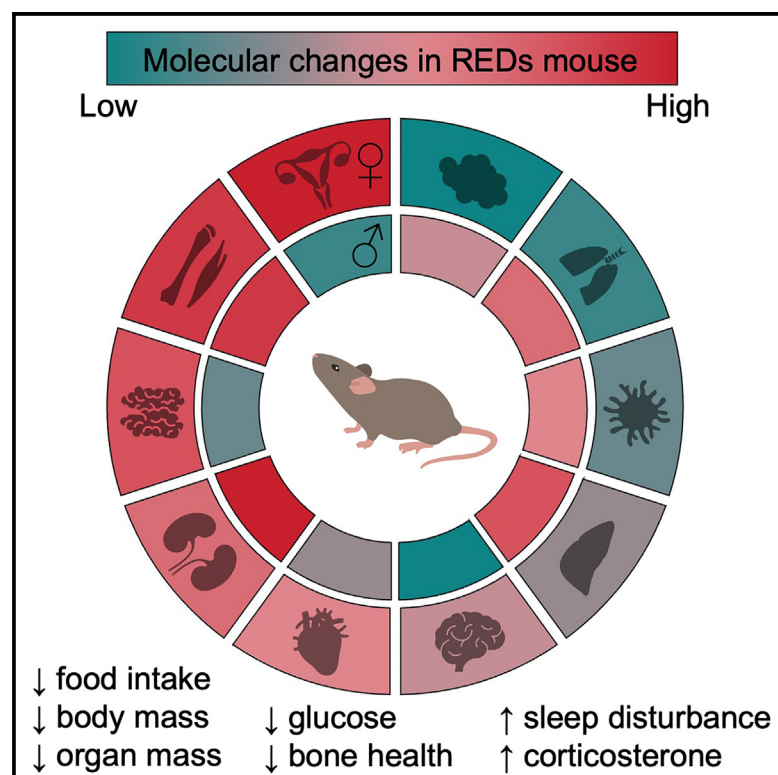


Cell Metabolism

Multi-organ transcriptome atlas of a mouse model of relative energy deficiency in sport

Graphical abstract



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In brief

van Rosmalen et al. present a transcriptome atlas of 19 metabolic/endocrine organs from mice under *ad libitum* and energy-deficient conditions. In response to energy deficiency, 30% of all genes show differential expression in at least one tissue. This extensive dataset will shed light on the molecular underpinnings of REDs.

Highlights

- We mimic behavior and physiology of REDs in mice using the “exercise-for-food” paradigm
- REDs affects extracellular matrix organization in multiple tissues
- In females, reproductive organs and muscles are largely affected by energy deficiency
- In males, kidneys are largely affected by energy deficiency



Article

Multi-organ transcriptome atlas of a mouse model of relative energy deficiency in sport

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SUMMARY

Insufficient energy intake to meet energy expenditure demands of physical activity can result in systemic neuroendocrine and metabolic abnormalities in activity-dependent anorexia and relative energy deficiency in sport (REDs). REDs affects >40% of athletes, yet the lack of underlying molecular changes has been a hurdle to have a better understanding of REDs and its treatment. To assess the molecular changes in response to energy deficiency, we implemented the “exercise-for-food” paradigm, in which food reward size is determined by wheel-running activity. By using this paradigm, we replicated several aspects of REDs in female and male mice with high physical activity and gradually reduced food intake, which results in weight loss, compromised bone health, organ-specific mass changes, and altered rest-activity patterns. By integrating transcriptomics of 19 different organs, we provide a comprehensive dataset that will guide future understanding of REDs and may provide important implications for metabolic health and (athletic) performance.

INTRODUCTION

Relative energy deficiency in sport (REDs), previously known as the female athlete triad, is defined by the International Olympic Committee (IOC) as a condition arising from an imbalance between excessive energy expenditure and insufficient energy intake (intentional or unintentional), resulting in an overall negative energy balance.^{1–4} Prolonged inadequate energy intake, insufficient to support vital (e.g., circulation and neural activity) and non-vital functions (e.g., reproduction, growth, and fat storage), under high training load causes detrimental effects on physiological function and health, including metabolic rate, reproductive function, bone health, and musculoskeletal and cardiovascular health.^{2,3,5,6} These adaptive changes triggered by an energy deficit may, over time, potentially lead to nutrient deficiencies, fatigue, and illness and may lead to reduced (athletic) performance, increased injury susceptibility, and an elevated risk of developing psychological disorders, including depression.^{7–9}

REDs is a complex syndrome affecting many organ systems, significantly impacting both health and performance.¹⁰ When body fat stores are too low, this causes serious health issues. Alterations in lipid/estrogen profiles (LDL, triglycerides, cholesterol, and estrogen) and endothelial dysfunction may put REDs

patients at higher risk for cardiovascular disease, including bradycardia, arrhythmias, and low blood pressure.^{11,12} REDs also has detrimental consequences for bone health, including reduced bone mineral density (BMD).^{13–15} Such changes in bone microarchitecture increase the risk of stress fractures, which can have long-term adverse effects.^{13,16} Low energy availability is a strong indicator of musculoskeletal injury risk.¹⁷ Furthermore, anorexia can lead to reduced kidney function, which may induce severe electrolyte disruption and changes in water balance.¹⁸ Reduced fat stores and reduced estrogen levels can also lead to menstrual dysfunction, such as functional hypothalamic amenorrhea, oligomenorrhea, anovulation, or luteal phase defects.^{2,19,20} Severe energy deficiency can disrupt the pituitary luteinizing hormone (LH) pulse frequency, impacting the hypothalamus-pituitary-gonadal (HPG) axis, resulting in suppression of the reproductive axis under starvation.^{21,22}

Approximately 40% of professional athletes are affected by REDs, and it is suspected that many more recreational active individuals who intentionally increase physical activity and do not ingest enough food to support energy demands may also experience aspects of REDs.^{7,23,24} The wide range of symptoms suggests that energy deficit impacts multiple organ systems and numerous molecular pathways, yet there is very little understanding of REDs at the molecular level. Diagnosing REDs is



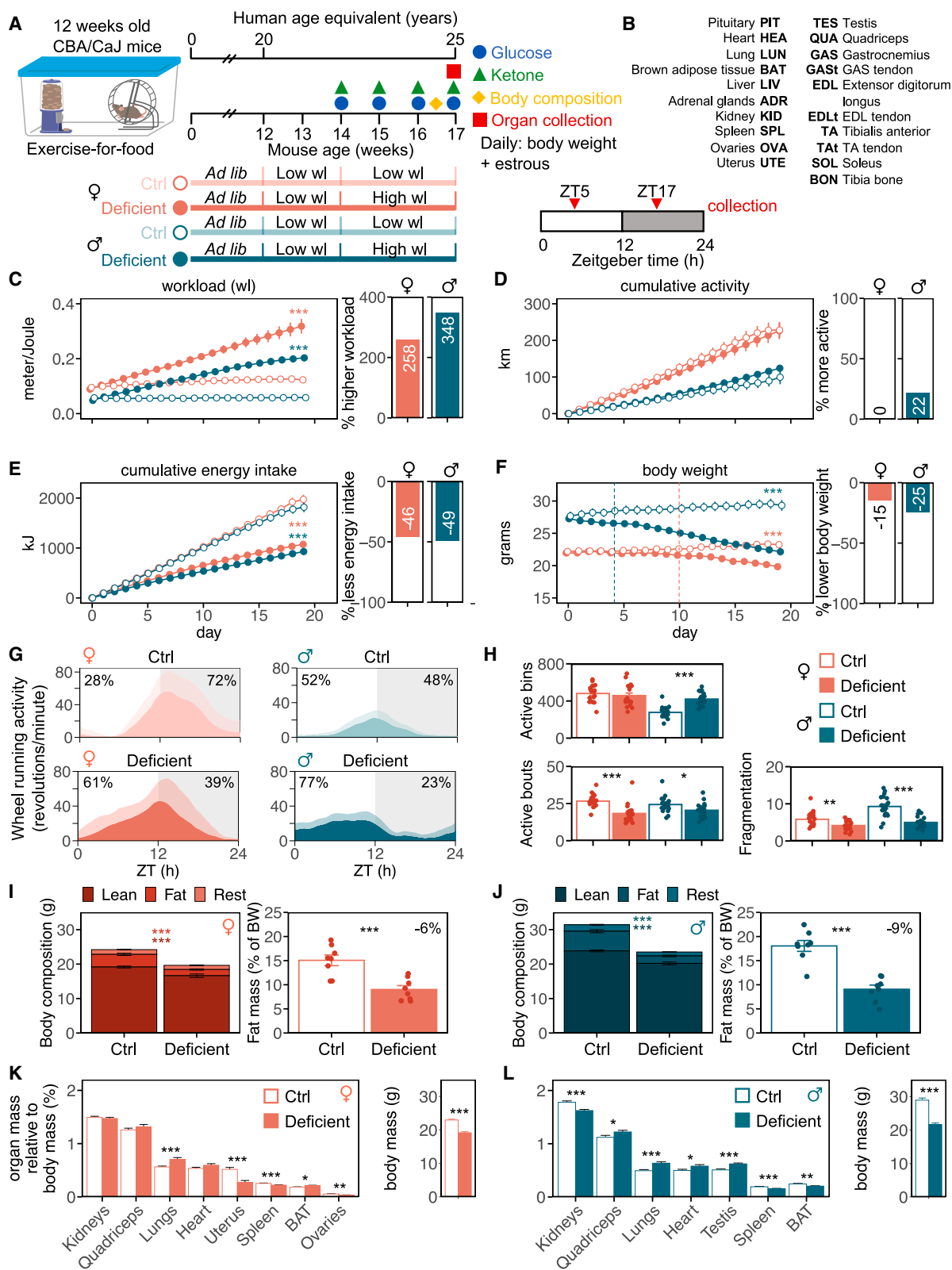


Figure 1. Physiology and behavior of relative energy deficiency in a mouse model

(A) Experimental set-up and design.

