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Metabolic stressors and signals differentially affect energy allocation between reproduction and immune function

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ABSTRACT

Most free-living animals have finite energy stores that they must allocate to different physiological and behavioral processes. In times of energetic stress, trade-offs in energy allocation among these processes may occur. The manifestation of trade-offs may depend on the source (e.g., glucose, lipids) and severity of energy limitation. In this study, we investigated energetic trade-offs between the reproductive and immune systems by experimentally limiting energy availability to female Siberian hamsters (*Phodopus sungorus*) with 2-deoxy-D-glucose, a compound that disrupts cellular utilization of glucose. We observed how glucoprivation at two levels of severity affected allocation to reproduction and immunity. Additionally, we treated a subset of these hamsters with leptin, an adipose hormone that provides a direct signal of available fat stores, in order to determine how increasing this signal of fat stores influences glucoprivation-induced trade-offs. We observed trade-offs between the reproductive and immune systems and that these trade-offs depended on the severity of energy limitation and exogenous leptin signaling. The majority of the animals experiencing mild glucoprivation entered anestrus, whereas leptin treatment restored estrous cycling in these animals. Surprisingly, virtually all animals experiencing more severe glucoprivation maintained normal estrous cycling throughout the experiment; however, exogenous leptin resulted in lower antibody production in this group. These data suggest that variation in these trade-offs may be mediated by shifts between glucose and fatty acid utilization. Collectively, the results of the present study highlight the context-dependent nature of these trade-offs, as trade-offs induced by the same metabolic stressor can manifest differently depending on its intensity.

1. Introduction

Animals are faced with the challenge of obtaining energetic resources. The energy available to most animals under natural conditions is finite and will depend on the quality and abundance of these energetic resources in the environment. Once an animal obtains energy, it is then faced with the challenge of balancing energy allocation among different physiological, biochemical, and behavioral processes (Ricklefs and Wikelski, 2002; Sheldon and Verhulst, 1996). The balancing of allocation toward diverse biological processes often results in energetic trade-offs among physiological systems. One commonly observed energetic trade-off occurs between the reproductive and immune systems (Demas et al., 2012; Fedorka, 2014), where increased investment into one system results in decreased investment to the other. Maintaining reproduction and activating and maintaining the immune system requires a substantial allocation of energy (Demas, 2004; Speakman, 2008). Therefore, the expression of constraints on these systems and trade-offs between them is often dependent on the animal's access to energetic resources in its environment. This dependence on the environment may explain why in some cases a reproduction-immune trade-off may be observed with relatively limited resources in the wild but is not observed in the laboratory where food is available ad lib (French and Moore, 2008; French et al., 2009b).

One possibility why the display of energetic trade-offs is often context-dependent is that the display of energetic limitations and trade-offs varies with the type of energy that is being used to fuel the physiological and behavioral processes. Energetic trade-offs may be manifested in different ways depending on an animal's access to both current food availability in the environment and stored energy availability in the form of body fat. For instance, food deprivation inhibits ovulation and estrous behavior in lean, but not fat, female Syrian hamsters (*Mesocricetus auratus*)

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(Schneider and Wade, 1989), suggesting that having large fat stores may be sufficient to overcome energetic deficits imposed by limited current energetic resources. Food restriction does not suppress antibody production in female Siberian hamsters (*Phodopus sungorus*) (Zysling et al., 2009); however, surgically removing body fat stores (i.e., lipectomy) from male prairie voles (*Microtus ochrogaster*) and male and female Siberian hamsters results in decreased antibody production (Demas et al., 2003; Demas and Sakaria, 2005). Collectively, these results provide support that different types of metabolic fuels (e.g., glucose from immediate food ingestion, free fatty acids from adipose tissue) may influence the expression of energetic trade-offs within and between these two systems.

One way to manipulate glucose availability is with treatment with 2-deoxy-D-glucose (2-DG). 2-DG is a non-metabolizable glucose analog, which causes a transient disruption of glycolysis by inhibiting glucose oxidation, resulting in a state of glucoprivation (Horton et al., 1973). Treating ad lib-fed female Syrian hamsters with high doses of 2-DG (1750 or 2000 mg/kg) induces anestrus, whereas a lower dose of 2-DG (750 mg/kg) induces anestrus only in hamsters that are food restricted (Schneider et al., 1993). Previous work in our lab has shown that treatment with 2-DG (750 mg/kg) results in reduced antibody production and reduced reproductive tissue mass in female Siberian hamsters (Zysling and Demas, 2007) and reduced splenocyte production in female deer mice (*Peromyscus maniculatus*) (Demas et al., 1997b). Thus, reducing glucose availability via 2-DG treatment suppresses energetic allocation to both reproduction and immunity.

While it is clear from these studies that reducing glucose utilization can suppress reproductive and immune responses, the context-dependent nature of some of these results (e.g., lower doses of 2-DG suppress reproduction in only food restricted animals, not ad lib-fed animals) illustrates that glucose is not the only fuel source that animals can utilize to power reproduction and immunity. For instance, anestrus can be induced in ad lib-fed hamsters treated with a lower dose of 2-DG (750 mg/kg) if fatty acid utilization is simultaneously blocked (via methyl palmoxirate treatment), suggesting that changes in reproductive status may be driven by availability of all metabolic fuels, rather than just individual metabolic fuel types (Schneider and Wade, 1989). White adipose tissue (WAT), a primary energy source for organisms, provides storage for lipids that can be liberated to free fatty acids and glycerol when triggered by glucagon. WAT is not only a source of fuel, but it is also an endocrine organ that synthesizes and releases hormones (Ahima and Flier, 2000; Cinti, 2007). The peptide hormone leptin is one such adipose hormone, and circulating levels of leptin are directly proportional to the mass of adipose tissue in mammals (Maffei et al., 1995). Thus, high levels of leptin indicate adequate energy stores, whereas low circulating levels of leptin are consistent with an energy deficit. Furthermore, leptin not only influences lipid metabolism but it also increases glucose metabolism, glucose uptake, glucose turnover, and glucose oxidation (Kamohara et al., 1997).

