissue; RWAT, retroperitoneal white adipose tissue; LPS, lipopolysaccharide.

Tissue masses were collected via necropsy at the conclusion of the experiment (5 days after LPS or saline injection).

Different letters indicate statistically significant differences between group means ($P < 0.05$); groups sharing the same letter are statistically equivalent.

composite fat mass, followed by the LD-FR groups, and then the SD-AL groups ($P < 0.02$ for all comparisons between groups).

DISCUSSION

The results of this study demonstrate that seasonal differences in body mass alone do not regulate all variation in sickness symptoms in Siberian hamsters; however, inducing body mass loss in long-day housed hamsters does result in animals displaying some symptoms that appear more short-day like. Specifically, long-day hamsters that were food restricted showed body mass loss in response to LPS that was attenuated in comparison to long-day *ad libitum* fed hamsters but comparable to short-day *ad libitum* fed hamsters. The attenuation of body mass loss in long-day food-restricted hamsters was likely due in part to attenuation of LPS-induced anorexia, as the long-day food-restricted hamsters showed patterns of anorexia intermediate between the short-day and long-day *ad libitum* fed groups but showed the lowest percentage of body mass loss of the three groups. We expected to see differences in the magnitude of LPS-induced decreases in nest building between the short- and long-day *ad libitum* fed groups; however, no differences were observed. Rather, we found that the long-day food-restricted group showed less of a decrease in nest building after LPS, suggesting that in this experimental context, the act of prior food restriction may have had a greater impact than photoperiod on this measure. In contrast to our predictions, the intensity of LPS-induced anhedonic behavior was not affected by food restriction, as both long-day *ad libitum* fed and long-day food-restricted animals showed LPS-induced decreases in saccharin solution intake at 0–6 h post-injection while short-day *ad libitum* fed animals did not.

All three LPS-treated groups showed hypothermia, rather than fever, from 4 or 6 h post-injection until the end of the measuring period, 24 h after injection. Hypothermic responses to sickness are not uncommon (Martin et al., 2008b; Owen-Ashley et al., 2008; Burness et al., 2010; French et al., 2013; Carlton and Demas, 2014) and can actually be beneficial to survival during severe inflammation or low energy availability (Romanovsky and Székely, 1998). For

instance, rats that receive high concentrations of endotoxin display hypothermic responses and also show decreased glucose levels compared with control-injected animals; however, rats that receive lower doses of endotoxin show fever and no hypoglycemia (Lang et al., 1985). As all three LPS-treated groups showed reduced glucose in comparison to saline-treated animals after injection, their glucose levels coupled with their hypothermic responses may suggest the animals were experiencing severe inflammation. In concordance with the photoperiodic influence on temperature, hamsters in the LPS-treated long-day *ad libitum* fed group had colonic temperatures that were lower than those of the short-day group at several time points, indicating that photoperiodic influences on temperature were maintained during the hypothermic response. In contrast, the LPS-treated long-day food-restricted group had temperature recordings that were lower than those of the LPS-treated short-day group only at 8 h post-injection, suggesting that hypothermia was attenuated in this group compared with the LPS-treated long-day *ad libitum* fed group.

We assessed correlations between pre-LPS baseline body mass and the maximum percentage body mass loss that was displayed after injection. We did not find a significant correlation between these measures, suggesting that the intensity of energetically costly sickness symptoms may not be entirely limited by a minimum body mass threshold that the animal cannot pass if it is to survive (Ashley and Wingfield, 2012). However, there were significant correlations between pre-LPS body mass and percentage body mass loss at days 3 and 4 post-LPS. Whereas maximum percentage body mass loss may not be correlated with pre-LPS body mass, correlations at days 3 and 4 post-LPS suggest that the length of time an animal can maintain body mass loss throughout sickness may be constrained by its pre-sickness body mass. In white-crowned sparrows (*Zonotrichia leucophrys*), baseline body mass is correlated with post-LPS percentage decreases in body mass, with initially heavier individuals showing the greatest decreases in body mass 24 h after LPS injection (Owen-Ashley et al., 2006, 2008). The differences in the time points at which these correlations are observed between hamsters and sparrows may reflect species differences in the amount of extra energy reserves each animal stores. For instance, if hamsters maintain greater surplus energy stores than sparrows, then the need to regulate sickness symptoms to avoid hitting a body mass where survival is risked may not occur until later into the sickness response. In the future, doing comparative studies across species with varying degrees of surplus energy stores may elucidate relationships between body mass and sickness intensity.