(B) List of the 20 tissues collected. Red arrows indicate the timing of tissue collection (ZT5 and ZT17). The shaded area indicates the dark phase (ZT12–ZT24).

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challenging given the variability and subtlety of early-stage symptoms.^{3,24,25} Current clinical assessments rely on symptom evaluation, blood assays, and dual-energy X-ray absorptiometry (DXA) scans to assess bone density.^{4,25,26} Furthermore, no early-detection biomarkers exist to date, and there is not a single validated diagnostic approach for REDs, given the syndrome's complex array of symptoms.⁴ The current REDs treatment focuses on nonpharmacological interventions to increase energy intake and reduce exercise.^{4,25,27,28} This treatment strategy is not always effective,^{3,29} primarily due to partial recovery,³⁰ long recovery periods,³¹ and high dropout rates,³² and certain symptoms like low BMD may only be partially reversible or irreversible with increased energy availability.^{30,33} This highlights the importance of an animal model for understanding molecular changes in response to an energy deficit.

Animal models are essential for understanding the molecular basis of REDs and for developing biomarkers and effective treatment strategies. Rodent models for REDs/activity-based anorexia have been developed in the past.^{34–37} However, the utility of those paradigms to study REDs was limited by high mortality rates and low exercise levels. In order to understand the molecular underpinnings of energy deficiency's impact on different organ systems, physiology, and health, we introduce the “exercise-for-food” paradigm^{38–41} as a mouse model of REDs. Our model mimics a REDs condition in a mouse, with a similar phenotype as human REDs patients: high activity levels, low energy intake, reductions in body/lean/fat/organ weights, disturbed activity-rest patterns, lowered blood glucose levels, and reduced bone health. We investigate relatively young animals (12–17 weeks old), equivalent to 20–25 years old in humans.⁴² This aligns with the typical age range of professional athletes (20–30 years), the age to develop eating disorders, the peak of BMD (females, 19–20 years; males, 20–24 years), and the peak of fertility.^{43,44}

In contrast to humans, animal models give us the unique opportunity to look into the molecular mechanisms of energy deficiency. This mouse model now allows us to comprehensively address these molecular changes in metabolic, endocrine, and musculoskeletal organs, which are challenging to access in human subjects. To this end, we conducted an extensive analysis of the transcriptome of 19 different metabolic tissues and plasma proteomics and metabolomics from energy-sufficient (Ctrl) and energy-deficient (deficient) female and male CBA/CaJ mice at two different time points (day versus night). Our study revealed that energy deficiency affects gene expression in multiple metabolic/endocrine organs. Reproductive tissues and muscles were most affected in females, while in males the kidneys were most affected. Overall, these findings provide insights into the molecular changes in response to a systemic energy deficit and focus

on crucial organs that play a role in governing metabolic processes related to both exercise and energy intake.

RESULTS

Physiology and behavior of relative energy deficiency in a mouse model

To model exercised-linked energy deficiency in mice, we used the exercise-for-food paradigm^{38–41} wherein running wheel activity was coupled to a food reward, phenotyped the mice, and collected tissue samples for further analyses (Figures 1A and 1B). The Ctrl mice had constant access to food, while the deficient mice experienced a gradual reduction in food reward over a 3-week period (Figure 1C). Both groups showed high levels of exercise, and deficient males increased exercise toward the end of the paradigm (Ctrl females, 14.6 ± 1.4 km/day [mean $\pm$ SEM]; deficient females, 12.2 ± 1.5 km/day; Ctrl males, 4.7 ± 0.6 km/day; deficient males, 8.4 ± 0.5 km/day; Figures 1D and S1A–S1K). Furthermore, females ran twice the distance during the 19-day protocol compared to males (Ctrl females, 228 ± 22 km [mean $\pm$ SEM]; deficient females, 226 ± 22 km; Ctrl males, 99 ± 19 km; deficient males, 124 ± 7 km). Over the 3-week period, the deficient mice consumed 46%–49% less food while maintaining a high level of wheel-running activity, resulting in a negative energy balance (Figure 1E). Throughout the paradigm, deficient mice lost body weight, with females (–15%) displaying a lesser degree of weight loss compared to males (–25%) (Figure 1F). Moreover, the onset of weight loss in females occurred later in the protocol (day 10) than in males (day 4). The energy deficit was accompanied by reduced blood glucose levels both during day and night, whereas ketone bodies did not significantly change (Figures S1L–S1O).

Analysis of the activity patterns revealed that deficient mice exhibited reduced fragmentation, characterized by a decrease in the number of active bouts while the number of active bins per day either remained the same (in females) or increased (in males) (Figures 1G and 1H). This indicates that the activity bouts were extended in duration, potentially leading to disrupted sleep, as mice typically exhibit polyphasic sleep patterns with short active and short sleep bouts (2–4 min).⁴⁵ Moreover, energy deficiency led to a prolonged active period, accompanied by increased daytime activity (61% in females, 77% in males) in mice that are typically night-active under *ad libitum* conditions (72% in females, 48% in males), providing additional evidence of sleep disruption (Figures 1G and S1). The link between energy deficiency and sleep disturbances has been observed in athletes at risk for low energy availability, as indicated by their both objective and subjective poor sleep quality.^{6,46–50} This link has also been observed in eating disorders such as anorexia.^{51–53}

(C–F) Daily changes in (C) workload (mJ), (D) cumulative running-wheel activity, (E) cumulative energy intake, and (F) body weight in Ctrl (open symbols) and deficient (closed symbols) female (red) and male (green) mice ($n = 20$ /group). The dashed lines indicate when female (red) and male (green) mice significantly lost body weight.

(G) Average activity profiles are depicted as 3-h running averages $\pm$ SD for the last 5 days of the protocol ($n = 20$ /group).

(H) Average number of active bins, number of active bouts, and fragmentation (active bouts/active bins $\times$ 100) over the last 5 days of the protocol.

(I and J) Body composition measured by echoMRI and fat mass (% of body weight) after 3 weeks of intervention in (I) female and (J) male mice ($n = 8$ /group).

(K and L) Fresh organ mass relative to body mass (%) is depicted for (K) females (red) and (L) males (green) ($n = 20$ /group).

One-way ANOVA was used to analyze the effect of an energy deficit on different physiological parameters between experimental groups. All data are presented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

See also Figures S1 and S2.

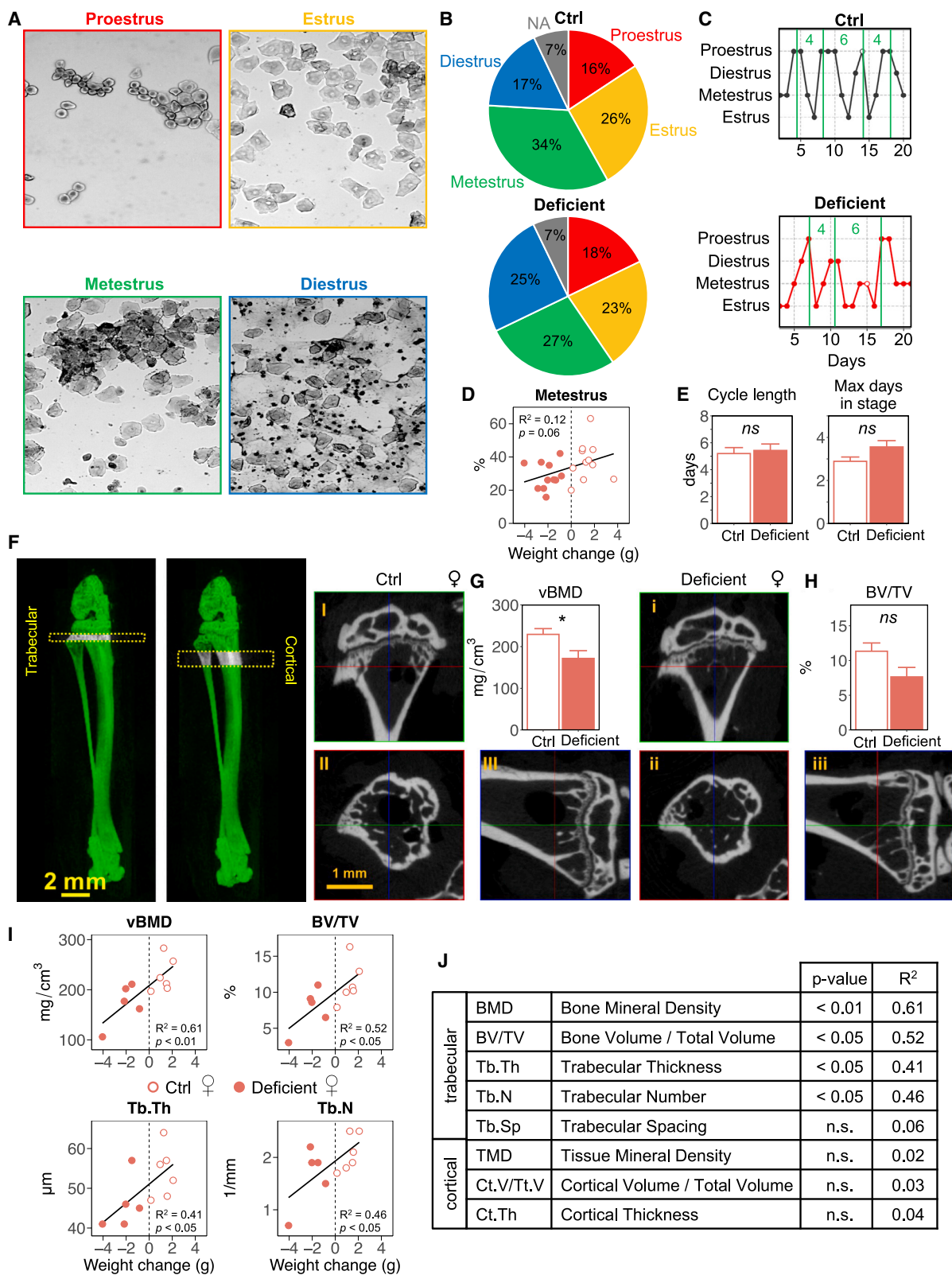


Figure 2. Effects of energy deficiency on the estrous cycle and bone morphology

(A) Images of vaginal smear cytology stained with crystal violet staining reflecting the different stages of the estrous cycle: proestrus (primarily nucleated epithelial cells), estrus (primarily cornified epithelial cells), metestrus (50% cornified epithelial cells and leukocytes), and diestrus (primarily leukocytes).