While leptin was first characterized for its role in food intake and adiposity, there is now ample evidence that leptin plays a role in mediating both reproduction (Caprio et al., 2001; Schneider et al., 2012) and immunity (Carlton et al., 2012; La Cava and Matarese, 2004; Lord, 2002). Treatment with leptin restores estrous cycling in fasted female Syrian hamsters, however, when hamsters are fasted and treated with 2-DG, leptin does not restore estrous cycling. As 2-DG inhibits glucose oxidation, these results suggest that leptin influences energy allocation to reproduction via effects on metabolic fuel oxidation rather than through signaling of available adipose stores (Schneider et al., 1998). Additionally, leptin treatment counteracts the fasting-induced suppression of cell-mediated immunity in mice (Lord et al., 1998), and it attenuates the suppressive effects of surgical lipectomy on antibody production in male Siberian hamsters (Demas and Sakaria, 2005). Male Siberian hamsters treated with 2-DG show reductions in antibody production, and providing exogenous leptin alleviates this suppression of humoral immunity (Drazen, 2001) suggesting that the effects of leptin on humoral immunity are at least in part mediated through changes in signals of fat availability (Drazen, 2001; Drazen et al., 2001). Therefore, leptin may act differently in how it regulates energy allocation to reproduction versus immunity when glucose utilization is impaired.

The goal of the present study was to determine how leptin, as a neuroendocrine signal, affects energy allocation between the reproductive and immune systems in female Siberian hamsters experiencing glucoprivation. Specifically, we experimentally reduced glucose utilization with either a low (750 mg/kg) or high dose (1750 mg/kg) of 2-DG, and then supplemented animals with exogenous leptin. We assessed reproductive (i.e., estrous cycling, reproductive tissue mass) and immune (i.e., serum bacterial killing, antibody production) indices in response to our treatments. We predicted that mild glucoprivation (low 2-DG dose) would reduce reproductive tissue mass and antibody production and that leptin treatment should restore antibody production and may restore reproductive tissue mass. Additionally, we predicted that at more severe glucoprivation (high 2-DG dose), animals would be more energy limited and would show halted estrous cycling in addition to reduced reproductive tissue mass, antibody production, and bacterial killing ability. Because the high dose of 2-DG provides significant energy limitation, we expected that leptin supplementation would only provide a sufficiently large energetic signal to restore one system (e.g., the immune system because it is most important for survival) and this restoration might occur at a potential additional energetic cost to the reproductive system. Finally, in order to assess potential causation behind trade-offs, we assessed serum triglyceride and cortisol concentrations to determine the physiological mechanisms that may mediate energy allocation.

2. Materials and methods

2.1. Animals and housing

Adult female (>60 days of age) Siberian hamsters ($n = 58$) were obtained from our breeding colony at Indiana University. The progenitors of these animals were generously provided by Dr. Randy Nelson (Ohio State University) and Dr. Timothy Bartness (Georgia State University). In order to minimize the effects of inbreeding, our animals are outbred approximately every 10 generations. All animals were initially group housed (2–5 with same sex siblings on weaning at 17–18 days of age) in long-day photoperiods (light:dark, 16:8). Temperature (20 ± 2 °C) and humidity (50 ± 10%) were maintained at constant levels. For the experiment, animals were all housed in the same room where they were maintained on long days (16:8) and individually housed in polypropylene cages (27.8 × 17.5 × 13.0 cm). Food (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO, USA) and tap water were available ad lib during the entire course of the experiment. Animals used in this experiment came from 18 different litters across 8 different breeding pairs. All animal methods were reviewed and approved by the Institutional Animal Care and Use Committee at Indiana University Bloomington (protocol No. 10-038).

2.2. Experimental methods

For 8 days prior to experimental treatments, vaginal cell samples were obtained by vaginal lavage (with 0.9% sterile saline) from each animal between 0930 h and 1130 h EST to determine estrous cycle stage (Scotti et al., 2007). For 5 days prior to experimental

treatments, body mass (to the nearest 0.1 g) and food consumption (to the nearest 0.1 g) were assessed daily between 0930 h and 1130 h EST to establish pre-treatment mean body mass and mean food intake baseline values. Food consumption was assessed by weighing the food pellets remaining in the hopper each day. Daily vaginal sampling and body mass and food consumption measurements were continued throughout the entirety of the experiment.

Animals were initially quasi-randomly selected (i.e., no more than one animal from each litter was placed into a single group) and assigned to one of three 2-DG treatment groups that received 0.1 ml injections of either a 0.9% sterile saline control or of one of two concentrations of 2-DG (750 mg/kg dissolved in 0.9% sterile saline or 1750 mg/kg dissolved in 0.9% sterile saline; Sigma–Aldrich, St. Louis, MO, USA), administered intraperitoneally (i.p.). The 750 mg/kg dose was used because a previous study in Siberian hamsters showed that this dose results in decreased antibody production and reduced reproductive tissue mass (Zysling and Demas, 2007). The 1750 mg/kg dose was used because this dose is the lowest dose of 2-DG that induces anestrus in ad lib-fed Syrian hamsters (Schneider et al., 1993). Additionally, these concentrations are lower than doses that induce torpor in Siberian hamsters (2500 mg/kg) (Dark et al., 1994; Zysling and Demas, 2007). Within each 2-DG treatment group, animals were further divided into one of two groups that received either control injections of 0.9% sterile saline or leptin. Control animals received a 0.2 ml injection of 0.9% sterile saline, while leptin-treated animals received a 0.2 ml injection of 45 μg recombinant murine leptin (Peprotech Inc., Rocky Hill, NJ, USA) dissolved in 0.9% sterile saline, administered i.p. Injections of 2-DG (or saline control) were administered every other day for 12 days, for a total of 6 injections while injections of leptin (or saline control) were administered every day for 12 days, for a total of 12 injections. All injections were provided between 1400 and 1500 h EST. Final group sample sizes were as follows: Veh/Veh ($n = 10$), Veh/Lep ($n = 10$), Low-2DG/Veh ($n = 10$), Low-2DG/Lep ($n = 8$), High-2DG/Veh ($n = 10$), High-2DG/Lep ($n = 10$).