Because we found that long-day food-restricted hamsters showed attenuation of some sickness symptoms, we sought to determine what energetic hormones could be acting as intermediaries between body mass and sickness. We previously showed that seasonal variation in leptin mediates seasonal variation in LPS-induced hypothermia but not other sickness symptoms (Carlton and Demas, 2014). In the present study, we found that even though the long-day food-restricted group showed comparable body masses to the short-day *ad libitum* fed group, long-day food-restricted animals had higher leptin levels than short-day *ad libitum* fed animals. Therefore, if leptin is the primary intermediary between body mass and sickness, we would have expected to see that sickness symptoms exhibited by the long-day food-restricted group were in between those of the short- and long-day *ad libitum* fed groups. The results of the present study are largely consistent with previous findings (Carlton and Demas, 2014), as the long-day food-restricted group showed an intermediate hypothermic response, exhibiting temperatures that fell in between those of the short-day and long-day

ad libitum fed groups at several time points. The long-day food-restricted group also showed an intermediate anorexic response. While the intensity of this measure is consistent with differences in leptin levels across groups, our previous work directly manipulating leptin levels does not support the hypothesis that leptin mediates sickness-induced anorexia in Siberian hamsters (Carlton and Demas, 2014). Although some research has linked leptin with sickness-induced anorexia (Sachot et al., 2004; Harden et al., 2006), other studies have suggested that these variables have little relation to each other (Faggioni et al., 1997; Lugarini et al., 2005). Thus, the role of leptin in modulating sickness-induced anorexia in Siberian hamsters and other species remains unresolved.

Suppression of the inflammatory response via circulating cortisol may be a promising mechanism to explain the attenuated sickness in the long-day food-restricted group. Glucocorticoids are released during sickness responses and are critical for regulating their intensity (Sapolsky et al., 2000). Rats that have their adrenal glands removed to prevent glucocorticoid production show greater body mass loss in response to LPS compared with sham-operated controls (Johnson et al., 1996), while administration of synthetic glucocorticoids concurrently with LPS attenuates sickness-induced decreases in food intake (Uehara et al., 1989). Both the saline- and LPS-treated long-day food-restricted groups in our study exhibited elevated cortisol levels relative to their respective long-day *ad libitum* fed counterparts. Thus, it is possible that increased circulating cortisol levels in the long-day food-restricted animals may have acted to suppress immunological modulators of sickness-induced anorexia and body mass loss.

Although the long-day food-restricted group exhibited elevated cortisol levels relative to the long-day *ad libitum* fed group, both the saline-treated and LPS-treated long-day food-restricted groups had similar cortisol levels to their respective short-day *ad libitum* fed counterparts. Siberian hamsters show increased circulating cortisol levels in short days compared with long days (Bilbo and Nelson, 2003; Ashley et al., 2013; Carlton and Demas, 2014). Therefore, it is possible that increased cortisol levels may be facilitating the attenuation of sickness symptoms in short-day animals. Previous studies have investigated the effects of melatonin, testosterone and leptin on seasonal variation in sickness intensity, but none have identified a seasonally modulated hormone that explains the majority of sickness response variation in this species (Bilbo and Nelson, 2002; Wen et al., 2007; Prendergast et al., 2008; Carlton and Demas, 2014). In addition, there may be seasonal changes in glucocorticoid receptors or binding proteins in this species that may affect regulation of sickness responses. Although very little is known about seasonal variation in glucocorticoid receptors and proteins in Siberian hamsters, there is evidence that they vary seasonally in other species and that seasonal variation in receptors can occur within immune tissues (Romero et al., 2006; Lattin et al., 2013). Thus, examining the roles that seasonal variation in glucocorticoid mechanism play in this phenomenon may be a promising next step.

While short-day hamsters displayed increased cortisol levels with *ad libitum* food access, we had to restrict food intake in long-day hamsters to achieve the same levels. Sustained moderate food restriction has been shown to increase cortisol levels in Siberian hamsters (Bilbo and Nelson, 2004; Zysling et al., 2009). Corticosterone is elevated to higher levels after LPS injection in food-restricted mice and rats than in non-food-restricted animals, and its elevation corresponds with lower levels of proinflammatory cytokines (Matsuzaki et al., 2001; MacDonald et al., 2014). Thus, it

is possible that increased cortisol levels generated by food restriction may be suppressing proinflammatory cytokines in long-day food-restricted hamsters in this study.