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Body composition analyses revealed that deficient mice lost both fat (females, −6%; males, −9%) and lean mass (females, −6%; males, −10%) (Figures 1I and 1J). Further investigation into the origin of this loss revealed that the deficient mice experienced a reduction in organ mass. In females, organs such as the uterus, spleen, and ovaries decreased both in absolute mass and relative to body mass, while in male kidneys, spleen and brown adipose tissue (BAT) reduced in mass (Figures 1K, 2L, S2A, and S2B). Interestingly, there was no reduction in absolute lung size, suggesting that deficient mice invested energy in preserving lung tissue, likely to adapt to the demand of high exercise loads.

We observed a time-of-day difference in muscle glycogen content, with elevated levels during the dark phase in both Ctrl and deficient mice (Figure S2C). Conversely, liver glycogen showed a significant interaction between group and time of day, with higher levels during the dark phase in deficient mice and higher levels during the light phase in Ctrl mice (Figure S2D). This discrepancy suggests a divergence in the time-dependent glycogen storage patterns between muscle and liver tissue. While liver glycogen levels adapt in mice, altering their activity rhythms due to energy deficiency, muscle glycogen patterns remained similar. This implies that the depletion of muscle glycogen reserves during the light phase may contribute to fatigue in deficient mice, particularly considering their diurnal activity pattern.

Effects of energy deficiency on the estrous cycle and bone morphology

In contrast to the ~28-day human menstrual cycle, the estrous cycle in mice typically lasts 4–5 days. In humans, reduced energy availability can cause menstrual disturbances (e.g., amenorrhea) aiming to prevent pregnancy to conserve energy for vital processes during periods of resource scarcity. By daily assessing the cell-type composition within vaginal smears (Figures 2A–2C), surprisingly we did not detect significant differences between the estrous cycle characteristics of Ctrl and deficient animals ($n = 11/\text{group}$) (Figures 2D and 2E). However, deficient mice showed a trend toward less frequently entering metestrus (34% versus 27%, −7%) and more frequently entering diestrus (17% versus 25%, +8%). Similarly, mice that lost more weight showed reduced frequencies of entering the metestrus phase (Figure 2D).

To assess bone health in deficient mice, we conducted 3D μCT scans of the female proximal tibia and quantified trabecular

(BMD, bone volume/total volume [BV/TV], trabecular spacing [Tb.Sp], trabecular number [Tb.N], and trabecular thickness [Tb.Th]) and cortical (tissue mineral density [TMD], cortical volume/total volume [Ct.V/Tt.V], and cortical thickness [Ct.Th]) bone morphology metrics (Figures 2F–2J). The tibia is the most common site of stress fractures in athletes (49%)^{54,55} and may be likewise affected in REDs mice. Female athletes are more prone to stress injuries⁵⁶; therefore, we decided to assess bone morphology in female mice. μCT datasets were visualized in orthogonal 2D planes to confirm image quality as confirmed by the clarity of individual trabeculae and cortical walls (Figures 2F and S2). Trabecular bone morphology of female mice ($n = 6/\text{group}$) revealed significant differences between deficient and Ctrl groups. Trabecular bone is porous with high bone marrow and fat content, facilitating vascularization, blood flow, and bone turnover (bone remodeling),⁵⁷ consequently resulting in a high impact on trabecular bone health after periods of deficiency. Deficient mice showed lower BMD than Ctrl (Ctrl, $229 \pm 14 \text{ mg/cm}^3$; deficient, $172 \pm 19 \text{ mg/cm}^3$, −25%; $p < 0.05$), which is linked to reduced bone calcium/mineral levels inducing bone weakness and increased fracture risk⁵⁸ and is consistent with previous studies in rodents when energy availability was low.³⁶ A trend toward lower BV/TV (Ctrl, $11\% \pm 3\%$; deficient, $8\% \pm 1\%$, −33%; $p = 0.07$) and Tb.Th (Ctrl, $54 \pm 3 \mu\text{m}$; deficient, $46 \pm 3 \mu\text{m}$, −15%; $p = 0.07$) in deficient mice was also observed (Figures 2G and 2H). Trabecular bone morphology significantly correlated with weight loss. Animals with more weight loss displayed lower BMD, BV/TV, Tb.Th, and Tb.N (Figures 2I and 2J). Similar relationships between tibia microarchitecture and low energy availability contribute to higher bone stress injury risk in REDs.^{14,15} In contrast, deficient mice did not show altered cortical bone metrics: TMD ($1,118 \pm 12$ versus $1,112 \pm 12 \text{ mg/cm}^3$), Ct.V/Tt.V ($55\% \pm 1\%$ versus $55\% \pm 1\%$), and Ct.Th (204 ± 3 versus $207 \pm 6 \mu\text{m}$) remained similar between Ctrl and deficient female mice. Overall, these findings show that mice subjected to an energy deficiency have trabecular morphology indicative of impaired bone formation and lower bone quality, which potentially can lead to increased injury susceptibility.

Energy deficiency affects gene expression across endocrine and metabolic organs

We investigated the overall impact of energy deficiency on gene expression in 419 samples representing various tissues (Figures 1B and S3A; Table S1). We implemented a differential expression analysis comparing transcripts of Ctrl and deficient

(B) Frequency plots for identified estrous stages during the last 7 days in Ctrl (top) and deficient mice (bottom) ($n = 10\text{--}11/\text{group}$).

(C) Examples of estrous cycle plots for individual Ctrl (top) and deficient mice (bottom).

(D) Correlation between frequency entering metestrus and weight loss.

(E) Bar graphs showing estrous cycle length and maximal consecutive days in one stage in Ctrl (white) and deficient mice (red).

(F) Trabecular and cortical bone morphology were analyzed in the regions 350–850 μm and 1,500–2,500 μm distal to the proximal GP, respectively. The region of analysis is indicated by the dashed yellow box. Trabecular bone in the (I and i) sagittal, (II and ii) transverse, and (III and iii) coronal view of Ctrl (I–III) and deficient (i–iii) female tibia ~0.5 mm distal to the proximal growth plate.

(G and H) Bar graphs showing (G) trabecular bone mineral density and (H) bone volume/total volume (BV/TV) stage in Ctrl (white) and deficient mice (red) ($n = 5\text{--}6/\text{group}$).

(I) Significant correlations between trabecular parameters and weight change.

(J) Definitions of morphologic parameters and p values and R^2 of correlations with weight change.

One-way ANOVA was used to analyze the effect of an energy deficit on different parameters between experimental groups. All data are presented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Linear regression models were fit by using the lm function in R to assess the correlation between different parameters. See also Figure S2.

