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Exogenous kisspeptin enhances seasonal reproductive function in male Siberian hamsters

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Summary

1. Animals living in temperate climates are faced with the challenge of reproducing only when environmental conditions are suitable for offspring survival. Environmental cues signalling current and future energy availability (e.g., food availability and photoperiod respectively) are used to appropriately time reproduction. The precise neuroendocrine mechanisms regulating reproduction in response to these cues are unknown.

2. The goal of the present study was to investigate a functional role for kisspeptin, a neuropeptide that shows promise as a key regulator of seasonal reproduction, in integrating multiple environmental cues to regulate reproduction in the Siberian hamster (*Phodopus sungorus*). Siberian hamsters undergo robust gonadal regression and terminate reproduction in unsuitable environments [short winter-like day lengths (photoperiods), low food availability]. Adult male hamsters were housed in short-day or intermediate photoperiods, received either *ad-libitum* access to food or mild food restriction, and were treated with either kisspeptin or a vehicle for 6 weeks to determine the ability of kisspeptin to attenuate gonadal regression.

3. Hamsters exhibited varying degrees of gonadal regression in response to inhibitory environments (short-day photoperiod, food restriction). Kisspeptin treatment successfully enhanced testis mass under these inhibitory conditions, but did not affect normal seasonal changes in body mass and food intake. Thus, kisspeptin specifically enhanced reproductive function without altering other, non-reproductive physiological responses to these environmental treatments.

4. The inhibitory environmental conditions used in this study caused little if any decline in serum luteinizing hormone (LH) and testosterone over the course of the experiment. Kisspeptin treatment tended to exacerbate the decline in LH within individuals, but there were no significant effects of kisspeptin when comparing changes in the hormone levels amongst groups. The interesting outcome that kisspeptin enhanced testis mass, apparently independently of hypothalamic endocrine mechanisms, suggests the possibility of local kisspeptin action within the gonads.

5. Overall, we show a functional role for kisspeptin in integrating complex environmental information to specifically support reproduction. Future work should focus on direct effects of kisspeptin at the gonadal level of the HPG axis as well as its interactions with the metabolic functions and other hormones involved in reproduction, e.g., gonadotropin-inhibitory hormone (GnIH).

Key-words: food restriction, gonadal regression, intermediate photoperiod, reproduction, RFamide, seasonality

Introduction

Animals inhabiting temperate climates experience strong seasonal fluctuations in environmental factors that influence survival and reproductive success. To cope with these fluctuations, individuals have evolved to limit reproduction to times of year when conditions are most favourable for survival of self and offspring (e.g., times of maximal energy availability) (Bronson 1985; Bronson & Heideman 1994; Goldman 2001). Environmental cues signalling fluctuating energy fall into two broad categories: initial predictive cues, that signal the likelihood of future energy availability

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(e.g., photoperiod), and supplementary cues, that are direct indicators of immediate energy availability (e.g., current food availability) (Wingfield & Farner 1980). Many rodents including Siberian hamsters (*Phodopus sungorus*, Pallas, 1773; Fig. 1) successfully synchronize reproduction with favourable conditions by detecting and integrating both types of cues. Siberian hamsters are reproductively active in long-day, summer-like (>14 h of light per day) photoperiods and undergo robust gonadal regression in short-day, winter-like (<12 h of light per day) photoperiods (Hoffmann 1982). Photoperiod information is encoded through a neuroendocrine signal of pineal melatonin; greater durations of melatonin secretion in longer dark periods of winter trigger gonadal regression (Bartness *et al.* 1993; Goldman 2001). To use photoperiod to determine the time of year, two pieces of information are required: the duration of melatonin release in the present as well as the change in this duration of release over time (whether duration of release becomes longer, indicating the onset of winter, or shorter, indicating the onset of summer) (Goldman 2001). In constant photoperiod extremes of >14 h or <10 h of light per day, similar to those surrounding the summer and winter solstices in temperate regions in nature, supplementary cues fail to influence reproductive physiology in Siberian hamsters because photoperiod provides an accurate indication of resource availability in the near future, even without information regarding how photoperiod is changing (Paul *et al.* 2009a, b). However, if hamsters develop within a constant photoperiod of intermediate length (13.5 h of light per day), which would occur close to the vernal and autumnal equinoxes in nature, photoperiod cannot provide an accurate indication of future energy availability without the additional information of change in duration of melatonin release. In these conditions, supplementary cues have a greater effect on seasonal reproductive processes. Recent work has shown that combining an intermediate photoperiod treatment in the lab with either mild food restriction or same-sex group housing—both of which signal impending limited energy availability—triggers gonadal regression, indicating that hamsters rely on supplementary cues when initial predictive cues are unreliable (Paul *et al.* 2009a, b). These physiological responses to fluctuating laboratory environments are a promising area of exploration in seasonal reproduction, as the neuroendocrine mechanisms underlying this integration of complex environmental information are not yet fully understood.

Fig. 1. Siberian hamster (*Phodopus sungorus*) long-day (bottom) and short-day (top) photoperiod reproductive morphs. Photographer: Aaron Jasnow.

The hypothalamo-pituitary-gonadal (HPG) axis integrates the large variety of environmental and internal signals that modulate reproductive function, including photoperiod, energetic state, stress level, social environment, and others. The hypothalamic gonadotropin-releasing hormone (GnRH) neurons control secretion of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland, thereby leading to changes in gonadal function (Nelson 2011). GnRH pulse frequency can be altered by either direct or indirect input from numerous signals, both external, such as photoperiod, and internal, such as nutritional status. For example, photoperiod-induced changes in melatonin control seasonal reproduction by altering GnRH pulse frequency. However, melatonin does not affect GnRH neurons directly (Malpaux *et al.* 2001), and so the specific neuroendocrine mechanisms that take place between the melatonin signal of photoperiod and the activation of pulsatile GnRH release have not yet been fully described.