On the day following the first 2-DG or saline injection, all animals received a single subcutaneous injection of 100 μg of keyhole limpet hemocyanin (KLH; Sigma–Aldrich, St. Louis, MO, USA) suspended in 0.1 ml sterile saline in order to generate a humoral immune response. KLH is an innocuous respiratory protein derived from the giant keyhole limpet (*Megathura crenulata*) that generates a robust, non-replicating antigenic response in rodents, but does not make the animals sick (i.e., no inflammation or fever) (Dixon et al., 1966).

2.3. Blood sampling and necropsies

On days 5 and 10 post KLH injection, a blood sample was drawn from all animals via the retro-orbital sinus for immediate measurement of blood glucose and later measurement of bacterial killing ability (days 5 and 10), KLH-specific antibodies (days 5 and 10), serum triglyceride concentrations (day 5), and serum cortisol concentrations (day 10). These time points were chosen to capture peak anti-KLH immunoglobulin G production during the course of the immune response (Demas et al., 1997a). Triglyceride concentrations and cortisol concentrations were only measured at single time points during the study due to limited serum availability. We chose to measure triglycerides at day 5 because animals that stopped estrous cycling did so no later than this date, so any effects of triglycerides on this measure could ideally be captured at this date. We assessed cortisol at day 10 because a previous study in our lab showed that cortisol concentrations do not differ in Siberian hamsters treated with 750 mg/kg 2-DG from day 5 to day 10 (Zysling and Demas, 2007). Briefly, animals were lightly anesthetized with isoflurane vapors and blood samples were drawn from the retro-orbital sinus between 1300 and 1400 h EST. Blood samples were allowed to clot at room temperature for 1 h, the clots were removed, and the 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. Each blood sample consisted of ~3.5% of the animal's total blood volume, and all blood samples were collected within 3 min of initial handling. Animals were euthanized and necropsies were performed at the completion of the study (day 11 post KLH injection). Uterine horns, ovaries, inguinal WAT (IWAT), parametrial white adipose tissue (PWAT), and retroperitoneal WAT (RWAT) were removed to determine the effects of 2-DG and leptin on these tissues. All tissues were 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.

2.4. Blood glucose measurement

Blood glucose levels were measured from the samples collected at 5 and 10 days after KLH injection. Immediately upon sampling, ~5 μl of whole blood was transferred onto the 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.

2.5. Vaginal cytology

After vaginal cell samples were obtained via vaginal lavage, samples were transferred to microscope slides, fixed with methanol, and stained with Giemsa. These samples were evaluated for estrous stage (diestrus I, diestrus II, proestrus, estrus) under 100× magnification. The following cellular characteristics were used to characterize the four stages of the estrous cycles: diestrus I (presence of many polymorphonuclear leucocytes and some nonnucleated keratinized cells), diestrus II (primarily secretory material and cellular debris, some parabasal cells), proestrus (clumps of lightly staining nucleated epithelial cells), estrus (many nonnucleated keratinized cells) (Moffatt-Blue et al., 2006; Scotti et al., 2007). In addition, anestrus was characterized by slides which contained a lot of cellular debris with cells that did not appear intact. Animals were determined to have stopped cycling if, after they had previously been cycling in the experiment, they entered anestrus and remained in anestrus until completion of the experiment. Two animals (one in the low dose 2-DG and vehicle group; one in the high dose 2-DG and leptin group) did not show normal estrous cycling during the 8 days prior to the start of injections, so they were excluded from the cycling analysis.

2.6. Bacterial killing

We used an ex vivo bacterial killing assay as a functional assessment of the innate immune system's ability to clear a relevant pathogen. This assay quantifies the relative number of *Escherichia coli* colony forming units (CFU) that grow after incubation with serum. Briefly, lyophilized *E. coli* (Epower™, ATCC #8739, Microbiologics, St. Cloud, MN, USA; 1 pellet = 10^7 CFU) was added to 40 ml 1 M sterile PBS warmed to 37 °C to create a bacterial stock solution. This solution was activated by incubation for 30 min at 37 °C. The stock bacteria solution (500,000 CFU/ml) was diluted 1:10 with sterile 1 M PBS to create a 50,000 CFU/ml working solution. Meanwhile, serum samples were diluted 1:20 in glutamine enriched CO_2-independent media (Invitrogen Corp., Carlsbad, CA, USA). For each sample, the bacterial working solution was added at a 1:10 ratio to the diluted serum sample. To generate a positive control (i.e., solution containing only media and bacteria), the bacterial

working solution was diluted 1:10 with glutamine enriched CO_2-independent media. The diluted samples and the positive control were incubated for 30 min at 37 °C to induce bacterial killing. After incubation, 50 μl of sample and the positive control was added to tryptic soy agar plates in duplicate. All plates were covered, inverted, and stored overnight at 37 °C. Following incubation, colony numbers were counted on each plate, and duplicates were averaged. Bactericidal capacity was calculated as a percent of bacteria killed relative to the positive control plates in which no killing occurred.