It cannot be ignored that, although we induced body mass loss in the long-day food-restricted group so that it mimicked natural short-day body mass loss, this pattern of body mass loss in long-day animals would likely be interpreted by the brain and periphery as ‘seasonally inappropriate’ (Mercer et al., 2001). Differences in neural interpretation of energetic state between the long-day food-restricted and short-day *ad libitum* fed groups may explain why the long-day food-restricted group showed attenuated body mass loss in response to LPS compared with the short-day group, despite exhibiting a longer duration of LPS-induced anorexia. It is possible that food restriction resulted in a slowing of metabolic rate in long-day food-restricted animals. Syrian hamsters (*Mesocricetus auratus*) recover from short-term food restriction by slowing down their resting metabolic rates, rather than increasing food consumption over normal pre-restriction levels (Borer et al., 1985). As Siberian hamsters also do not recover from energy deficit by increasing food consumption (Bartness and Clein, 1994), long-day food-restricted animals in this current study may have had reduced metabolic rates as well. Even though the long-day food-restricted group may have shown a greater intensity of LPS-induced anorexia than the short-day *ad libitum* fed group, if their metabolic rates were slower, they could have exhibited reduced body mass loss. The food restriction treatment may also explain why the long-day food-restricted group displayed lesser decreases in nest building. Having access to a nest decreases food intake in Siberian hamsters housed in low temperatures by 18%, suggesting that nesting is an energy conservation strategy (Kauffman et al., 2003). Although the long-day food-restricted group was provided with access to 100% of their pre-restriction mean food intake, they may have still been in energy conservation mode, explaining why they did not decrease nest building as much as the two other groups.

In conclusion, our data show that food restricting long-day Siberian hamsters to mimic short-day body mass loss results in the attenuation of some sickness symptoms, rendering them more short-day like. However, our results do not provide conclusive evidence that all seasonal variation in sickness intensity can be attributed to seasonal changes in energy stores, as the intensity of some symptoms remained unchanged or intermediate between those of the short- and long-day *ad libitum* fed groups. One promising mediator of seasonal variation in sickness intensity may be glucocorticoids, as glucocorticoids act to suppress sickness and can vary both seasonally and with food restriction. Future work should target understanding the role of glucocorticoids in modulating seasonal sickness responses and work toward understanding how natural variation in energy stores within and across species and seasons contributes to sickness intensity.

MATERIALS AND METHODS

Animals and housing conditions

Adult male (>60 days of age; average age 156 days) Siberian hamsters ($N=90$) were obtained from our breeding colony at Indiana University. All animals were initially group housed (2–5 per cage with same-sex siblings on weaning at 17–18 days of age) in long-day photoperiods (16 h:8 h light:dark), and then individually housed in polypropylene cages (27.8×17.5×13.0 cm) for 1 week prior to experimental housing. Food (Laboratory Rodent Diet 5001, LabDiet, St Louis, MO, USA) and water were available *ad libitum* prior to the start of the experiment. Temperature (20±2°C) and humidity (50±10%) were maintained at constant levels. All animal methods were reviewed and approved by the Institutional Animal Care and Use Committee at Indiana University.

Experimental methods

Animals were assigned to one of three groups matched for initial body mass. The first group was housed in short days (8 h:16 h light:dark) and was fed *ad libitum* throughout the entire experiment (SD-AL; $N=46$). Measures of body mass (to the nearest 0.1 g) and food consumption (to the nearest 0.1 g) were collected every other day for 10 weeks to track short-day-induced changes in body mass and food intake and photoperiodic responsiveness to short days (described below). Food intake was assessed by weighing the food pellets remaining in the hopper each day.

The second and third groups were housed in long days (16 h:8 h light:dark) for the duration of the experiment. The second group was fed *ad libitum* throughout the entire experiment (LD-AL; $N=20$). Body mass and food intake were measured every other day for 10 weeks. The third group was provided *ad libitum* access to food for the first 10 days and then food restricted for the following 60 days (LD-FR; $N=24$). During the first 10 days, body mass and food intake were measured every other day to establish pre-restriction mean values for these measures. During the next 60 days, these animals were allocated a pre-measured amount of food each day at the start of their active dark phase (16:00 h) that ranged in quantity from 65% to 100% of the animal's pre-restriction mean food intake (Mauer and Bartness, 1997). Body mass was recorded every other day for this group, and food access was adjusted so that the LD-FR group mean body mass tracked the SD-AL group mean. We modulated food availability to the LD-FR group to target the pattern of body mass loss in the SD-AL group, rather than providing food quantities that were pair matched to SD-AL food intake, because body mass loss in short days is influenced by other factors in addition to food intake (Wade and Bartness, 1984).