In recent years, the RFamide neuropeptides kisspeptin and gonadotropin-inhibitory hormone (GnIH, also known as RFamide-related peptide 3, RFRP-3) have emerged as important neuroendocrine regulators of reproduction, and show promise as components of the neuroendocrine system controlling seasonal reproduction in mammals (Revel *et al.* 2007; Greives *et al.* 2008b; Clarke & Caraty 2013; Henson, Carter & Freeman 2013; Simonneaux *et al.* 2013; Kriegsfeld *et al.* 2015). Kisspeptin in particular is an ideal candidate for integrating multiple types of environmental and internal signals to initiate appropriate changes in reproductive physiology. Kisspeptin neurons are located in the arcuate (ARC) and anteroventral periventricular (AVPV) nuclei of the hypothalamus, and exert robust stimulatory action on GnRH neurons (Gottsch *et al.* 2004; Greives *et al.* 2007; d'Anglemont de Tassigny & Colledge 2010; Abbara *et al.* 2013). Kisspeptin increases the pulse frequency of GnRH neurons—subsequently triggering the release of LH and, in some species, FSH, which in turn increases gonadal steroid release—in every mammalian species investigated (reviewed in Abbara *et al.* 2013). ARC kisspeptin neurons are sensitive to both photoperiod and food availability cues, showing lower expression of kisspeptin mRNA and protein in short-day photoperiods and

in conditions of food restriction (Castellano *et al*. 2005; Revel *et al*. 2006; Greives *et al*. 2007; Luque, Kineman & Tena-Sempere 2007; Mason *et al*. 2007; Paul *et al*. 2009b; Ansel *et al*. 2010). Short-day photoperiods are also associated with decreased secretion of LH and testosterone, which affects kisspeptin expression in conjunction with photoperiod; kisspeptin expression in the AVPV is directly, but not completely, modulated by testosterone secretion in male Siberian hamsters (Greives *et al*. 2007, 2008a). Short-day photoperiod and declining testosterone therefore both lower kisspeptin expression in the brain, which leads to the suppression of GnRH and LH secretion, overall resulting in gonadal regression. Kisspeptin neurons also express receptors for the adipose tissue hormone leptin, an important indicator of body fat reserves, indicating their ability to directly detect internal energy availability (Smith *et al*. 2006; Luque, Kineman & Tena-Sempere 2007). Overall, we hypothesize that kisspeptin functions as a 'gatekeeper' to reproductive function by integrating and responding to multiple types of cues (e.g., environmental signals, internal energetic state) and initiating appropriate control of the HPG endocrine axis.

The goal of this study was to assess the role of kisspeptin in integrating photoperiod and food availability cues. To accomplish this, we administered exogenous kisspeptin to hamsters exposed to combinations of photoperiodic and food availability cues that were expected to initiate gonadal regression and assessed the hamsters' physiological and endocrine responses to these combinations of treatments. Exogenous kisspeptin has previously been administered to Siberian hamsters in short-day photoperiods in an unsuccessful attempt to block the normal process of gonadal regression, indicating that strong, initial predictive photoperiod cues alone are too robust for kisspeptin to override (Greives, Kriegsfeld & Demas 2008). Therefore, we hypothesized that exogenous kisspeptin would be successful in blocking regression in response to the supplementary cue of food restriction in an intermediate photoperiod, thus demonstrating the integration of photoperiodic and food availability cues to organize appropriate reproductive responses in complex environmental conditions. Specifically, we expected kisspeptin to prevent the loss of testis mass as well as the decline in HPG axis activity in the face of inhibitory supplementary cues.

Materials and methods

ANIMALS AND HOUSING

Adult (>60 days of age) male Siberian hamsters ($n = 74$) were obtained from 29 litters produced by 10 breeding pairs in our intermediate-day photoperiod (13.5 : 10.5 h light : dark cycle, lights on at 01.30 h Eastern Standard Time, EST) breeding colony at Indiana University. Animals were weaned at 18 days of age, and subsequently housed either individually or with one to four same-sex littermates before entering the experiment. All hamsters were individually housed in polypropylene cages (27·5 × 17·5 × 13·0 cm) with Sani-chip bedding material for 1 week before the start of experimental treatments. Animals received *ad-libitum* access to food (Lab Diet 5001; PMI Nutrition, St. Louis, MO, USA) throughout development before the experiment, and *ad-libitum* access to tap water at all times. Temperature and humidity were maintained at 20 ± 2 °C and 50 ± 10%, respectively. All animal procedures were reviewed and approved by the Indiana University Bloomington Institutional Animal Care and Use Committee.

EXPERIMENTAL DESIGN

Hamsters were randomized by breeding pair source and litter into eight experimental groups, in a full factorial design by photoperiod [intermediate-day (ID) or short-day (SD), 8 : 16 h light : dark cycle, lights on at 07.00 h EST), food availability (*ad-libitum*, AL or food restriction, FR), and injection treatment {10 μM kisspeptin [KiSS-1 (112-121) Amide/Kisspeptin-10/Metastin (45–54) Amide (Human), Phoenix Pharmaceuticals, Burlingame, CA, USA] or the vehicle in which kisspeptin was dissolved [0·1 M sterile phosphate-buffered saline, PBS]}. After 1 week of individual housing and monitoring of baseline food intake, SD animals were transferred to the SD photoperiod, with food and injection treatments within both photoperiods beginning on the same day. Daily food and injection treatments, in addition to weekly monitoring of body mass and estimated testis volume (ETV), continued for 6 weeks, at the end of which animals were euthanized for tissue collection at 10.00 h EST.

FOOD RESTRICTION

For 5 days immediately before the start of experimental treatments, *ad-libitum* food intake was measured to assess a baseline level of intake for each animal. Each hamster's average daily intake across 5 days was used to calculate individual food-restriction rations, which were provided just before lights out each day (15.00 h EST). For FR animals in the ID photoperiod, animals were provided a gradually decreasing ration, in which they received 90% of their baseline intake (w/w, ±0·1 g) during weeks 1–4 of the experiment, and 80% of their baseline intake during weeks 5–6, to simulate naturally-decreasing food availability in the declining photoperiods of autumn (Paul *et al*. 2009a, b). FR animals in the SD photoperiod were given rations reflective of their AL counterparts, because food intake naturally decreases during reproductive regression in SD photoperiods. To accomplish this, SD-AL hamsters' food intake was monitored daily and averaged as a group. Each SD-FR hamster received 90% of the mean SD-AL intake each day, unless an individual's baseline intake was less than the SD-AL mean, in which case he received 90% of his original baseline intake to maintain the mild caloric restriction. SD-FR intake remained 90% of SD-AL intake throughout the experiment; it was not decreased to 80% for the final 2 weeks as with the ID-FR rations. In both photoperiods, upon providing FR rations, any remaining ration in the cage food hopper from the previous day was collected and weighed (±0·1 g) to gain a better approximation of actual intake, although it was not feasible to differentiate calorie consumption from hoarding or cheek pouch storage.

REPRODUCTIVE REGRESSION MONITORING

Body mass (±0·1 g) and estimated testis volume (ETV) were assessed weekly to track reproductive responses to experimental treatments (Paul *et al*. 2009a, b). Hamsters typically lose 15–20% of their adult body mass in response to SD photoperiod treatment alone (Bartness & Wade 1985). Those which do not are part of a genetic subset of Siberian hamsters termed SD non-responders;

these hamsters maintain a reproductively active phenotype regardless of photoperiod treatment (Prendergast, Kriegsfeld & Nelson 2001). FR treatment was expected to enhance mass loss in the SD photoperiod and to trigger a SD-AL-like pattern of mass loss in the ID photoperiod.