2.7. Anti-KLH enzyme-linked immunosorbent assay (ELISA)

To assess humoral immunity to KLH injection, serum anti-KLH immunoglobulin G (IgG) concentrations were assayed using an enzyme-linked immunosorbent assay (ELISA) (Demas et al., 2003). Microtiter plates were coated with KLH by incubating overnight at 4 °C with 0.5 mg/ml KLH in sodium bicarbonate buffer (pH 9.6). Plates were washed with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-T; pH 7.4), then blocked with 5% non-fat dry milk in PBS (to reduce non-specific binding), and then washed again with PBS-T. Thawed serum samples were diluted 1:20 with PBS-T, and 300 μL of each serum dilution was added to the plate wells in duplicate. Positive control samples (i.e., pooled sera from hamsters previously shown to have high anti-KLH antibody responses) and negative control samples (i.e., pooled sera from KLH-naïve hamsters) were also diluted 1:20 with PBS-T and added to the plate wells in duplicate. Plates were incubated at 37 °C for 3 h and then washed with PBS-T. 150 μL of secondary antibody (alkaline phosphatase-conjugated-anti Syrian hamster IgG diluted 1:500 with PBS-T; Rockland, Gilbertsville, PA, USA) was added to the wells and the plates were incubated for 1 h at 37 °C. Plates were then washed again with PBS-T and 150 μL of the enzyme substrate *p*-nitro-phenyl phosphate (Sigma–Aldrich, St. Louis, MO, USA; 0.1 mg/ml in diethanolamine substrate buffer) was added to each well. The absorbance of each well was measured (Bio-Rad iMark Microplate Reader, Hercules, CA, USA) at 405 nm. The mean for each sample was calculated and expressed as a percentage of the positive control mean (% plate positive).

2.8. Triglyceride assay

Total serum triglyceride concentrations were assessed with a commercially prepared colorimetric assay kit (Triglyceride Colorimetric Assay Kit, Cayman Chemical Co., Ann Arbor, MI, USA). Prior to testing samples, we validated the appropriate dilution (1:4) so that triglyceride concentrations lay within the detectable range of the assay. Some samples expressed triglyceride levels at the 1:4 dilution that ran off the curve, so these samples were rerun at a 1:10 dilution. Intra-assay variabilities were 3.2% and 3.9%; inter-assay variability was 0.7%.

2.9. Cortisol enzyme immunoassay (EIA)

Cortisol is the predominant glucocorticoid in Siberian hamsters, with concentrations ~100× that of corticosterone (Reburn and Wynne-Edwards, 2000). Serum cortisol concentrations were determined in multiple enzyme immunoassays (EIAs) from a commercially prepared kit (Cortisol EIA Kit; Enzo Life Sciences, Inc., Farmingdale, NY, USA). This assay was previously validated for use in Siberian hamsters (Demas et al., 2004) and is highly specific for cortisol; cross-reactivity with corticosterone is 27.7% and <4.0% for other steroid hormones. The sensitivity of the assay is 56.72 pg/ml. Samples were diluted to 1:40 with assay buffer and run in duplicate. Intra-assay variabilities were 3.0% and 3.5%; inter-assay variability was 14.0%.

2.10. Statistical analyses

All statistical tests were performed using JMP 10 (SAS Institute Inc., Cary, NC, USA), and a value of $P < 0.05$ was considered to be statistically significant. Data were checked for normality and homogeneity of variance and those data that were non-normally distributed were transformed. Glucose, cortisol, and triglyceride concentrations were not normally distributed and were log transformed to best meet the assumptions of parametric tests. Bacterial killing ability was not normally distributed and was reverse square root transformed to meet the assumptions of parametric tests. All differences in measurements collected at only one time point (i.e., fat tissue masses, reproductive tissue masses, triglyceride concentrations, cortisol concentrations) were assessed via a two-way (2-DG treatment (3) × leptin treatment (2)) analysis of variance (ANOVA). Body mass at the time of sampling was included as a covariate in the models for the fat tissue masses, reproductive tissue masses, and triglyceride concentrations to control for the effect of body mass on these dependent variables. Differences in repeated measures (i.e., body mass, food intake, antibody levels, bacterial killing ability, glucose concentrations) were assessed via repeated-measures ANOVAs with time as a within-subjects variable. Initial body mass was included as a covariate in the model for food intake levels to control for the effect of body mass on this measure. The within-subject comparisons for body mass and food intake violated assumptions of sphericity and were Greenhouse-Geisser (GG)-corrected. Treatment effects on the frequencies of animals remaining in estrus throughout the experiment were assessed with Fisher's Exact Test because expected values in some cells were less than 5. Post-hoc comparisons between pair-wise means were conducted using Fisher's LSD tests when the overall ANOVAs were statistically significant.

3. Results

3.1. Body mass, food intake, and body fat

There were no effects of 2-DG ($F_{2,52} = 0.63$, $P = 0.535$; Table 1) or leptin ($F_{1,52} = 0.62$, $P = 0.434$) on final body mass. While treatment did not affect final body mass, body mass decreased over the course of the experiment (within subjects, $F_{1.9,103.9} = 8.89$, $P < 0.001$, GG-corrected). Food intake over the course of the experiment was related to the initial body mass of the animals ($F_{1,51} = 13.89$, $P < 0.001$). After controlling for the effect of body mass, there were no effects of 2-DG ($F_{2,52} = 1.02$, $P = 0.369$; Table 1) or leptin ($F_{1,52} = 0.06$, $P = 0.812$) on daily food intake. Additionally, food intake did not change over the course of the experiment (within subjects, $F_{7.7,393.1} = 1.27$, $P = 0.258$, G-G-corrected). IWAT, PWAT, RWAT, and composite adipose tissue mass were all related to the body mass of the animal at the time of tissue collection ($P < 0.001$ in all cases). After controlling for the effect of body mass, there were no treatment effects on any of these fat tissue measures ($P > 0.05$ in all cases; Table 1).

3.2. Blood glucose

Blood glucose levels did not differ across sampling times within individuals ($F_{1,51} = 2.38$, $P = 0.129$; Table 1), nor did levels differ among groups (2-DG, $F_{2,51} = 0.12$, $P = 0.886$; leptin, $F_{1,51} = 0.16$, $P = 0.693$; 2-DG × leptin, $F_{2,51} = 0.58$, $P = 0.564$).

3.3. Estrous cycling

Estrous cycling was impaired in animals treated with the low 2-DG dose (Fig. 1A). Fifty-six percent of the animals that received

Table 1
Effects of 2-DG dose and leptin treatment on mean (±SEM) final body mass, food intake on the last day of the experiment, composite body fat mass, blood glucose levels, and paired ovary mass. No statistically significant differences between group means ($P < 0.05$) were found for any of these measures.