The SD-AL group was housed in experimental conditions 6 weeks prior to the LD-AL and LD-FR groups because a subset of the hamsters housed in short days fail to show reproductive responsiveness to prolonged exposure to this photoperiod (i.e. they do not display gonadal regression or reductions in body mass and fat stores). These individuals are referred to as photoperiodic non-responders (Puchalski and Lynch, 1986). Because we wanted to directly match body mass loss of the SD-AL and LD-FR groups, we needed to exclude the photoperiodic non-responders from our calculations of the SD-AL body mass loss trajectory (Mauer and Bartness, 1997). By observing body mass loss patterns in the short-day hamsters during the 6 weeks prior to housing the LD-FR group, we could remove animals who were not losing weight (and were likely photoperiodic non-responders) from the SD-AL body mass group means. Paired testes mass was collected at the end of the experiment in order to confirm short-day responsiveness (defined as a paired testes mass <0.15 g) (Greives et al., 2008). Twelve animals were determined to be photoperiodic non-responders and were removed from the experiment.

Starting on experimental day 70 for each group, body mass and food intake measurements were collected daily for the next 5 days to establish pre-injection baseline values for these measurements. During these 5 days, at the start of the dark phase, LD-FR animals were provided with daily food allocations equal to 100% of their pre-restriction means in order to relieve the effects of food restriction but not result in excessive food hoarding. After periods of food restriction, Siberian hamsters do not increase their food intake above normal levels when provided *ad libitum* access to food but do increase food hoarding (Bartness and Clein, 1994). In order to avoid complications with increased hoarding, we only allowed hamsters access to their normal food intake rather than access to excess food that would be hoarded rather than consumed.

On the fifth day of baseline measurement collection, half of the animals in each group were injected i.p. ~ 15 min before the onset of darkness with 25 μ g LPS (LPS from *Salmonella enterica* serotype typhimurium; Sigma-Aldrich, St Louis, MO, USA; Carlton and Demas, 2014) suspended in 0.1 ml sterile 0.9% saline. The remaining animals were injected i.p. with 0.1 ml sterile 0.9% saline. Sickness responses were assessed throughout the 4 days following injections.

Sickness response measurements

Fever, body mass loss and anorexia

Colonic temperatures (to the nearest 0.1°C) were collected immediately before injection and 2, 4, 6, 8, 10, 12, 16, 20 and 24 h after injection using a MicroTherma 2T thermometer (ThermoWorks, Alpine, UT, USA) and a

lubricated RET-3-ISO thermocouple probe (Physitemp Instruments, Inc., Clifton, NJ, USA) inserted ~ 12 mm into the rectum. To assess body mass loss and anorexia, daily body mass and food intake measurements continued until the end of the study. Hamsters in the SD-AL and LD-AL groups received *ad libitum* access to food until the end of the experiment, while hamsters in the LD-FR group continued to receive daily food allocations equal to 100% of their pre-restriction mean values.

Hedonic behavior

To assess the effects of our treatments on hedonic behavior, we provided hamsters with a highly palatable sodium saccharin solution (Baillie and Prendergast, 2008). Saccharin is a non-caloric artificial sweetener. As such, differences in ingestion between the groups would not interfere with our abilities to control energy intake. Beginning 5 days before injection, for the first 6 h of the dark phase (16:00 h to 22:00 h) hamsters were provided with a fluid bottle containing a solution of 0.1% sodium saccharin (saccharin sodium salt hydrate, Sigma-Aldrich) dissolved in tap water (Baillie and Prendergast, 2008). The saccharin solution bottles were weighed (to the nearest 0.1 g) before they were given and after they were collected from the hamsters each day. Presentation of saccharin solution continued daily to day 3 post-injection.

Nest-building behavior

To assess the effects of our treatments on thermoregulatory behavior, beginning 5 days before injection, each hamster was provided with a compressed cotton nestlet weighing ~ 2.5 g (Ancare, Bellmore, NY, USA) for the first 6 h of the dark phase (Baillie and Prendergast, 2008). The nestlet was weighed (to the nearest 0.1 g) before presentation, and the unshredded portion was weighed after presentation. When provided with a nestlet, hamsters quickly start shredding the cotton to build a nest. Nest building is an adaptive behavior to enhance energy conservation in low temperatures; however, hamsters readily build nests in room temperature (20–23°C) (Puchalski et al., 1988). Presentation of nestlets continued daily to day 3 post-injection.

Blood sampling and necropsies

Blood samples (~ 250 μ l) were drawn from each animal 4 h after the onset of darkness at two time points (3 days before injection and on the day of injection) to assess circulating blood glucose, leptin and cortisol concentrations. Briefly, animals were lightly anesthetized with isoflurane vapors, and blood samples were drawn from the retro-orbital sinus. Blood samples were allowed to clot at room temperature for 1 h, clots were removed, and samples were centrifuged at 4°C for 30 min at 2500 rpm. Serum aliquots were aspirated and stored in sealable polypropylene microcentrifuge tubes at -20°C until assayed. All blood samples were collected within 3 min of initial handling. Animals were killed 5 days after LPS injection and necropsies were performed. Testes, IWAT, EWAT and RWAT were removed, cleaned of connective tissues, and weighed to the nearest 0.1 mg. A composite adipose tissue score was calculated by summing the individual WAT pad masses.