Reproductive response was tracked more directly with the ETV measure. Each week, hamsters were lightly anaesthetized with isoflurane vapours, fur covering the scrotal area was moistened with 70% ethanol to facilitate visualization of testes, and calipers were used to measure the length and width of the right testis (±0·01 mm). ETV was calculated as the length × width^2, which is directly correlated with testis mass and spermatogenesis; an ETV of 400 mm^3 indicates a mass of approximately 200 mg; the critical mass for production of viable spermatids is ~350–400 mg (Gorman & Zucker 1995; Schlatt *et al*. 1995).

KISSPEPTIN TREATMENT

Each hamster received one daily 100 μL intraperitoneal (i.p.) injection of either 10 μM kisspeptin suspended in 0·1 M sterile PBS or the PBS vehicle alone in the afternoon (13.00 h EST) (Greives, Kriegsfeld & Demas 2008). Kisspeptin was stored at 4 °C in lyophilized 200 μg aliquots. To maintain homogeneity of the solution for the 6-week experimental period, an excess of lyophilized kisspeptin was reconstituted each day and added to the previous day's solution. To verify the efficacy of the remaining peptide at the end of the experiment, it was administered (100 μL i.p. injection) to four non-experimental male hamsters, from whom blood samples were collected both just prior to and 30 min following the injection. Serum luteinizing hormone (LH) and testosterone were measured in both blood samples to confirm the post-injection surge in circulating reproductive hormones (Fig. 2).

BLOOD SAMPLING AND TISSUE COLLECTION

Two blood samples (one during the individual housing period, 5 days prior to the start of the experiment, and one just before euthanasia and tissue collection on the final day) were collected at 09.00 h EST to monitor changes in reproductive hormones in response to experimental treatments. Hamsters were lightly anaesthetized with isoflurane vapours and blood (~3·5% of the animal's total blood volume) was drawn from the retro-orbital sinus and collected into microcentrifuge tubes. 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 5000 *g*. Serum was collected from the tubes and transferred to a −20 °C freezer for storage until performing hormone assays.

On the last day of the experiment after the final blood sample was collected, hamsters were deeply anaesthetized in isoflurane vapours and necropsies were performed to collect testes and epididymal white adipose tissue (EWAT), a fat pad that surrounds the testis, promotes spermatogenesis, and exhibits expression of kisspeptin mRNA and possibly its cognate receptor (GPR54), although published confirmation of the presence of GPR54 is needed (Brown *et al*. 2008; Chu *et al*. 2010; Brown, Imran & Wilkinson 2011). Tissues were weighed to determine reproductive and energetic status as a result of experimental treatments.

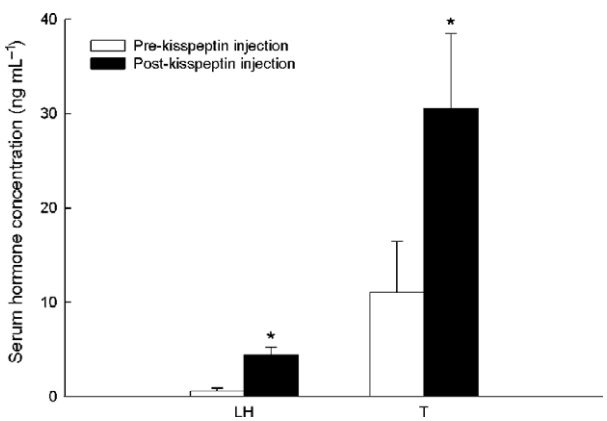

Fig. 2. Mean (±SEM) circulating luteinizing hormone (LH) and testosterone (T) before and after administration of remaining kisspeptin peptide at the end of the 6-week experimental period. *Indicates significant differences between pre- and post-injection circulating hormone levels ($P < 0·05$).

HORMONE MEASUREMENT

Serum LH concentrations were determined via a radioimmunoassay (RIA) described previously (Legan *et al*. 2009) with slight modifications. The standard (rat LH, RP-3) and purified LH for iodination were obtained from Dr. A.F. Parlow at the National Hormone and Peptide Program, Torrance, CA, USA. Because detection of basal levels required a large volume of serum (200 μL), only single aliquots of each sample were diluted in 0·05 M phosphate-buffered saline (PBS) containing 0·1% gelatin (gel-PBS). The primary antibody was CSU 120 (provided by Dr. Terry Nett, Colorado State University, Fort Collins, CO, USA), diluted 1 : 10 000 in 1 : 100 normal rabbit serum (Millipore, St. Charles, MO, USA). The tubes were incubated for 24 h at 22 °C after the addition of 100 μL primary antibody, and after adding radiolabelled LH (~60 000 counts min^{-1}/100 μL gel-PBS, iodinated by the iodogen method), and again following addition of the secondary antibody (anti-rabbit gamma globulin, diluted 1 : 50 in gel-PBS; Millipore). The LH results reported herein were obtained from four assays for which the mean sensitivity was 0·05 ng mL^{-1}, determined as two standard deviations below the maximum binding. Two replicates each of two standard serum pools from male hamsters that inhibited binding on average to 71·4 and 83·7% were analysed at the beginning, middle and end of each assay for determination of inter- and intra-assay coefficients of variation (CVs). The inter-assay CV was 13·4% and the mean intra-assay CV was 8·6%.

Serum testosterone was measured via a commercially available enzyme immunoassay (EIA) kit (Testosterone ELISA Kit, Enzo Life Sciences, Inc., Farmingdale, NY, USA). Serum samples were diluted for measurement on the linear phase of the standard curve and were run in duplicate; any set of duplicates with a CV >10% was re-assayed. All results reported herein were obtained from five assays. The sensitivity of the assay was 7·81 pg mL^{-1}, determined as the lowest concentration of standard. Based on the testosterone concentrations of two replicates each of four standard serum samples from male hamsters that were analysed in each assay and inhibited binding on average to 28·6, 28·6, 42·6, and 64·6%, the inter-assay CV was 14·0% and the mean intra-assay CV was 5·2%.