	Vehicle	Leptin
Final body mass (g)		
Vehicle	38.7 ± 1.9	36.1 ± 2.3
Low	40.1 ± 2.4	37.9 ± 2.4
High	38.9 ± 1.6	41.0 ± 3.0
Food intake (g/day)		
Vehicle	4.8 ± 0.2	4.7 ± 0.5
Low	5.0 ± 0.5	4.9 ± 0.4
High	4.7 ± 0.2	4.7 ± 0.3
Composite body fat (g)		
Vehicle	1.59 ± 0.21	1.43 ± 0.31
Low	1.97 ± 0.25	1.55 ± 0.30
High	1.55 ± 0.19	1.83 ± 0.35
Day 10 blood glucose (mg/dl)		
Vehicle	115.3 ± 6.5	113.2 ± 9.2
Low	110.4 ± 4.3	120.5 ± 7.2
High	113.3 ± 6.3	106.9 ± 5.4
Paired ovary mass (mg)		
Vehicle	18.40 ± 2.69	18.36 ± 0.58
Low	21.95 ± 2.69	14.80 ± 1.22
High	18.37 ± 1.61	23.23 ± 2.97

the low 2-DG dose became anestrus by the end of the experiment, whereas in all other groups, 20% or fewer of the animals were anestrus at the end of the experiment. This difference in anestrus between the low 2-DG dose group and the other five groups was significant ($P = 0.003$).

3.4. Reproductive tissue masses

Uterine horn and ovarian masses were affected by the body mass of the animal at the time of tissue collection (uterine horns: $F_{1,51} = 7.36$, $P = 0.009$; ovaries: $F_{1,51} = 8.68$, $P = 0.005$). After controlling for body mass, there were effects of both 2-DG ($F_{2,51} = 3.36$, $P = 0.024$) and leptin ($F_{1,51} = 6.60$, $P = 0.013$) but no effect of the 2-DG × leptin interaction ($F_{2,51} = 2.25$, $P = 0.116$) on uterine horn mass (Fig. 1B). Specifically, uterine horn masses in all treatment groups were significantly lower than those of the vehicle-treated controls (for all comparisons, $T \geqslant 2.36$, $P \leqslant 0.022$). Conversely, there were no treatment effects on ovarian mass (2-DG, $F_{2,51} = 0.59$, $P = 0.558$; leptin, $F_{1,51} = 0.06$, $P = 0.811$; 2-DG × leptin, $F_{2,51} = 2.92$, $P = 0.063$; Table 1).

3.5. Antibody production

In all groups, anti-KLH IgG levels increased from day 5 to day 10 (within subjects, $F_{1,52} = 64.34$, $P < 0.001$; Fig. 2A). There was a main effect of 2-DG dose (between subjects, $F_{2,52} = 3.37$, $P = 0.042$), such that animals treated with the high dose of 2-DG had lower IgG levels than animals treated with just the vehicle ($P = 0.012$). There were no main effects of leptin ($F_{1,52} = 2.01$, $P = 0.162$) or the 2-DG × leptin interaction ($F_{2,52} = 2.79$, $P = 0.071$) on IgG levels. Specifically, this high 2-DG suppression of IgG levels was driven by animals treated with the high 2-DG dose and leptin showing lower IgG levels than animals treated only with leptin (and no 2-DG) (day 5, $T = 2.99$, $P = 0.004$; day 10, $T = 2.79$, $P = 0.007$).

3.6. Bacterial killing

Bacterial killing ability did not change across blood sampling time points (within subjects, $F_{1,52} = 0.16$, $P = 0.688$; Fig. 2B). However, there was a main effect of leptin ($F_{1,52} = 11.37$, $P = 0.001$), but no effects of 2-DG ($F_{2,52} = 1.36$, $P = 0.266$) or the 2-DG × leptin interaction ($F_{2,52} = 0.16$, $P = 0.0755$) on bacterial killing ability. Specifically, at both sampling points, bacterial killing was elevated in animals treated with leptin but no 2-DG as compared to the vehicle-treated controls (day 5, $T = 2.54$, $P = 0.014$; day 10, $T = 2.29$, $P = 0.026$).

3.7. Serum triglycerides

Serum triglyceride levels were positively related to the body mass of the animal at the time of sampling ($F_{1,57} = 11.53$, $P = 0.001$). After controlling for the effect of body mass, there was an effect of 2-DG ($F_{2,57} = 5.18$, $P = 0.009$; Fig. 3A), but no effects of leptin ($F_{1,57} = 3.51$, $P = 0.067$) or the 2-DG × leptin interaction ($F_{2,57} = 0.88$, $P = 0.421$) on triglyceride levels. Specifically, animals treated with the high 2-DG dose and either vehicle ($T = 3.07$, $P = 0.004$) or leptin ($T = 3.36$, $P = 0.002$) showed decreased triglyceride levels as compared to vehicle-treated controls.

3.8. Serum cortisol

Cortisol did not differ among groups (Fig. 3B). There were no significant effects of 2-DG ($F_{2,50} = 1.67$, $P = 0.199$), leptin ($F_{1,50} = 0.06$, $P = 0.805$), or the 2-DG × leptin interaction ($F_{2,50} = 0.16$, $P = 0.854$) on serum cortisol levels.