Blood glucose measurement

Blood glucose was measured from the samples collected 4 h after injection. Immediately upon collection, ~ 5 μ l of whole blood was transferred onto test strips of a blood glucose monitoring system (ReliOn, Micro Blood Glucose Monitoring System, Arkray USA, Inc., Minneapolis, MN, USA), and the readout was recorded. The meter was previously calibrated using an internal standard provided by the manufacturer.

Leptin ELISA

We assessed circulating leptin levels in the samples collected 3 days prior to injection to determine whether the groups showed differing serum concentrations of this hormone. Leptin levels were assayed via commercially prepared mouse leptin ELISA kits (Crystal Chem, Downers Grove, IL, USA). This kit has previously been validated in Siberian hamsters (Carlton and Demas, 2014). Samples were diluted 1:4 and run in duplicate. Intra-assay coefficients of variability were 6.8%, 12.5% and 1.8%.

Cortisol enzyme immunoassay (EIA)

We assessed circulating cortisol levels to determine whether the groups differed in the magnitude of baseline and LPS-induced activation of the HPA axis. Cortisol is the predominant glucocorticoid in Siberian hamsters (Reburn and Wynne-Edwards, 2000). Serum cortisol concentrations were determined in multiple EIAs from a commercially prepared kit (Enzo Life Sciences, Inc., Farmingdale, NY, USA). This assay was previously validated for use in Siberian hamsters (Demas et al., 2004). Samples were diluted 1:80 with assay buffer and run in duplicate. Intra-assay variabilities were 3.1%, 0% and 0.6%.

Statistical analyses

All statistics were performed using JMP 10 (SAS Institute Inc., Cary, NC, USA). Residuals were checked for normality and homogeneity of variance, and those data that were non-normally distributed were transformed with the function that best fitted the data. Three animals were excluded from the final analyses: one from the saline-treated SD-AL group because it exhibited sickness symptoms despite receiving no LPS, one from the LPS-treated LD-AL group because it showed abnormal body mass loss despite no food restriction, and one from the saline-treated LD-AL group because it displayed abnormal food hoarding. The final sample sizes were as follows: SD-AL saline ($N=16$), SD-AL LPS ($N=17$), LD-AL saline ($N=9$), LD-AL LPS ($N=9$), LD-FR saline ($N=12$) and LD-FR LPS ($N=12$).

Pre-injection baseline values were calculated for body mass, food intake, saccharin solution intake and percentage nesting material shredded by averaging the three daily measurements immediately prior to injections (days 72–74). We did not include days 70–71 in this mean because measurements on these days were more variable as animals were adjusting to changes in food allocation and the presence of saccharin solution and nestlets. To determine whether there were group effects on pre-injection baseline body mass, baseline food intake, leptin levels and baseline saccharin solution intake, one-way ANOVA were performed. Pre-injection baseline saccharin solution intake was log transformed and leptin concentration was square-root transformed. Pre-injection baseline percentage nesting material shredded could not be transformed to meet the assumptions of normality, so a Kruskal–Wallis test was performed.

Because group affected pre-injection food intake, body mass, saccharin solution intake and percentage nesting material shredded (see Results), post-injection changes in these measurements were expressed as percentages of each animal's baseline values. Repeated measures ANOVA were performed on post-injection percentage changes in food intake, body mass and saccharin solution intake and on colonic temperature. The within-subject comparisons for percentage change in body mass, colonic temperature and percentage change in saccharin solution intake violated the assumptions of sphericity and were Greenhouse–Geisser corrected. Post-injection percentage change in saccharin solution intake was square-root transformed. Post-injection changes in percentage nesting material shredded could not be transformed to meet the assumptions of normality, so a Kruskal–Wallis test was performed. Differences in glucose, cortisol and tissue mass among the groups were assessed with two-way ANOVA. Glucose was square-root transformed, while cortisol and the tissue masses were log transformed. Correlations between pre-injection baseline body mass and percentage changes in body mass were assessed for LPS-treated animals using ANCOVA. *Post hoc* comparisons were conducted using Fisher's least significant difference tests when ANOVA were statistically significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

E.D.C. contributed to project design, and carried out data collection and analysis, interpretation of results and manuscript writing. G.E.D. contributed to project design and provided comments on the manuscript.

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