STATISTICAL ANALYSES

Statistical tests were performed using JMP 12.0.1 (SAS Institute Inc., Cary, NC, USA). A value of $P < 0·05$ was considered to be statistically significant for all tests. Two animals were excluded from analysis after observing their final reproductive state and determining that their measurements lay outside two standard deviations from the group mean. One animal in the SD-AL-kisspeptin group was a SD non-responder (final paired testes mass of 0·441 g), and one animal in the ID-FR-kisspeptin group had severely underdeveloped testes, smaller than those of most SD

individuals (0·049 g). This testis mass did not result from gonadal regression, as it was accompanied by chronically high LH throughout the experiment, presumably because of the lack of negative feedback on the HPG axis by testosterone (final LH concentration was 2·13 ng mL^{-1}, an order of magnitude higher than other measurements); the process of gonadal regression includes deactivation of the HPG axis. These hamsters were excluded because, in addition to qualifying as statistical outliers, their aberrant phenotypes would not be appropriate to compare with those of other individuals in their experimental groups. Final sample sizes for each group were as follows: SD-AL-vehicle: 10; SD-AL-kisspeptin: 8; SD-FR-vehicle: 10; SD-FR-kisspeptin: 9; ID-AL-vehicle: 8; ID-AL-kisspeptin: 9; ID-FR-vehicle: 9; ID-FR-kisspeptin: 8. Data distributions were checked for homogeneity of variance and for normality of model residuals. Those distributions with unequal variances or producing non-normal residuals were transformed to best meet these assumptions for parametric tests. Raw body mass, LH concentration values, and final paired testes mass were log-transformed, whilst percent change in body mass and percent change in ETV were square root-transformed. Differences in LH concentration over the experimental period were assessed by calculating [log(final concentration) – log(initial concentration)]. For measurements of body mass, food intake, ETV, and LH concentrations over time, a repeated measures analysis of variance (ANOVA) was used to detect the effects of photoperiod, food availability, and injection treatments within and between subjects, with time as a within-subjects variable. Within-subjects comparisons for body mass, restricted food intake, and ETV violated the assumptions of sphericity and were Greenhouse-Geiser (G-G) corrected. Effects of treatments on one-time (paired testes and EWAT masses; individual time points of body mass, food intake, and LH concentration) or summary (percent change in body mass/ETV, differences in LH concentration) measurements were assessed through ANOVA models including the three treatments in a full factorial design. Comparisons of group means in these measures were assessed through one-way ANOVAs with group as the primary predictor. Percent change in body mass was included as a covariate in analysis of percent change in ETV and final body mass was included as a covariate in final paired testes and EWAT masses. When these one-way ANOVAs were statistically significant, *post-hoc* comparisons of group means were assessed through individual Student's *t*-tests, which do not correct for multiple comparisons. For analysis of testosterone concentration, Welch's ANOVA was used as an alternative test compatible with heterogeneity of variances; this was necessary because variance heterogeneity led to differences amongst groups in initial testosterone levels. Initial concentration, final concentration, and change in serum testosterone were assessed using Welch's ANOVA with Games-Howell *post-hoc* tests. Because the heterogeneity of variance was so pronounced in initial testosterone concentrations, it was not appropriate to conduct repeated measures analysis for testosterone.

Results

BODY MASS AND FOOD INTAKE

There were no significant treatment effects or differences between groups in baseline body mass ($F_{7,63} = 0{\cdot}38$, $P = 0{\cdot}91$). Body mass decreased over time in all animals in the SD photoperiod and in food-restricted animals in the ID photoperiod (within-subjects time × photoperiod effect, G-G corrected: $F_{1{\cdot}74,109{\cdot}63} = 76{\cdot}75$, $P < 0{\cdot}001$; within-subjects time × food effect, G-G corrected: $F_{1{\cdot}74,109{\cdot}63} = 33{\cdot}64$, $P < 0{\cdot}001$), but was unchanged during the course of the experiment in the ID-AL groups (Fig. 3a). Food treatment first affected body mass as early as week 2 ($F_{1,63} = 4{\cdot}57$, $P = 0{\cdot}037$) and SD photoperiod treatment showed a significant effect on mass by week 3 ($F_{1,63} = 10{\cdot}66$, $P = 0{\cdot}002$). Total percent change in body mass was affected by photoperiod ($F_{1,63} = 83{\cdot}98$, $P < 0{\cdot}001$) and food ($F_{1,63} = 50{\cdot}46$, $P < 0{\cdot}001$) treatments (Fig. 3b); thus loss of body mass was increased by food restriction and shorter day length. Kisspeptin treatment did not affect changes in body mass ($F_{1,63} = 0{\cdot}21$, $P = 0{\cdot}65$).

There were no significant treatment effects or differences between groups in baseline food intake ($F_{7,63} = 0{\cdot}59$, $P = 0{\cdot}76$). SD photoperiod treatment decreased *ad-libitum* food intake gradually throughout the experiment (effect of photoperiod between subjects: $F_{1,31} = 6{\cdot}44$, $P = 0{\cdot}016$; effect of time × photoperiod within subjects: $F_{5,27} = 17{\cdot}08$, $P < 0{\cdot}001$; Fig. 4a); thus the SD-AL hamsters ate

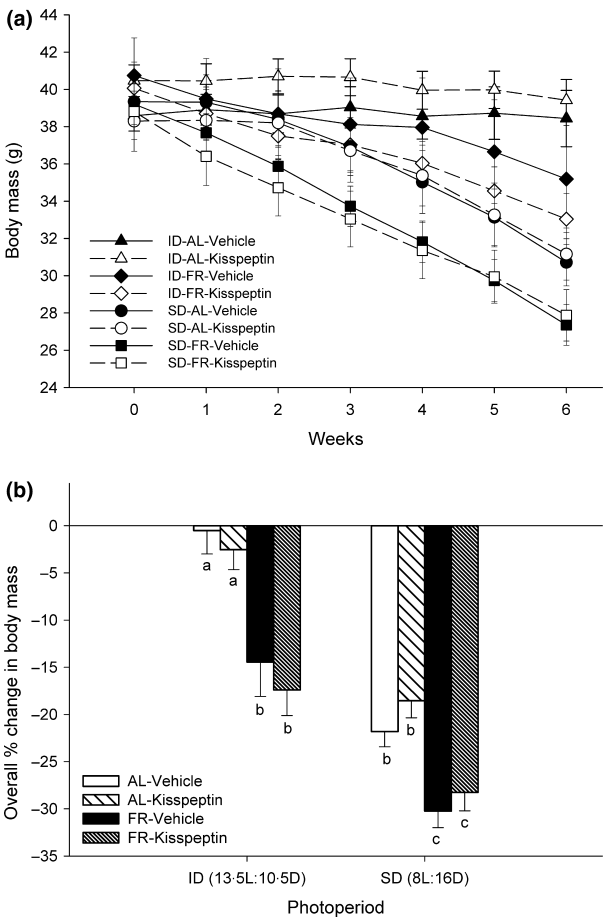

Fig. 3. Mean (±SEM) (a) body mass over time and (b) overall % change in body mass as a result of photoperiod, food availability, and injection treatments. (a) Except for the ID-AL groups, body mass decreased within individuals over time according to photoperiod and photoperiod × food treatments. (b) Groups with different letters indicate statistically significant differences between group means ($P < 0{\cdot}05$); groups sharing the same letter are not significantly different.