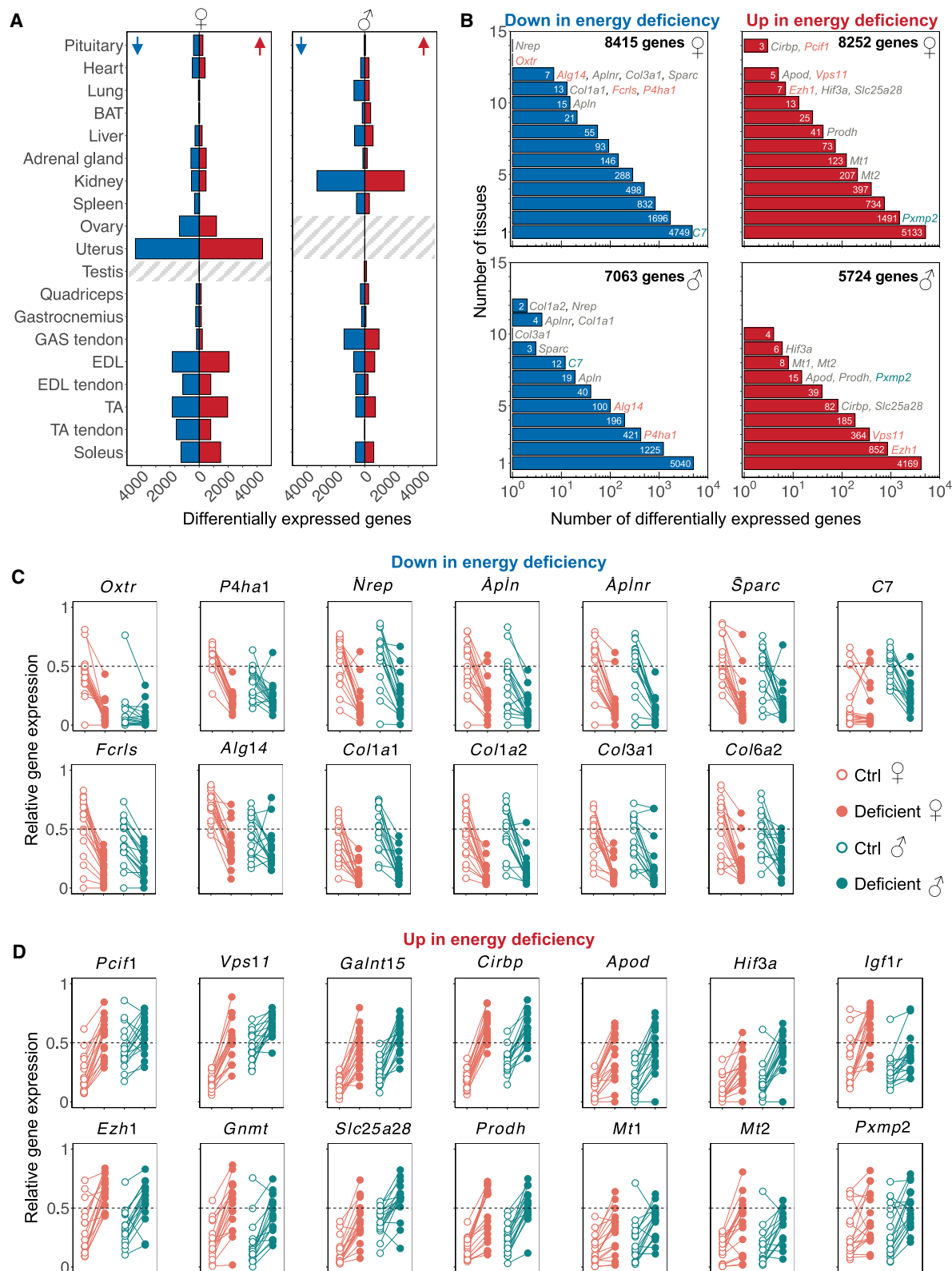


Figure 3. Energy deficiency affects gene expression across endocrine and metabolic organs

(A) Number of differentially expressed (DE) genes from time-independent analyses for all tissues in female and male mice. Genes downregulated in deficient mice are shown in blue and upregulated are shown in red.

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mice, irrespective of the time of day. We found substantial variation in the number of differentially expressed (DE) genes across different tissues, showing tissue-specific responses to an energy deficiency (Figure 3A; Table S2). Importantly, comparison of DE genes in the liver or muscle from our study with the respective organs from mice under calorie restriction or exercised showed minimal overlap,^{59,60} thus supporting the notion that the REDs represents a molecular state that is not largely due to calorie deficit or exercise alone. In total, 14,837 uniquely DE genes (protein-coding and non-coding) were detected in any one tissue in females and 11,326 in males. However, there were also changes shared among ≥ 5 tissues ($>25\%$ of analyzed tissues) of deficient mice; 497 [females]/154 [males] genes were upregulated and 640 [females]/181 [males] genes were downregulated; Figure 3B; Table S3). A greater proportion of DE genes was found exclusively in females (6,153, 41%) compared to males (2,641, 23%). This sex-specific gene expression suggests the contribution of sex-linked genes and sex hormones. 643 of these female-specific DE genes contained an estrogen receptor response element (ERE) within -10 kb to $+5$ kb from the mRNA 5' end,⁶¹ indicating that changes in estrogen may be responsible for changes in the expression of these genes. Moreover, a transcription factor (TF) enrichment analysis revealed that 11%–28% (mean = 17%) of the DE genes per tissue contain a response element for ER (Figure S3B; Table S4). Reduced circulating estradiol levels have been found in chronic deficient rodents and humans.^{21,36} In the following paragraphs, we focus on transcripts that were DE in multiple (≥ 5) tissues in deficient mice.

Several genes were predominantly downregulated in tissues of deficient females. For example, the oxytocin receptor (*Oxtr*) was downregulated in 13/18 female tissues (Figure 3C). Oxytocin, a peptide hormone produced in neurons of the paraventricular nucleus (PVN) of the hypothalamus projecting to the pituitary to release oxytocin to the bloodstream, plays a crucial role in regulating various physiological processes, including social behavior, pair bonding, eating behavior, metabolism, and the initiation of labor and lactation.^{62–64} Previous studies have shown that individuals with symptoms of REDs exhibit lower plasma oxytocin levels, which is associated with changes in bone microarchitecture, as this hormone is anabolic to the bone.^{65–68} Moreover, estrogen, through the activation of the ER β TF, modulates oxytocin expression.⁶³ Our findings indicate that low energy availability decreases oxytocin signaling throughout the body, possibly due to reduced estrogen levels. Furthermore, these results highlight the multi-tissue effects of oxytocin in the body, indicating its functional relevance and link with energy balance beyond the uterus and mammary glands.

P4ha1, a key enzyme involved in collagen synthesis, was downregulated in 11/18 female tissues. This finding aligns with the downregulation of multiple genes related to extracellular matrix (ECM) organization (*Nrep*, *ApIn*, *ApInr*, and *Sparc*), including various collagens (*Col1a1*, *Col1a2*, *Col3a1*, and *Col6a2*) in both females and males (≥ 10 tissues). Considering the importance of collagen in ECM organization,⁶⁹ these findings suggest that

low energy availability leads to reduced tissue strength and support, leading to musculoskeletal weakness. Also, several transcripts coding for proteins involved in immune response (*C7* and *Fcrls*) were downregulated with energy deficiency. This is in agreement with the observation that REDs patients are more susceptible to infections due to decreased immunity.^{6,70,71}

Both apelin (*ApIn*) and its receptor (*ApInr*) were downregulated in multiple tissues. Apelin is an adipokine (factor secreted by adipose tissue to signal energy metabolism: insulin secretion, glucose uptake, mitochondrial biogenesis, fatty acid oxidation, and lipolysis) that is increased in obesity.⁷² The significant reduction in fat mass in deficient mice may have led to lower secretion of adipokines such as apelin, which may have induced adaptive changes in glucose and lipid metabolism. *Alg14*, involved in protein N-glycosylation, was downregulated in multiple tissues.

A smaller subset of genes was significantly upregulated upon energy deficiency (Figure 3D). Among them, *Pcif1*, an mRNA methyltransferase that plays an important role in gene expression regulation,⁷³ was primarily upregulated in female tissues (14/18 female, 1/17 male). The upregulation of the growth-promoting receptor, *Igf1r* (10/18 female, 3/17 male), suggests altered IGF signaling in deficient mice. Consistent with these findings, reduced IGF-1 levels have been associated with energy deficit in humans.^{5,74}

Several other genes, including *Pcif1*, *Vps11*, *Galnt15*, *Cirbp*, *Apod*, *Hif3a*, *Igf1r*, *Ezh1*, *Gnmt*, *Slc25a28*, *Prodh*, *Mt1*, *Mt2*, and *Pxmp2*, were upregulated in multiple tissues of both females and males. Cold-inducible RNA-binding protein (*Cirbp*) was induced in various tissues and plays an important role in the adaptive response to hypothermia. Indeed, both mice and humans lower their core body temperature as an adaptive response to reduce energy expenditure during periods of food scarcity.^{38,75–78} Apolipoprotein D (*Apod*), a component of high-density lipoprotein (HDL) and a member of the lipocalin family that transports small hydrophobic molecules and binds to steroids and bilirubin, showed increased expression in several female and male tissues. Hypoxia-inducible factor *Hif3a* is a TF that regulates processes in response to hypoxia by regulating gene transcription.⁷⁹ This finding indicates that deficient mice exhibit signs of tissue hypoxia, which may be a consequence of hemoglobin deficiency anemia, leading to impaired oxygen binding and transport having detrimental effects. Prolonged hypoxia induces bone acidosis, leading to reduced BMD when exercising.⁸⁰ Prolonged malnutrition in individuals with REDs increases their susceptibility to iron deficiency.⁸¹ Upregulation of the mitochondrial iron transporter *Slc25a28* in deficient mice suggests an iron deficiency in these mice. Furthermore, upregulation of the transporter *Slc10a6* for large neutral amino acids in multiple muscle tissues indicates inadequate nutrient supply to the muscles. Furthermore, the expression of metallothionein (*Mt1* and *Mt2*) was upregulated in various tissues. Besides its role in regulating essential metal balance (zinc and copper), it can also be induced by glucocorticoids,⁸² which are elevated in the plasma of deficient mice.³⁹ Interestingly, a TF enrichment analysis revealed that 4%–24% (mean = 17%) of

(B) Distribution of DE genes and the number of tissues in which they are downregulated or upregulated in females (top) and males (bottom).

(C and D) Normalized expression for a selection of (C) downregulated genes and (D) upregulated genes in Ctrl (open symbols) and deficient (closed symbols) females (red) and males (green) across tissues. Connecting dots represent the same tissues.

See also Figure S3 and Tables S2 and S3.