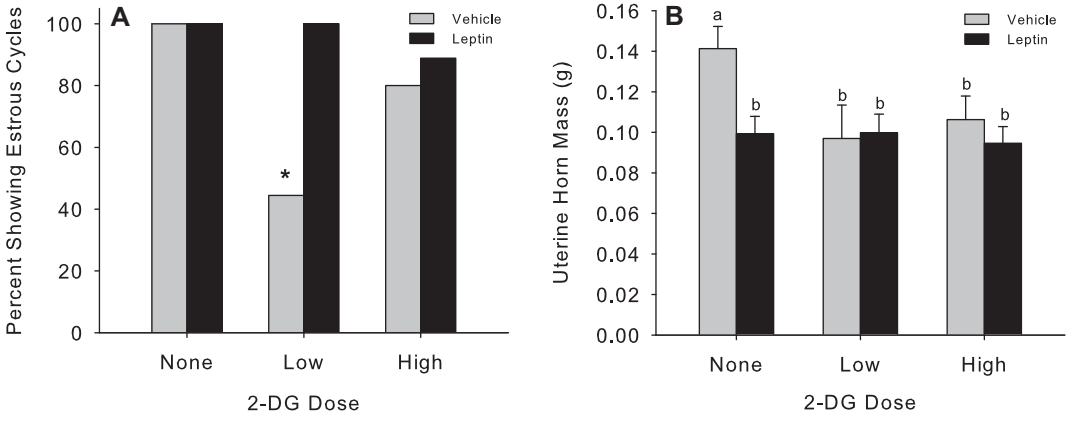

Fig. 1. Effects of 2-DG dose and leptin treatment on (A) the percentage of hamsters showing normal estrous cycles and (B) mean (±SEM) uterine horn mass. In (A), groups that differ significantly ($P < 0.05$) from the vehicle/vehicle-treated control are indicated by an asterix (*). In (B), groups with different letters indicate statistically significant differences between group means ($P < 0.05$); groups sharing the same letter are statistically equivalent.

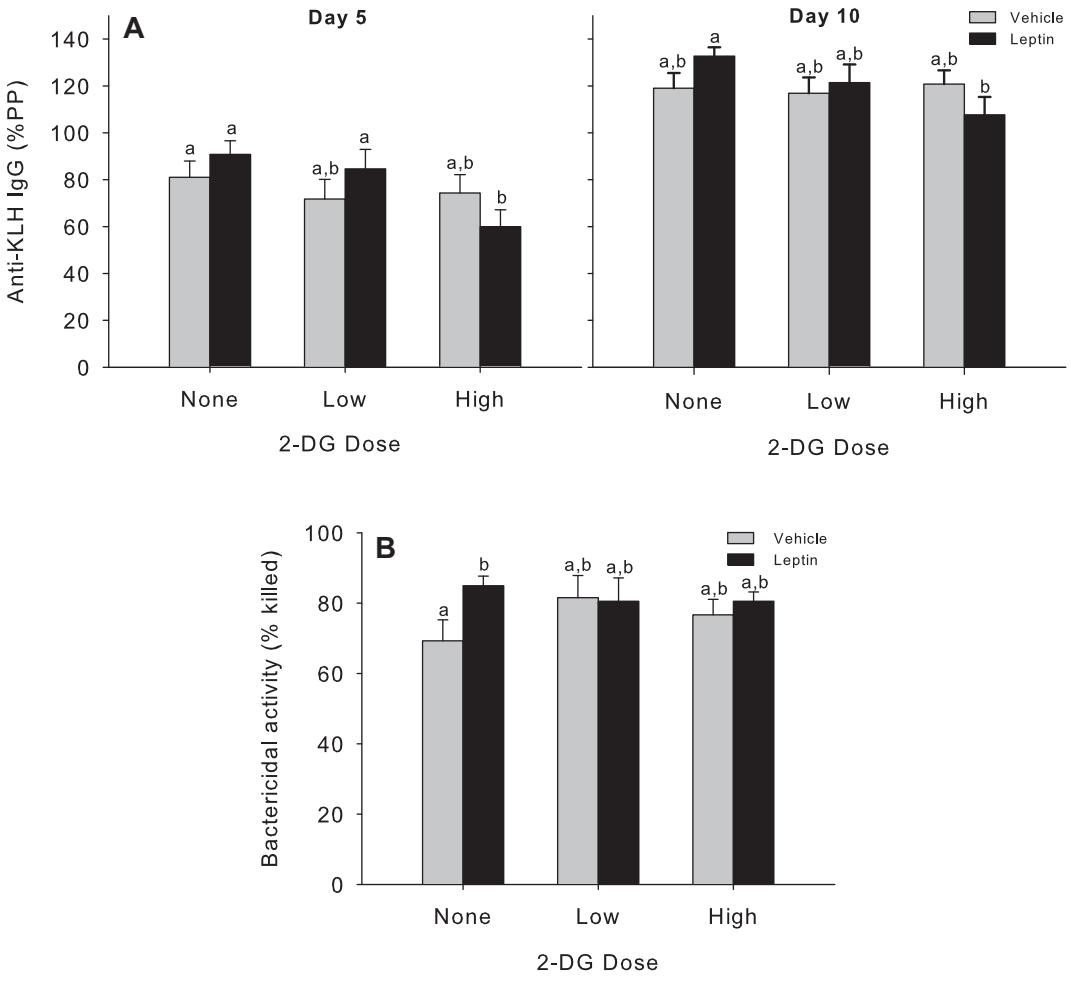

Fig. 2. Effects of 2-DG dose and leptin treatment on mean (±SEM) (A) serum anti-KLH immunoglobulin G (IgG) at day 5 (left) and day 10 (right) after KLH inoculation and (B) serum bacterial killing ability at day 10 after KLH inoculation (as there was no effect of time on this measure, only data from day 10 are shown). Groups with different letters indicate statistically significant differences between group means ($P < 0.05$); groups sharing the same letter are statistically equivalent.