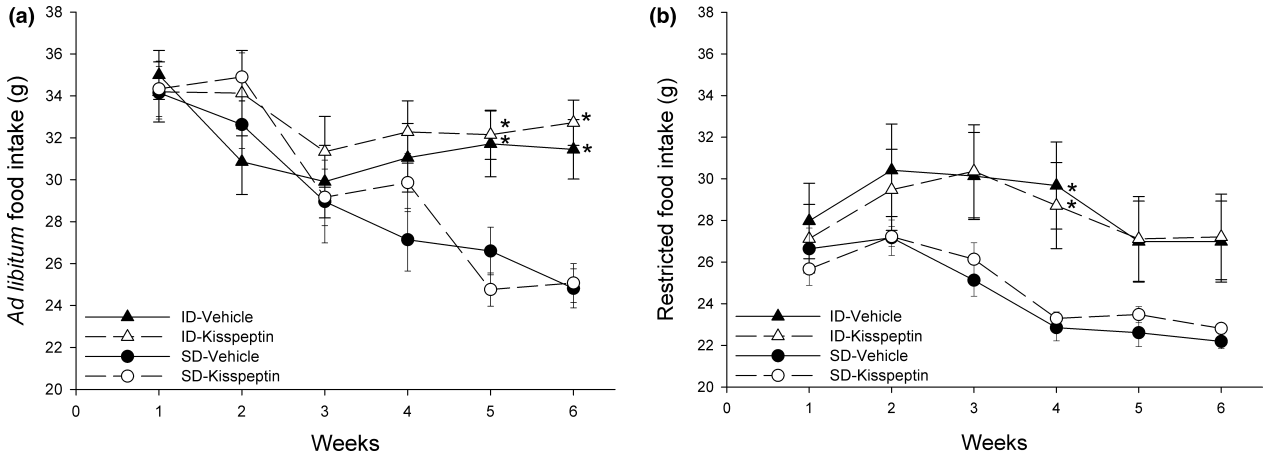

Fig. 4. Mean (±SEM) (a) *ad-libitum* and (b) restricted food intake across the 6-week experimental period. *Indicates significant differences between ID- and SD-treated animals' intake ($P < 0{\cdot}05$).

significantly less than the ID-AL hamsters in weeks 5 and 6. The SD-FR animals' consumption of their restricted food rations similarly declined over time (effect of time within subjects, G-G corrected: $F_{2{\cdot}36,75{\cdot}60} = 22{\cdot}96$, $P < 0{\cdot}001$). ID-FR animals' restricted food consumption was higher than that of the SD-FR animals only in week 4, just before the shift from 90 to 80% restriction in weeks 5–6 (effect of photoperiod between subjects: $F_{1,32} = 8{\cdot}89$, $P = 0{\cdot}005$; effect of time × photoperiod within subjects, G-G corrected: $F_{2{\cdot}36,75{\cdot}60} = 6{\cdot}79$, $P = 0{\cdot}001$; Fig. 4b). Kisspeptin treatment did not affect *ad-libitum* or restricted food intake (AL: $F_{1,31} = 0{\cdot}613$, $P = 0{\cdot}44$; FR: $F_{1,32} = 0{\cdot}0001$, $P = 0{\cdot}99$).

ESTIMATED TESTIS VOLUME

There were no significant treatment effects or differences between groups in baseline ETV ($F_{8,62} = 0{\cdot}33$, $P = 0{\cdot}94$). Changes in ETV over time were highly reflective of changes in body mass, with time × photoperiod (G-G corrected $F_{4{\cdot}86,306} = 25{\cdot}76$, $P < 0{\cdot}001$), time × food (G-G corrected $F_{4{\cdot}86,306} = 11{\cdot}14$, $P < 0{\cdot}001$), and time × photoperiod × food (G-G corrected $F_{4{\cdot}86,306} = 2{\cdot}41$, $P = 0{\cdot}038$) exerting the strongest effects within subjects; thus SD photoperiod and food restriction triggered a decline in ETV over time (Fig. 5a). Total percent change in body mass significantly co-varied with total percent change in ETV ($F_{1,62} = 17{\cdot}77$, $P < 0{\cdot}001$), with photoperiod ($F_{1,62} = 9{\cdot}47$, $P = 0{\cdot}003$) and food ($F_{1,62} = 6{\cdot}12$, $P = 0{\cdot}016$) also playing a role (compare Figs 3b and 5b). Kisspeptin treatment had no effect on changes in ETV ($F_{1,62} = 1{\cdot}42$, $P = 0{\cdot}24$).

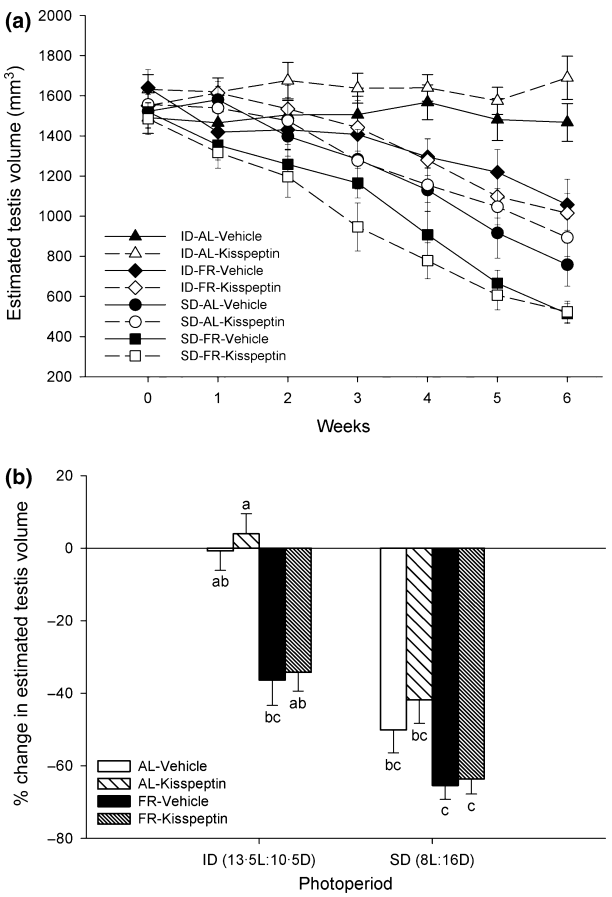

Fig. 5. Mean (±SEM) (a) estimated testis volume (ETV) over time and (b) overall % change in ETV as a result of photoperiod, food availability, and injection treatments. (a) ETV decreased over time within individuals according to photoperiod, food, and photoperiod × food treatments. (b) Groups with different letters indicate statistically significant differences between group means ($P < 0{\cdot}05$) with % change in body mass as a covariate; groups sharing the same letter are not significantly different.