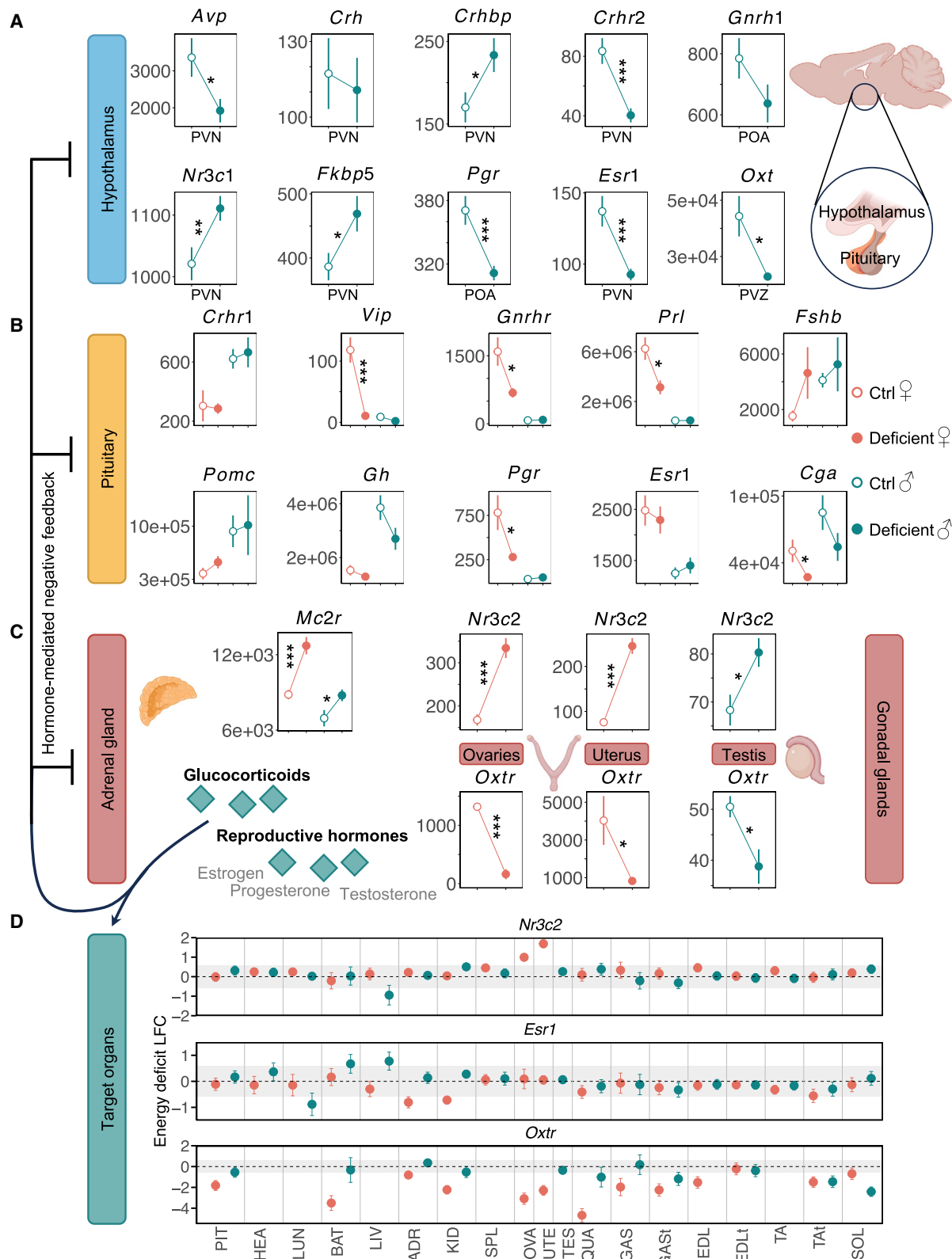


Figure 4. HPA axis and HPG axis gene expression changes in deficient mice

(A–C) Normalized expression for a selection of candidate genes involved in (A) hypothalamic (PVN, paraventricular nuclei; POA, preoptic area; PVZ, periventricular zone) from a previously published dataset,⁴¹ (B) pituitary, and (C) adrenal/gonadal axis signaling.

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the DE genes per tissue contain a glucocorticoid receptor element (GRE) to bind glucocorticoids and subsequently regulate transcription (Figure S3B; Table S4). Furthermore, the *Ptbp2* gene, a locus identified in genome-wide association studies (GWASs) as having links to both anorexia nervosa and BMI, was upregulated in 6 tissues of deficient female mice.^{83,84} In summary, these changes in gene expression shared among multiple tissues irrespective of time of day indicate a systemic effect of energy deficiency on the whole body for several pathways.

Time-of-the-day-specific gene expression changes

In addition to the time-independent analyses, we also analyzed the transcriptome datasets for expression changes at specific times of day (day, zeitgeber time [ZT]5 or night, ZT17) (Figure S3C; Table S5). In every organ, we found DE genes that were specific to either ZT5 or ZT17, and <30% of DE genes were shared between both time points. Similar functional annotation of DE genes in each tissue at ZT5 or ZT17 yielded time-specific as well as shared GO terms (Table S5). Such strong temporal effects on DE genes may arise from changes in the phase of the circadian clock. This experimental paradigm of activity-dependent food availability also changes the phase of the molecular circadian clock in most brain regions and peripheral tissues,⁴¹ which parallels a change in diurnal activity-rest pattern (Figure S1). However, a change in phase of the clock may not fully explain gene expression changes that are specific to only day or night. For example, colony-stimulating factor 1 (*Csf1*), a gene encoding for a cytokine that controls macrophages, was decreased in male lungs during energy deficiency only at ZT17 (Figure S3D). Glycogen synthase 2 (*Gys2*) in male liver was higher in Ctrl mice at ZT5. Nuclear receptor subfamily 4 group A member 3 (*Nr4a3*), encoding a nuclear hormone receptor, was elevated in deficient female adrenal glands at ZT5. Insulin-like growth factor binding protein 1 (*Igfbp1*) was increased in deficient mice at ZT17, and the taurine transporter (*Slc16a6*) was higher in the extensor digitorum longus (EDLs) of Ctrl mice at ZT17. In males, the reduction in BAT size aligns with reduced BAT activity observed in male endurance athletes.⁸⁵ The decreased uncoupled protein 1 (*Ucp1*) expression, a mitochondrial regulator of non-shivering thermogenesis within BAT⁸⁶ at ZT5 in males (Figure S3D), suggests attenuated BAT activity during energy deficiency. These findings align with previous research that demonstrated reduced core body temperature during inactivity in deficient male mice.³⁸ In contrast, female mice did not change BAT size or *Ucp1* expression. This is in line with their lower amplitude core body temperature rhythms and relatively high temperatures during the inactive phase under deficient conditions.⁸⁷

HPA axis and HPG axis gene expression changes in deficient mice

Next, we conducted a targeted analysis to investigate the impact of energy deficiency on endocrine function, as observed in athletes experiencing relative energy deficiency.⁵ Hypothalamic-pi-

uitary-adrenal/gonadal/thyroid (HPA, HPG, and HPT) axis activity plays a critical role in REDs.⁵ While the expression of the key stress response regulator, corticotropin-releasing hormone (*Crh*), in the PVN of the hypothalamus remained unaffected, another releasing factor, corticotropin-releasing hormone binding protein (*Crhbp*), was upregulated, and the corticotropin-releasing hormone receptor (*Crhr2*) was downregulated (Figure 4A). Elevated *Crhbp* expression in several brain sites can be triggered by various stressors, including food deprivation and treadmill running.⁸⁸ Moreover, the expression of arginine vasopressin (*Avp*), a controller of adrenocorticotrophic hormone (ACTH) secretion from the anterior pituitary, was reduced in deficient mice.

Moving downstream to the pituitary gland, ACTH is cleaved from proopiomelanocortin (POMC); however, the expression of both *Crhr1* and *Pomc* did not change upon energy deficiency, suggesting a potential involvement of glucocorticoid-dependent negative feedback (Figure 4B). It has been observed that plasma cortisol levels are elevated in response to fasting and in individuals with REDs,^{89–91} and a corresponding increase in plasma corticosterone (the equivalent hormone in rodents) was also described in this exercise-coupled feeding paradigm.³⁹ Additionally, the *Acth* receptor *Mc2r* was upregulated in the adrenal glands of deficient mice (Figure 4C). The glucocorticoid-mediated negative feedback acts on the hypothalamic GR complex (*Nr3c1* and *Fkbp5*), which increased in expression during energy deficiency (Figure 4A). The observed changes in genes associated with the HPA axis suggest activation of the neural circuitry linked with stress in deficient mice.

Optimal functioning of the HPG axis is required for fertility and reproduction. Previous studies have shown the tight link between energy balance and disruption in the reproductive axis.²² The key regulator of the reproductive axis, gonadotropin-releasing hormone (*Gnrh1*) neurons, located in the preoptic area (POA) of the hypothalamus, showed a tendency toward lower expression levels in deficient mice (Figure 4A). In the pituitary gland, genes coding for HPG axis releasing factor receptors, gonadotropin subunits, and steroid hormone receptors changed in expression in deficient females (Figure 4B). The expression of the gene encoding the GnRH receptor (*Gnrhr*) was reduced in female pituitary. Vasoactive intestinal polypeptide (*Vip*), which stimulates prolactin secretion from the pituitary,⁹² and prolactin (*Prl*) itself were both reduced in deficient females (with log2FC values of −3.5 and −1.0, respectively), but not in males. This indicates sex-dependent responses of genes within the reproductive axis in response to energy deficiency. Glycoprotein hormone alpha subunit (*Cga*), which binds to FSHb, LHb, or TSHb to form active dimers, was downregulated with energy deficiency, while *Fshb*, *Lhb*, and *Tshb* did not significantly change. Reduced *Cga* expression limits the pituitary gonadotropins (FSH, LH, and TSH) to activate downstream signaling pathways in target organs throughout the peripheral body during periods of food scarcity. Furthermore, *Oxtr* expression was downregulated and *Nr3c2* upregulated in gonadal

(D) Energy deficit-induced log2 fold changes (log2FC ± SEM) of receptors in target organs in Ctrl (open symbols) and deficient (closed symbols) females (red) and males (green).