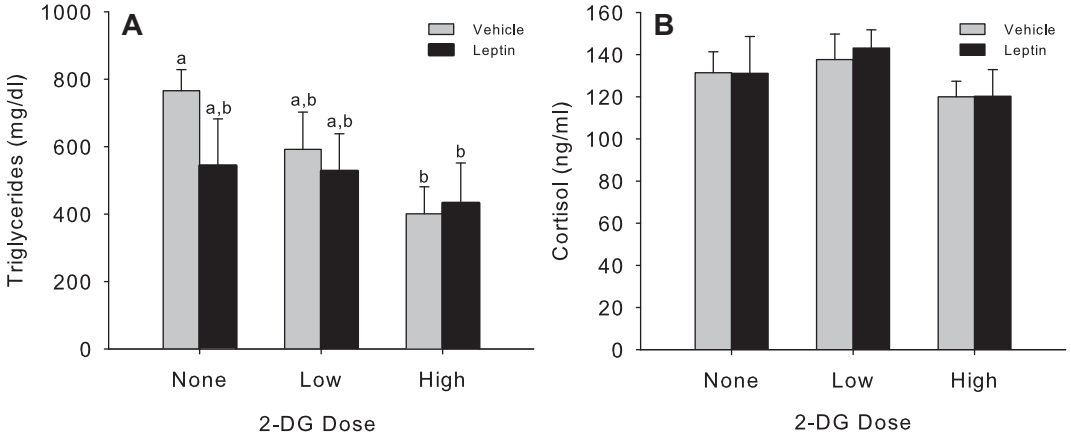

Fig. 3. Effects of 2-DG dose and leptin treatment on mean (±SEM) (A) serum triglyceride concentrations at day 5 after KLH inoculation and (B) serum cortisol concentrations at day 10 after KLH inoculation. Groups with different letters indicate statistically significant differences between group means ($P < 0.05$); groups sharing the same letter are statistically equivalent.

4. Discussion

Our findings demonstrate key trade-offs between the reproductive and immune systems in female Siberian hamsters and that these trade-offs depend on the severity of energy limitation and exogenous leptin signaling. We found that the majority of the animals experiencing mild glucoprivation (low dose 2-DG) entered anestrus, whereas leptin treatment restored estrous cycling in these animals. Surprisingly, virtually all animals experiencing more severe glucoprivation (high dose 2-DG) maintained normal

estrous cycling throughout the experiment and did not show anestrus. In addition, we found that animals experiencing any level of glucoprivation displayed reduced uterine horn mass, and exogenous leptin not only did not restore this mass, but it may have contributed to its decrease. In contrast to previous work in this species (Zysling and Demas, 2007), mild glucoprivation did not suppress antibody production in the current study. Alternatively, we found that animals experiencing more severe glucoprivation while receiving leptin supplementation showed reduced antibody production at both days 5 and 10 after KLH inoculation. The measure of innate immunity, serum bacterial killing ability, was not affected by glucoprivation treatment, although leptin treatment may have enhanced this measure. Taken together, our results suggest that at mild glucoprivation, female Siberian hamsters show reduced energy allocation to the reproductive system (via inhibition of estrous cycling) but that this reduction in energy allocation can be alleviated with an increased signal of fat stores. At a more severe level of glucoprivation, female Siberian hamsters do not reduce energy allocation to estrous cycling; however, providing these animals with a simultaneous signal of increased fat stores results in decreased antibody production, suggesting that these competing signals of energy availability may be resulting in energy being allocated to other physiological processes or systems not assessed in this study (e.g., thermoregulation, motor activity, self-maintenance).

In order to understand the potential mechanisms mediating energy allocation to the reproductive and immune systems during glucoprivation and leptin supplementation, we assessed circulating cortisol and triglyceride concentrations during the experiment. We measured circulating cortisol levels because 2-DG is often used in experimental procedures to induce metabolic stress, as its glucoprivic effects are often accompanied by increases in circulating glucocorticoid levels (Demas et al., 1997b; Weidenfeld et al., 1994). Stress can have both enhancing (e.g., acute stress) and suppressive (e.g., chronic stress) effects on immunity, while it acts mainly to suppress reproduction (Dhabhar, 2002; Sapolsky et al., 2000; Weil and Nelson, 2012). We found no differences in cortisol levels among the groups in our study. Similarly, previous studies in female Siberian hamsters found no differences in cortisol levels between animals treated with a 750 mg/kg dose of 2-DG as compared to controls (Zysling and Demas, 2007) and between hamsters food restricted to 70% of ad lib intake in comparison to controls (Zysling et al., 2009). Chronic stress from our daily injections could have contributed to the lack of differences in cortisol (and blood glucose) among groups, as all animals may have shown elevated baseline cortisol levels as a product of injection-related stress. The cortisol values measured in this study are ~50 ng/ml higher than those of female Siberian hamsters who have not been given daily injections or daily handling (Zysling et al., 2009). However, this large difference in cortisol levels between the hamsters in our study and the hamsters in the earlier study (Zysling et al., 2009) is more likely due to changes in the sensitivity of the cortisol antibody, as a previous study applying similar injection and handling methods to ours found cortisol levels similar to baseline levels of non-injected animals (Zysling and Demas, 2007). Additionally, it is possible that we did not see differences in cortisol levels among the groups because we took blood samples prior to the day's injection of 2-DG (i.e., 23 h from the last injection of 2-DG), and cortisol levels were not chronically elevated by our 2-DG treatment. Alternatively, the differential effects of our treatments on reproduction and immunity may not be caused by glucocorticoids but rather may be mediated by the utilization of different energy forms or energy reallocation.

We measured serum triglyceride levels to assess whether animals were potentially tapping into lipid energy stores while their glucose stores were less accessible during glucoprivation. We were motivated to probe this mechanism when we discovered that estrous cycling was only suppressed in the group receiving the low 2-DG dose and not in the group that received the high 2-DG dose. This result is in contrast to work in Syrian hamsters that showed that in animals that are provided ad lib access to food, estrus is only suppressed when animals receive a dose of at least 1750 mg/kg 2-DG and not suppressed in animals receiving a dose of 750 mg/kg (Schneider et al., 1993). One hypothesis for why we observed no suppression of estrous cycling in hamsters treated with the high 2-DG dose could be that this high dose inhibited glucoprivation so greatly that it required animals to shift their main source of fuel to free fatty acids (FFA). It is well established that when oxidation of one fuel type is inhibited, oxidation of the other fuel type is enhanced (Friedman, 1998), and FFAs can be used to fuel reproduction and immunity (Pond, 1996; Schneider, 2004). Furthermore, administration of 2-DG results in increases in circulating FFA in rats and Siberian hamsters (Brito et al., 2008; Yamamoto et al., 1984). Consistent with this hypothesis, animals that were treated with the high 2-DG dose had the lowest levels of circulating triglycerides, possibly indicating that these animals were showing greater breakdown of triglycerides into FFA and glycerol (i.e., undergoing lipolysis). If hamsters experiencing more severe glucoprivation increase lipolysis to a greater extent than hamsters experiencing less severe glucoprivation, this increase in FFA availability may be great enough in the high 2-DG group to fuel estrous cycling despite the reduction in useable glucose. Furthermore, in ad lib fed Syrian hamsters, estrous cycling is only halted in animals experiencing both glucoprivation and lipoprivation, not in animals that are only experiencing one of the two (Schneider and Wade, 1989). Thus, it is possible that an increase in lipolysis could have counteracted the estrus-suppressing effects of glucoprivation.