FINAL REPRODUCTIVE MASS

Short-day photoperiod treatment stimulated gonadal regression ($F_{1,62} = 283{\cdot}19$, $P < 0{\cdot}001$), with paired testes mass undergoing a dramatic ~80–90% decrease compared with ID-AL control groups. Kisspeptin injection treatment inhibited full regression in SD-treated animals and enhanced testis mass in ID-treated animals ($F_{1,62} = 29{\cdot}67$, $P < 0{\cdot}001$), such that final testis mass was driven by

photoperiod and injection, but not food ($F_{1,62} = 0{\cdot}22$, $P = 0{\cdot}64$), treatments. A comparison of group means, after controlling for final body mass, revealed a significant effect of kisspeptin injection on final paired testes mass of SD-AL, SD-FR and ID-FR, but not ID-AL, hamsters ($F_{8,62} = 91{\cdot}54$, $P < 0{\cdot}001$; Fig. 6a).

Short-day photoperiod treatment decreased final EWAT mass ($F_{1,62} = 7{\cdot}14$, $P = 0{\cdot}010$); however, *post-hoc* comparisons between experimental groups fell short of significance after controlling for final body mass ($F_{8,62} = 1{\cdot}84$, $P = 0{\cdot}096$; Fig. 6b). EWAT mass was unaffected by food ($F_{1,62} = 0{\cdot}44$, $P = 0{\cdot}51$) and injection ($F_{1,62} = 0{\cdot}48$, $P = 0{\cdot}49$) treatments.

SERUM LH AND TESTOSTERONE

Serum LH concentrations did not differ amongst groups in the initial blood sample ($F_{7,63} = 0{\cdot}39$, $P = 0{\cdot}90$), and changed within subjects according to time ($F_{1,63} = 57{\cdot}57$, $P < 0{\cdot}001$); thus, basal LH levels generally decreased over time. In the final blood sample, there were significant effects of photoperiod ($F_{1,63} = 4{\cdot}75$, $P = 0{\cdot}033$), injection ($F_{1,63} = 13{\cdot}81$, $P < 0{\cdot}001$), and food × injection ($F_{1,63} = 6{\cdot}27$, $P = 0{\cdot}015$). Thus, in the final sample, serum LH concentrations averaged 0·25 ng mL^{-1} lower in kisspeptin-treated FR hamsters than in vehicle-treated FR hamsters, and 0·11 ng mL^{-1} lower in SD than ID hamsters (Fig. 7a). These latter two effects may be because of the final LH levels in the ID-FR-vehicle group, which were about 4-fold higher than those in all other groups. In this regard, it is noteworthy that the smallest decline occurred in ID-AL hamsters, in which no change was expected. *Post-hoc* comparisons of the total change in LH between experimental groups fell just short of significance ($F_{7,63} = 2{\cdot}12$, $P = 0{\cdot}054$; Fig. 7c).

Initial testosterone concentrations were highly variable amongst individuals, particularly in the ID-FR-kisspeptin and SD-AL-kisspeptin groups. Welch's ANOVA analysis of the initial sample of serum testosterone produced a significant result ($F_{7{\cdot}25,65} = 2{\cdot}40$, $P = 0{\cdot}049$), but *post-hoc* tests did not show any significant differences between individual groups (the closest difference to significance was that between the SD-AL-vehicle and SD-AL-kisspeptin groups, $P = 0{\cdot}14$). Treatment effects in the final blood sample were limited to a significant effect of photoperiod, with SD animals exhibiting less circulating testosterone (by 2·20 ng mL^{-1} on average) than ID animals, as expected ($F_{1,63} = 17{\cdot}23$, $P < 0{\cdot}001$) (Fig. 7b). Welch's ANOVA analysis of the total change in the testosterone amongst groups produced a significant result ($F_{7{\cdot}25,72} = 2{\cdot}95$, $P = 0{\cdot}021$), but similar to the initial testosterone concentrations, *post-hoc* analysis revealed no differences between individual groups (the closest difference to significance was between the ID-AL-kisspeptin and SD-AL-kisspeptin groups, $P = 0{\cdot}058$) (Fig. 7d).

Discussion

The goal of the present study was to examine the functional role of kisspeptin in driving reproductive responses to environmental cues that signal changes in energy availability. We accomplished this by measuring the effectiveness of the peptide in inhibiting the process of gonadal regression (i.e., preserving testis mass normally lost during regression in response to the environment) in male Siberian hamsters in complex environmental treatments. We predicted that exogenous kisspeptin treatment would preserve reproductive physiology and endocrine function in environments in which hamsters are solely reliant on supplementary cues (e.g., food restriction in intermediate photoperiod), but would fail to overcome environmental conditions that strongly inhibit reproduction (short-day photoperiod). We measured reproductive function by estimating testis volume throughout the experiment,

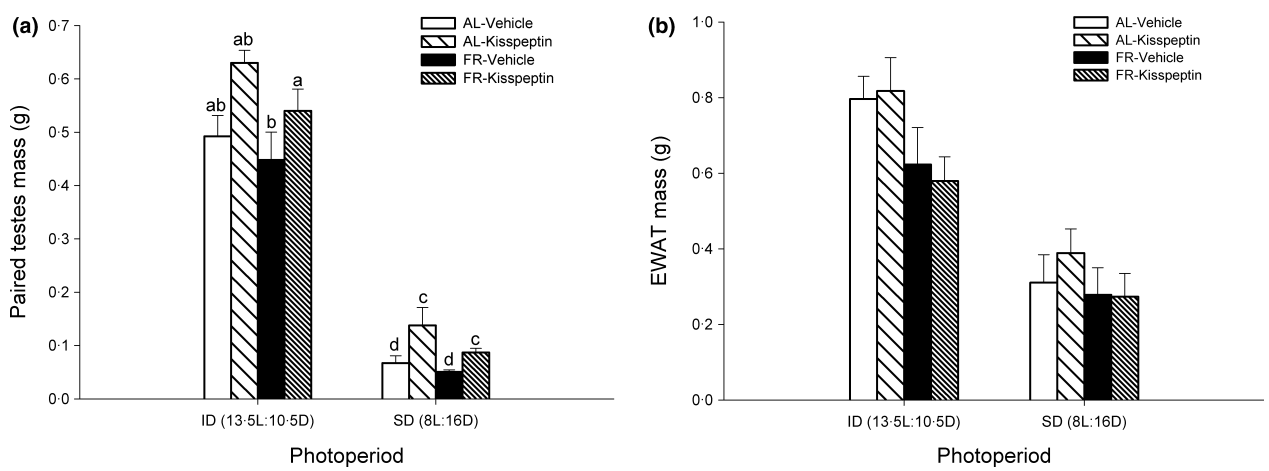

Fig. 6. Mean (±SEM) (a) final paired testes mass and (b) final epididymal white adipose tissue (EWAT) mass as a result of photoperiod, food availability, and injection treatments. Groups with different letters indicate statistically significant differences between group means with final body mass as a covariate ($P < 0{\cdot}05$); groups sharing the same letter are not significantly different. EWAT mass did not differ significantly amongst groups after controlling for body mass ($P = 0{\cdot}096$).