Data from all time points were combined in this analysis. All data are presented as mean ± SEM, **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

See also Figure S3 and Table S2.

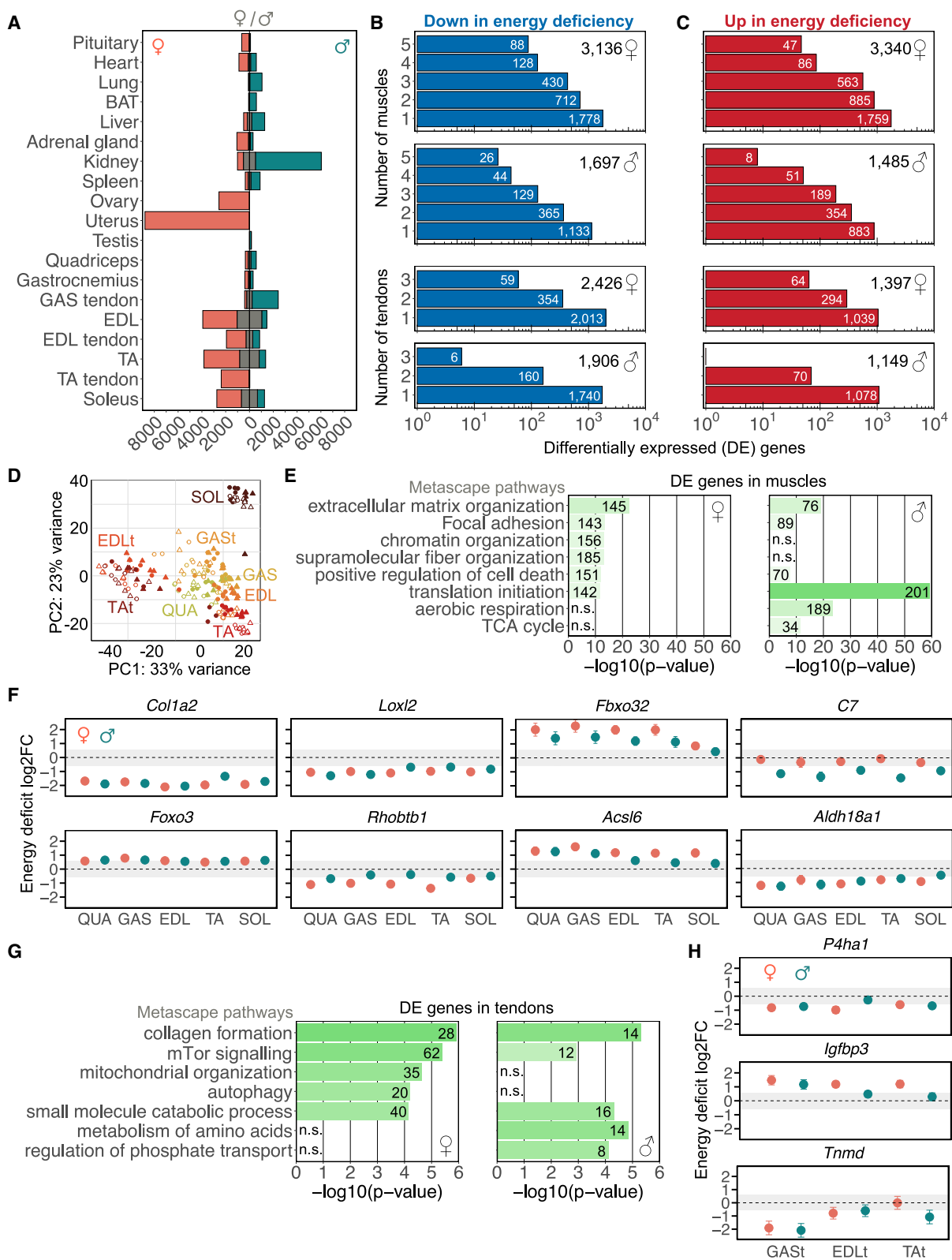


Figure 5. Energy deficiency affects gene expression in multiple muscles and tendons

(A) Number of differentially expressed (DE) genes from time-independent analyses in energy deficiency for all tissues in females and males. Female DE genes are shown in red, male DE genes in green, and overlapping genes between females and males in gray.

(legend continued on next page)

glands. This implies reproductive tissues change the sensitivity to glucocorticoids and oxytocin under a negative energy balance to regulate downstream physiological processes accordingly (Figures 4C and 4D). Hormone-mediated negative feedback induced alterations in the expression of several hypothalamic steroid hormone receptors, progesterone receptor (*Pgr*) and estrogen receptor (*Esr1*), in response to energy deficiency. Taken together, these findings suggest activation of the HPA axis and de-activation of the HPG axis activity, primarily in females, induced by a negative energy balance.

Energy deficiency affects gene expression in multiple muscles and tendons

Athletes who suffer from REDs are more prone to musculoskeletal injuries.⁹³ To investigate the effects of insufficient energy intake while excessively exercising on the molecular dynamics of the musculoskeletal system, we focused on DE transcripts in several muscles and tendons, most of which are challenging to access in human participants. Muscles and tendons were more affected in females than in males (Figures 5A and S4). In female muscles, 6,476 DE genes were identified, with 3,136 downregulated and 3,340 upregulated (Figures 5B and 5C; Table S3). In male muscles, 3,182 DE genes were detected, with 1,697 downregulated and 1,485 upregulated. Principal component analysis (PCA) based on whole-transcriptome levels showed clustering of (1) tendons (EDLt, GAS, and TAt), (2) glycolytic muscles (EDL, GAS, TA, and QUA), and (3) oxidative muscle (SOL) (Figure 5D). Functional annotation of these transcripts revealed distinct biological processes affected by energy deficiency in female and male mice. The top five enriched GO terms in female muscles were ECM organization, focal adhesion, chromatin organization, supramolecular fiber organization, and positive regulation of cell death (Figure 5E; Table S2). In male muscles, the top five enriched GO terms were translation initiation, aerobic respiration, ECM organization, focal adhesion, and TCA cycle.

Collagen, a prominent structural protein within the ECM of skeletal muscle, comprises approximately 1%–10% of dry muscle mass.⁹⁴ Over 50% of the 46 collagen-coding genes were downregulated in at least one muscle tissue under conditions of energy deficiency: 25 genes in females and 23 in males (Figures 5F and S5). The fibril-forming collagen types I, III, and XI were most affected across muscle tissues (Figure S5). Collagen provides strength and support to (muscle) tissue. Our findings suggest that energy deficiency may lead to reduced collagen content in connective tissues, potentially resulting in structural alterations and weakening of muscles and tissue support, thereby increasing the vulnerability to injuries. Notably, the decrease in muscle collagen content aligns with aging.⁹⁵ Insufficient dietary intake leads to limited availability of collagen precursors (glycine and proline), which reduces the synthesis of

muscle collagen fibers, potentially contributing to the elevated risk of injuries in REDs. Reduced collagen production within connective tissues has been previously demonstrated under conditions of undernutrition.⁹⁶

Foxo3, the main regulator of protein synthesis and degradation in muscle, was upregulated in all muscles (Figure 5F). A catabolic state has been associated with elevated expression of *Foxo3* in human and mouse skeletal muscle, promoting autophagy.⁹⁷ Lysyl oxidase (*Lox12*), an enzyme responsible for catalyzing the formation of collagen crosslinks, was downregulated in all muscles under conditions of energy deficiency. *Rhobtb1*, which provides instructions for making a protein involved in actin filament organization, was downregulated primarily in females experiencing energy deficiency. *Fbxo32*, known to promote muscle protein degradation, was upregulated in deficient muscles, particularly in females and muscles containing glycolytic fiber types. Acyl-CoA synthetase (*Acs16*), responsible for catalyzing the formation of acyl-CoA from fatty acids, was upregulated in deficient muscles. *C7*, encoding a protein involved in the complement system of the innate immune system, was downregulated exclusively in males, indicating increased susceptibility to bacterial infections.⁹⁸ *Aldh18a1*, encoding a protein important for mitochondrial function and proline formation, was downregulated in deficient muscles.

In female tendons, a total of 3,823 DE genes were identified, with 2,426 downregulated (Figure 5B) and 1,397 upregulated (Figures 5B and 5C; Table S3). Similarly, in male tendons, 3,055 DE genes were found, with 1,906 downregulated and 1,149 upregulated. The top five GO terms enriched in female tendons were collagen formation (*P4ha1*), mTOR signaling (*Igfbp3*), mitochondrial organization, autophagy, and small molecule catabolic process (Figures 5G and S6; Table S2). In male tendons, the top five enriched GO terms included collagen formation, metabolism of amino acids, small molecule catabolic process, regulation of phosphate transport, and mTOR signaling. For example, downregulation of *P4ha1*, an enzyme involved in collagen synthesis, was observed in response to energy deficiency (Figure 5H). Additionally, *Igfbp3*, which inhibits cell proliferation by binding to IGF, was upregulated in the tendons of deficient mice. Last, tenomodulin (*Tnmd*), a specific marker for tendon maturation, was downregulated in the gastrocnemius tendon. Interestingly, this gene has been associated with reduced running performance in mice.⁹⁹

The transcriptomes of the uterus, ovaries, and male kidneys are most affected by energy deficiency

In females, the uterus and ovaries were the most responsive tissues to a negative energy balance. These tissues showed the highest number of DE genes: 8,785 in the uterus (4,407 down- and 4,378 upregulated; Figures 6A–6C) and 2,563 in the ovaries (1,368 down- and 1,195 upregulated; Figures 6D–6F). In males,

(B and C) Distribution of DE genes and the number of muscles and tendons in which they are (B) downregulated (blue) or (C) upregulated (red).