While estrous cycling was suppressed in hamsters treated with the low 2-DG dose, concurrent treatment with leptin restored cycling. This result is in contrast to work in Syrian hamsters that shows that leptin does not restore normal estrous cycling in animals that are fasted while receiving treatment with 2-DG (Schneider et al., 1998). However, our study differs from this study because our animals were fed ad lib, and leptin treatment restores normal estrous cycling in Syrian hamsters that are fasted but do not receive 2-DG (Schneider et al., 1998). Because leptin supplementation cannot override the suppressive effects of glucoprivation on estrous cycling in fasted animals, Schneider and colleagues suggest that leptin influences reproductive function by indirectly affecting fuel oxidation (i.e., the "metabolic hypothesis"), not by acting as a signal of available fat stores (Schneider et al., 1998, 2012; Schneider and Zhou, 1999). In non-food restricted animals, however, it seems plausible that leptin supplementation may be able to overcome some of the energy limitations of glucoprivation since leptin increases intracellular oxidation of fatty acids (Shimabukuro et al., 1997). Thus, in our study, because animals were not fasted while being treated with 2-DG, leptin treatment may have been able to increase intracellular oxidation of fatty acids enough so as to compensate for the estrus-suppressing effects of reduced intracellular glucose oxidation at the low dose of 2-DG (although if entirely the case, we may have seen lower triglyceride levels in the group that received low 2-DG and leptin).

It is not surprising that our measure of innate immunity, serum bacteria killing, was not affected by any of our treatments, as maintaining the innate immune system comes at a fairly low energetic cost (Klasing, 2004). We did expect that 2-DG treatment (at both doses) would result in decreased antibody production due to the energetic costs of antibody generation, but instead, we found that 2-DG treated animals only showed a reduction in anti-KLH IgG production at the high dose. It is possible that, although we did not see reduced IgG production at the low dose, we may have seen

reduced production of a different antibody type that we did not measure (e.g., IgM). It is also possible that because energy was allocated away from investment in reproduction (i.e., all groups experiencing glucoprivation showed decreased uterine horn mass), then energy allocation toward humoral immunity could be maintained. For instance, other work in Siberian hamsters has shown that males that are treated with 2-DG show decreased testes mass but no reduction in delayed-type hypersensitivity (DTH) immune response, while females show no reduction in uterine mass but do show decreased DTH response (Martin et al., 2008). In our study, the group that received the high dose of 2-DG and leptin showed a reduction in IgG levels as compared to the group that only received leptin. This result was contrary to our predictions but may be the result of leptin treatment causing energy to be allocated to other energetically-costly physiological processes that we did not quantify in this study (e.g., thermoregulation, motor activity, self-maintenance). For example, treating pregnant Siberian hamsters with leptin results in increased allocation to reproduction but a subsequent decrease in innate immune function (French et al., 2009a). In our study, it is quite possible that leptin treatment increased energy allocation to another physiological process. This shunting of energy toward another process, coupled with already limited energy due to glucoprivation, may have resulted in less energy available for immune function. These results highlight the importance of expanding our investigation from dichotomous trade-offs to trade-offs among several systems in future studies of this sort.

It is important to note that much of the work on energetic regulation of estrous cycling has been performed in Syrian hamsters. Syrian and Siberian hamsters are both seasonally breeding rodents that reproduce in long-day lengths and shut down reproduction in short photoperiods; however, Syrian hamsters lose weight while transferring from short to long photoperiods, while Siberian hamsters gain weight during this transition (Bartness and Wade, 1985). In addition, these species show differences in how 2-DG treatment affects other metabolically-sensitive processes like food consumption and torpor (Bartness et al., 1995; Dark et al., 1994; Ritter and Balch, 1978; Schneider et al., 1993). Thus, it is not surprising that these animals may show differences in their reproductive responses to glucoprivation. In our study and a previous study from our lab (Zysling and Demas, 2007), we induced glucoprivation by injecting animals with 2-DG every other day. Alternatively, many of the studies that we have cited in this paper have induced glucoprivation by injecting animals with 2-DG more frequently (e.g., every 6 hours over the course of 2 days (Schneider et al., 1993); every 24 h for three consecutive days (Demas et al., 1997b)). These differences in the frequency of injections may be reflected in the differences in the effects of glucoprivation across these studies. Although we provided 2-DG less frequently than in some of these other studies, we are still confident that we were inducing glucoprivic effects because this same injection protocol has been previously shown to cause glucoprivation and affect immune responses in rats at a lower dose than the doses we chose (Chou et al., 1996).

In conclusion, our data support the presence of trade-offs between the reproductive and immune systems, which are regulated distinctly based upon the severity of glucoprivation and leptin signaling that the animal experiences. Because varying components of the reproductive and immune systems were suppressed under different levels of glucoprivation, these results highlight how trade-offs induced by the same energetic stressor can manifest in opposite ways depending on the intensity of the stressor. In addition, the context-dependent nature of these trade-offs support the idea that animals should preferentially allocate energy to processes that support survival or reproduction based upon their internal energetic environment. Collectively, these data showcase the complexities underlying the metabolic mechanisms facilitating energetic trade-offs and highlight that future studies should examine trade-offs among several physiological systems to elucidate how metabolic fuels are being allocated during energetic stress.

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