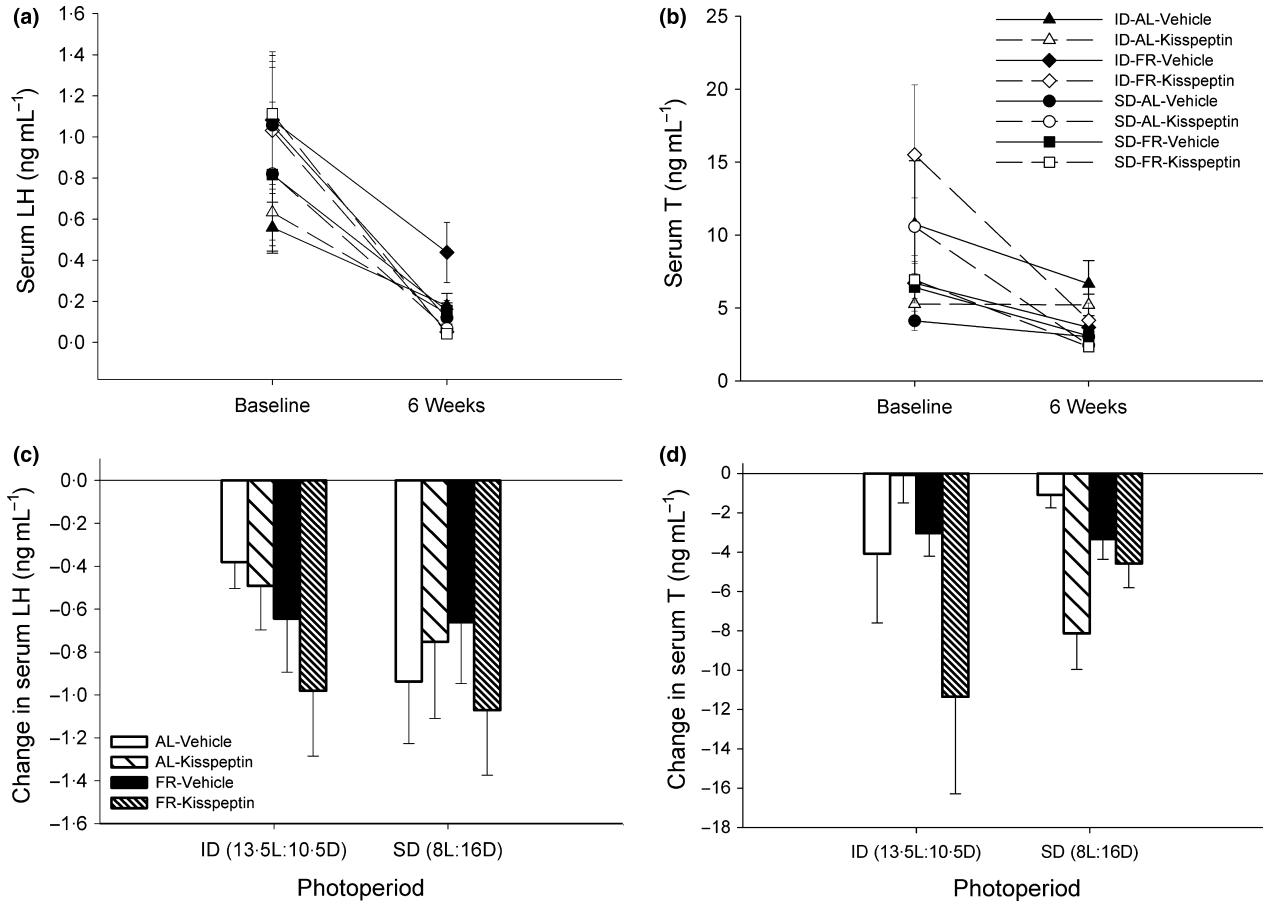

Fig. 7. Mean (±SEM) (a) serum luteinizing hormone (LH) and (b) serum testosterone (T) at the beginning and end of the experiment, and (c) difference in serum LH and (d) difference in serum T between the initial and final serum samples as a result of photoperiod, food availability, and injection treatments. (a) Serum LH declined over time in response to injection and food × injection treatments. (b) Serum T was highly variable amongst individuals and primarily showed effects of photoperiod in the final sample. (c) There were no significant differences amongst group means for the overall change in LH ($P = 0{\cdot}054$). (d) Welch's ANOVA was significant when comparing the change in T amongst groups ($P = 0{\cdot}021$), but there were no significant *post-hoc* differences between groups (closest difference to significance was between ID-AL-kisspeptin and SD-AL-kisspeptin groups, $P = 0{\cdot}058$).

measuring testis mass at the end of the experiment, and assessing reproductive hormone (LH, testosterone) concentrations before and after the experimental treatments.

In Siberian hamsters, gonadal regression is a remarkably robust and well-established response to environmental conditions, most notably photoperiod; this enables researchers to track reproductive function accurately by measuring tissue mass in comparison with hamsters unexposed to photoperiod change (Gorman & Zucker 1995; Prendergast, Gorman & Zucker 2000; Goldman 2001; Revel *et al.* 2006; Greives *et al.* 2007; Greives, Kriegsfeld & Demas 2008; Scherbarth & Steinlechner 2010; Ansel *et al.* 2011; Henson, Carter & Freeman 2013). Our estimated testis volume measure predicted regression in all groups except ID-AL animals, but was highly correlated with body mass, such that virtually no differences between groups could be seen when controlling for body mass. We therefore consider testis mass and circulating reproductive hormones our main indicators of reproductive function. Contrary to our predictions, kisspeptin partially preserved testis mass in both types of inhibitory environments. Hamsters exposed to SD photoperiods are considered gonadally regressed when mass of the paired testes is <0·1 g (Greives, Kriegsfeld & Demas 2008). SD hamsters receiving kisspeptin exhibited a larger paired testes mass compared with their vehicle-treated counterparts in both food treatment groups, and in SD-AL-kisspeptin hamsters, final paired testes mass exceeded this 0·1 g threshold, thus indicating that kisspeptin treatment partially maintained testis functionality. Food-restricted ID hamsters, whilst not differing from AL-treated groups in final paired testes mass, showed a similar enhancement of testis mass when treated with kisspeptin. Thus, providing an exogenous source of kisspeptin was effective in preserving testis mass, one indicator of reproductive function, despite exposure to inhibitory environmental conditions. Importantly, kisspeptin treatment did not affect the other physiological processes associated with seasonal change; body mass loss and decreased food intake proceeded normally, whilst kisspeptin specifically targetted the process of gonadal regression. It is well-established that Siberian hamsters and other seasonally breeding rodents undergo a suite of physiological changes in

response to relevant seasonal cues, including alterations of metabolism, pelage colour, and immune function (reviewed in Nelson *et al.* 2002; Scherbarth & Steinlechner 2010). Our results indicate that kisspeptin functions to coordinate reproductive responses to changing environments without altering non-reproductive responses, supporting hypotheses that kisspeptin is a crucial neuroendocrine component regulating seasonal reproduction (reviewed in Clarke & Caraty 2013).