(D) Principal component analysis (PCA) was performed on all muscle and tendon tissues. Different colors represent tissue type, open symbols represent Ctrl, closed symbols represent deficient, triangles represent females, and circles represent males.

(E and G) Bar graph of common Metascape-annotated GO biological process pathways for DE genes in (E) >1 muscle or (G) >1 tendon.

(F and H) Energy deficit-induced log fold changes (log2FC ± SEM) for a selection of genes in females (red) and males (green) across (F) muscles and (H) tendons. All data are presented as mean ± SEM.

See also Figures S4–S6 and Tables S2 and S3.

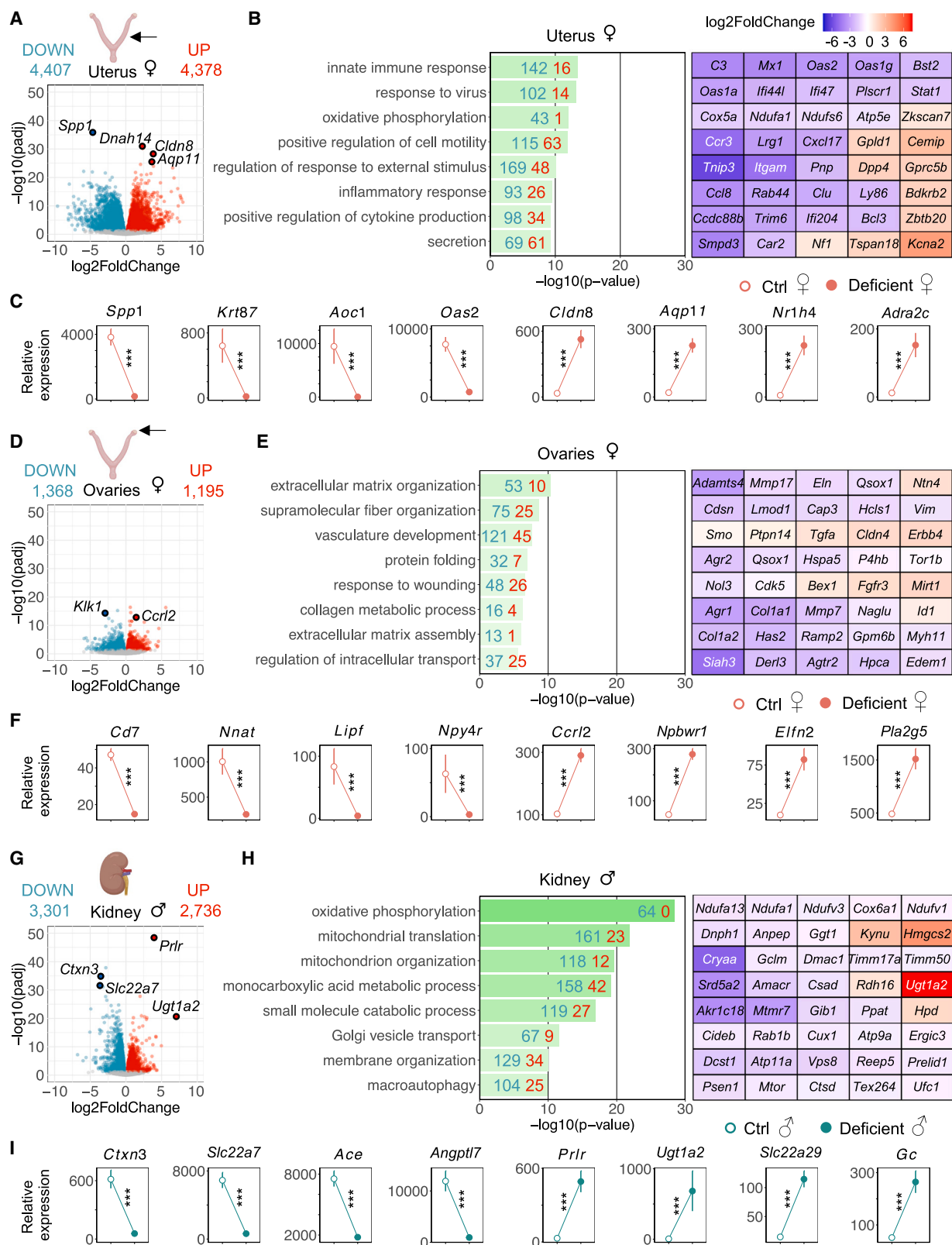


Figure 6. The transcriptomes of the uterus, ovaries, and male kidneys are most affected by energy deficiency

(A, D, and G) Volcano plots of differentially expressed (DE) genes from time-independent analyses in the (A) uterus, (D) ovaries, and (G) male kidney. Down-regulated genes in deficient mice (blue), upregulated genes (red).

(legend continued on next page)

the kidney was the most responsive tissue to a negative energy balance, showing 6,037 DE genes (3,301 down- and 2,736 upregulated; Figures 6G–6I). Interestingly, these organs also showed the greatest degree of shrinkage, implying that during periods of low energy availability, mice strategically allocate less energy to these organs to reduce energy expenditure (Figures 1K, 1L, S2A, and S2B).

Functional annotation of DE genes within the uterus revealed that genes linked to immune response (*C3*, *Mx1*, and *Oas2*), oxidative phosphorylation (*Cox5a*, *Ndufa1*, and *Atp5e*), and the positive regulation of cell motility (*Ccr3*, *Lrg1*, and *Cemip*) were most altered (Figure 6B). The endometrium of the female reproductive tract contains many immune cells and forms a barrier between the host and its environment to prevent pathogen entry.¹⁰⁰ The majority of genes associated with immune function were downregulated, such as complement components (*C3*, *Cfi*, and *C4b*), antigens (*Bsst2*, *Ly86*, and *Ly6a*), and chemokines (*Ccr3*, *Ccl8*, and *Cxcl17*). These findings indicate that the innate immune system in the uterus is impaired in response to energy deficiency. This aligns with previous findings demonstrating a positive relationship between *C3* levels and BMI.¹⁰¹ Given the high energy costs of immune responses, immune functionality depends on the host's energetic status.¹⁰² Furthermore, 43/44 genes associated with oxidative phosphorylation were decreased in expression. Several genes encoding mitochondrial cytochrome c oxidase (*Cox*), NADH dehydrogenase (*Nduf*), and ATP synthase genes were downregulated in deficient mice. This indicates reduced ATP (energy) synthesis, resulting in reduced metabolic activity that, in turn, contributes to energy preservation. The majority of genes associated with the positive regulation of cell motility were downregulated. This is associated with reduced cellular movement and decreased contractility, which indicates impaired preparations of the endometrium for embryo implantation due to energy deficit, diminishing the likelihood of successful implantation under conditions of food scarcity.

Osteopontin (*Spp1*), an ECM molecule and cytokine implicated in remodeling of the luminal epithelium for successful implantation,^{103,104} was dramatically downregulated in the uterus of deficient mice ($\log_2FC = -4.6$; Figure 6C). Furthermore, 17 genes encoding keratins (*Krt*), which serve as fibrous structural proteins, were downregulated. Uterine keratins have previously been shown to facilitate implantation.¹⁰⁵ Amine oxidase copper-containing 1 (*Aoc1*), responsible for the inactivation of histamine in pregnancy to protect mother and baby,¹⁰⁶ was also downregulated in the uterus ($\log_2FC = -8.0$). In contrast, Claudin 8 (*Cldn8*), a tight junction protein, was upregulated in the uterus of deficient mice ($\log_2FC = 4.0$). This protein can be modulated by steroids and is elevated in cervical carcinoma.¹⁰⁷ Another increased gene encodes for Aquaporin 11 ($\log_2FC = 3.6$; *Aqp11*), which regulates the water balance in the reproductive tract and plays a critical role in egg maturation/fertilization.¹⁰⁸ *Nr1h4*, a key regulator in bile acid metabolism, was also upregulated ($\log_2FC = 4.9$). Finally, the adrenoceptor (*Adra2c*), a nega-

tive regulator of uterine muscle contraction, was upregulated ($\log_2FC = 3.6$). Together, our findings indicate lower chances of successful implantation and subsequent pregnancy under conditions of energy deficiency.