We hypothesized that one mechanism by which kisspeptin may specifically regulate reproduction in response to the environment is through maintaining activation of the HPG axis in inhibitory environments. We assessed activation of the HPG axis at both the pituitary and gonadal levels by determining circulating LH and testosterone concentrations before and after experimental treatments. Serum LH responded to photoperiod, food × injection, and injection treatments, such that LH tended to be lower in SD animals and FR-kisspeptin animals at the end of the experiment. Repeated measures analysis indicated an effect of injection treatment within individuals, with kisspeptin-treated animals tending to exhibit greater declines in LH compared with vehicle-treated animals. In ID-AL hamsters, LH levels were not expected to fall, and indeed this group had the smallest decrease, averaging <0·5 ng mL^{-1}. However, the decreases in serum LH within individuals were not significantly different amongst groups. This is likely because basal LH levels are very low (near assay sensitivity) and pulsatile, and small differences could not be detected based on only two samples obtained on two separate days. Indeed, even after exposing male Siberian hamsters to 8 weeks of LD or SD photoperiods in a previous study that resulted in ~five-fold and 20-fold decreases in T and paired testes mass, respectively, there were no differences in LH levels (Greives *et al.* 2007). Serum testosterone declined over the 6 weeks of the present study in response to SD treatment, as photoperiod accounted for most differences between groups in circulating testosterone at the end of the experiment. Similar to LH, testosterone levels were not expected to change in the ID-AL hamsters, and these groups showed very little change in circulating testosterone. The ID-FR-kisspeptin group showed the greatest decline in testosterone, which is most likely due in part to the high initial testosterone concentrations in this group. In sum, based on minimal sampling, the HPG axis activity appeared to decline little, if at all, in either the pituitary or gonads in response to the inhibitory environmental treatments.

The foregoing results indicate that kisspeptin successfully preserved testis mass in inhibitory environmental conditions, but appeared to have done so largely independently of affecting HPG axis function. Kisspeptin may therefore have acted directly in the testes to prevent full regression. This possibility is supported by steadily mounting evidence for expression and action of kisspeptin in the testis in several mammalian species; however, kisspeptin and its cognate receptor (GPR54)'s precise function in the periphery, particularly in seasonally breeding species, is unknown, and evidence of their presence in the testis thus far appears to differ between species and varies throughout development (Anjum *et al.* 2012; Tariq *et al.* 2013; Hsu *et al.* 2014; Salehi *et al.* 2015). Thus, confirming the presence and specific sites of kisspeptin and GPR54 expression and action in the gonads of seasonally breeding mammals, as well as their responses to cues such as photoperiod and/or food availability, is necessary before drawing strong inferences about its role in coordinating gonadal regression. Based on findings in other mammalian species, possibilities for a role of kisspeptin in the gonad include mediating gonadotropin receptor expression, supporting spermatogenesis/sperm maturation, or other peripheral actions such as interacting with metabolic processes, thus providing a direct link between gonadal and metabolic function (Pinto *et al.* 2012; Wahab, Atika & Shahab 2013; Hsu *et al.* 2014; Salehi *et al.* 2015). This presents an exciting avenue of future exploration, as direct action of kisspeptin in the testes could potentially explain the interesting interactions between gonadal status and endocrine function across environmental contexts found in this study.

Another factor to be considered is the action of GnIH at multiple levels of the HPG axis. Generally, GnIH functions to impede HPG axis signalling and therefore acts in an opposite manner to kisspeptin (Bentley, Tsutsui & Kriegsfeld 2010). Kisspeptin and GnIH are thus hypothesized to co-regulate reproduction in response to environmental conditions, and have been shown to alter their expression in the brain according to photoperiod and food availability specifically in Siberian hamsters (Greives *et al.* 2008b; Paul *et al.* 2009b; Simonneaux *et al.* 2013). GnIH is also present in mammalian gonads and fluctuates across the spermatogenic cycle, but similarly to kisspeptin, its precise peripheral function is unknown (Zhao *et al.* 2010; Anjum *et al.* 2012). There is also evidence for involvement of GnIH in metabolic functions, as food restriction activates GnIH neurons in conjunction with impaired sexual behaviour in Syrian hamsters (Klingerman *et al.* 2011). Interactions between kisspeptin and GnIH are likely crucial to effective integration of multiple environmental cues.

Results from the current experiment differ from previous studies that have focused on both exogenous kisspeptin administration as well as reproductive regression within an intermediate photoperiod treatment. The present results are supported by previous work in Syrian hamsters, another seasonally breeding rodent species, in which peripheral administration of kisspeptin successfully stimulated testis recrudescence after regression had taken place (Revel *et al.* 2006; Ansel *et al.* 2011). However, in Siberian hamsters, kisspeptin treatment has previously not been successful in blocking the process of gonadal regression or initiating recrudescence (Greives, Kriegsfeld & Demas 2008). Differences in both experiment length and timing of kisspeptin treatment (the previous study did not begin kisspeptin treatment until 2 weeks after housing hamsters in the SD

photoperiod) likely contributed to the present results revealing a functional role for kisspeptin to inhibit gonadal regression in Siberian hamsters. Additionally, previous utilization of intermediate photoperiod treatment in Siberian hamsters has produced more clear evidence of gonadal regression in response to food restriction than in the present study (Paul *et al*. 2009a, b). In the current study, final paired testes mass in ID-FR hamsters did not differ from AL-treated hamsters. This difference is again most likely related to the timeline of the current study; past work with intermediate photoperiod gonadal regression has allowed the process to occur over 12 weeks, whereas the present study ended after 6 weeks (Paul *et al*. 2009a, b). Regardless, kisspeptin treatment clearly enhanced final paired testes mass in FR-treated hamsters compared with the AL treatment, indicating the ability of kisspeptin to support reproductive function in an inhibitory environment.

Collectively, the present findings demonstrate that kisspeptin plays an important functional role in integrating complex environmental information in Siberian hamsters to specifically support reproductive functionality. Mechanisms by which seasonally breeding animals successfully recognize and integrate cues to appropriately time reproductive activity are complex and not yet fully understood. Further exploration of kisspeptin's gonadal actions as well as its interactions with metabolic signals and other neuropeptides such as GnIH will be essential to further elucidating the complexities of achieving reproductive success within fluctuating environments.

Authors' contributions

A.M.B. and G.E.D. conceived the ideas and designed methodology; A.M.B. and S.J.L. collected the data; A.M.B. analysed the data and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Data accessibility

Data deposited in the Dryad Digital Repository https://doi.org/10.5061/dryad.kv011 (Bailey, Legan & Demas 2017).

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