Among the DE genes in the ovaries, several encoded for proteins associated with ECM organization (*Adams4*, *Mmp17*, and *Ntn4*) and protein folding (*Agr2*, *Qsox1*, and *Hspa5*) (Figures 6D and 6E). Cluster of differentiation 7 (*Cd7*), a member of the immunoglobulin superfamily, was downregulated in deficient mice ($\log_2FC = -1.7$) (Figure 6F). Neuronatin (*Nnat*), playing a role in oocyte aging,¹⁰⁹ was downregulated in deficient mice ($\log_2FC = -2.0$). Furthermore, genes involved in the digestion of dietary triglycerides (*Lipf*) and the receptor for neuropeptide Y (*Npy4r*) were also downregulated in deficient ovaries. On the other hand, *Ccr12*, a chemokine receptor recruiting immune cells to inflammatory sites, was upregulated ($\log_2FC = 1.5$). *Pla2g5*, a gene involved in fatty acid metabolism and implicated in polycystic ovary syndrome (PCOS),¹¹⁰ was also increased ($\log_2FC = 1.6$). *Npbwr1*, the receptor for neuropeptide B, which is implicated in lipid and glucose metabolism,¹¹¹ was increased in the ovaries of deficient mice ($\log_2FC = 2.6$). Last, *Elfn2* was also upregulated ($\log_2FC = 3.1$); interestingly, this gene has been implicated in ovarian cancer.¹¹²

Functional annotation of DE genes in the male kidney revealed that changed genes in deficient mice were associated with oxidative phosphorylation (*Ndufa13*, *Ndufa1*, and *Ndufv3*), catabolic process (*Akr1c18* and *Hpd*), and macroautophagy (*Psen1*, *Mtor*, and *Ctsd*) (Figures 6G and 6H). Starvation can induce autophagy in the kidney. Interestingly, kidney size was dramatically reduced by energy deficiency, particularly in males (females, 348 ± 6 versus 282 ± 8 mg, 19%, $p < 0.001$; males, 527 ± 14 versus 354 ± 11 mg, 33%, $p < 0.001$), reflecting the high degree of molecular changes in male kidneys. Several organic anion transporters were changed in expression (*Slc22a7* and *Slc22a29*), suggesting potential shifts in renal secretion and reabsorption due to lower nutrient/protein concentrations in the blood (Figure 6I). Additionally, angiotensin-converting enzyme (*Ace*), a pivotal regulator of blood pressure and fluid-salt balance, was downregulated ($\log_2FC = -2.1$), potentially contributing to the decrease in blood pressure previously observed under deficient conditions in both rodents and humans.^{113–115} In contrast, the prolactin receptor (*Prlr*) was upregulated in deficient male kidneys. Prolactin is known to inhibit Na^+/K^+ -ATPase activity in the proximal tubular.¹¹⁶ Thus, increased *Prlr* expression indicates reduced sodium reabsorption. Finally, vitamin D-binding protein (*Gc*), a marker for renal interstitial damage and fibrosis,¹¹⁷ was upregulated ($\log_2FC = 2.4$) and aligns with the large reduction in male kidney size after deficient conditions. Taken together, these findings highlight the sex-specific effects of energy deficiency on distinct organs. In females, reproductive organs were highly affected, whereas in males the kidney was mostly affected by energy deficiency.

(B, E, and H) Bar graphs of the top 8 GO biological processes for DE genes in (B) uterus, (E) ovaries, and (H) male kidney. The number of downregulated genes (blue) and upregulated genes (red) for each GO term is depicted in bar graphs. Heatmaps showing $\log_2FC$ for a subset of genes for each GO term.

(C, F, and I) Energy deficit-induced changes for a subset of genes in (C) uterus, (F) ovaries, and (I) male kidney.

All data are presented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

See also Tables S2 and S3.

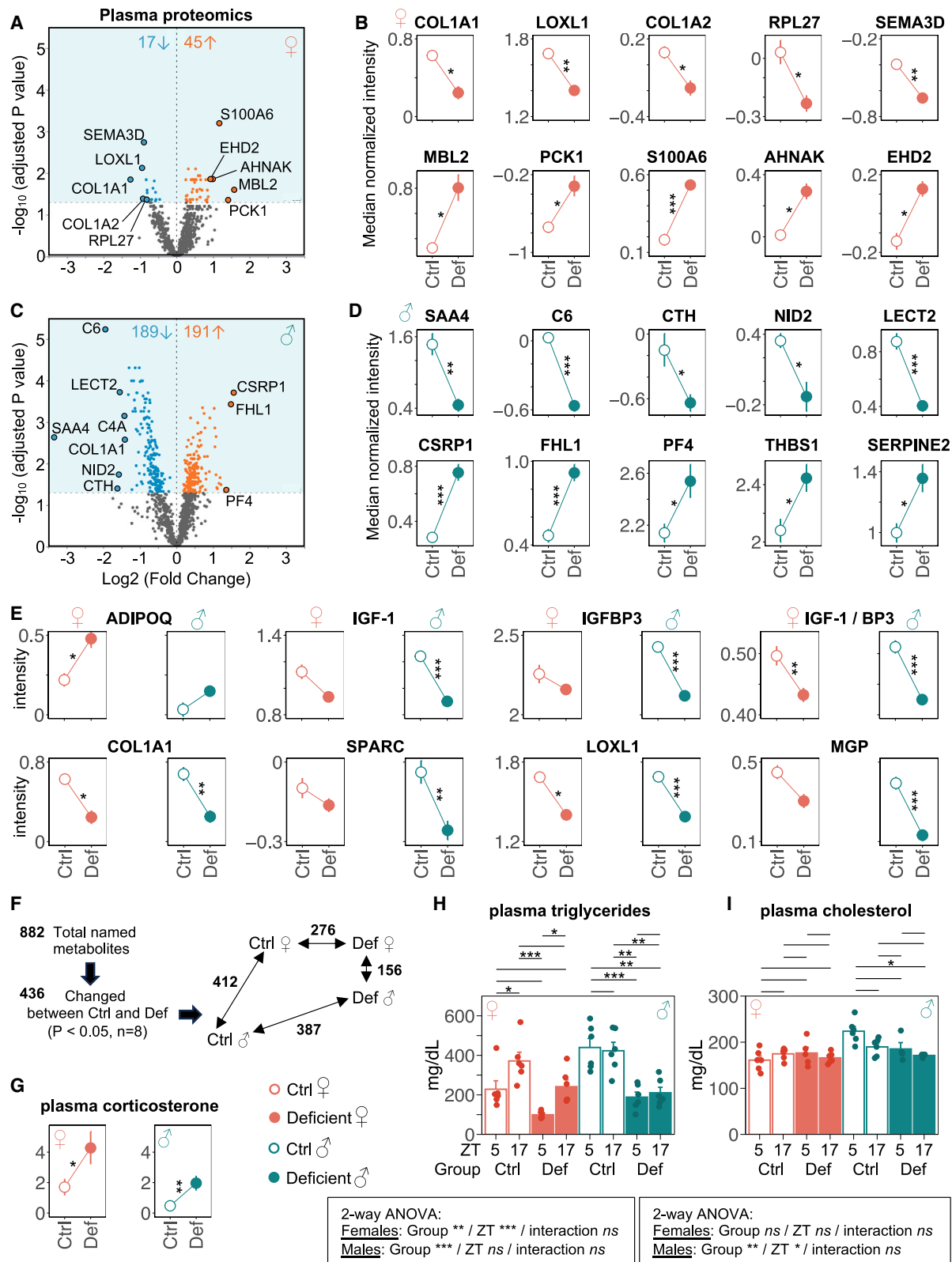


Figure 7. Plasma proteomics and metabolomics in deficient mice

(A and C) Volcano plots of differentially abundant proteins in (A) female and (C) male plasma. Proteins downregulated in deficient mice (blue), upregulated (red). (B and D) Top 10 differentially expressed plasma proteins are shown for (B) females and (D) males.

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Plasma proteomics and metabolomics in deficient mice

We used an untargeted plasma proteomics approach to identify changes in the circulation in response to an energy deficiency. We identified a total of 21,144 unique peptides mapping to 3,035 unique proteins, with an average of 10,107 peptides and 1,975 proteins per sample. By filtering the proteins detected in $\geq 50\%$ of the samples and using imputed normalized intensity values, we identified 62 significantly changed proteins in females (17 down- and 45 upregulated) and 380 changed proteins in males (189 down- and 191 upregulated) (Figures 7A–7E; Table S6). We conducted a manual examination of all 442 altered proteins to highlight the most relevant changes based on prior knowledge of energy metabolism. 31 DE proteins were overlapping between females and males. In females, the top 10 downregulated proteins were COL1A1, LOXL1, COL1A2, RPL27, SEMA3D, SLC6A4, RPL18, IGFALS, RPS9, and SERBP1, and the top 10 upregulated proteins were MBL2, PCK1, AHNK, EHD2, ADIPOQ, LAP3, AMY2A, VAT1, GLUD1, and ACLY (Figure 7B). In males, the top 10 downregulated proteins were SAA4, C6, CTH, NID2, LECT2, C4A, COL1A1, EGFR, SEMA3D, and KAZALD1, and the top 10 upregulated proteins were CSRP1, FHL1, PF4, THBS1, SERPINE2, ANGPT1, CSRP3, LTBP1, AK1, and PTMS (Figure 7D).

In agreement with our findings at the transcript level in several organs (Figure 3C), plasma levels of several ECM proteins were decreased. For example, COL1A1 protein also decreased in plasma of deficient mice (Figure 7E). Similarly, other collagens (COL1A2, COL3A1, COL11A2, COL14A1, COL6A3, and COL16A1), procollagen (PLOD3), and a collagen cross-linking enzyme (LOXL1) were also decreased in plasma (Figures 7B and 7E), matching with transcript levels in various tissues (Figure 3C). Exercise induces secretion of the collagen-binding ECM protein SPARC (Osteonectin) in both rodents and humans.^{118,119} In contrast, under deficient conditions, SPARC levels were reduced in both plasma protein levels (Figure 7E) and at the transcript level in multiple organs of mice (Figure 3C). Furthermore, several factors related to the immune system, such as mannose-binding lectin (MBL2), serum amyloid A4 (SAA4), and complement component 6 (C6), were altered in the plasma of deficient mice.

Increased adiponectin (ADIPOQ) levels in females were detected (Figure 7E). ADIPOQ is an anorexigenic adipokine regulating glucose levels and fatty acid metabolism. Increased circulating ADIPOQ levels were also found in women with REDs symptoms.^{120,121} In males, insulin-like growth factor (IGF-1) levels were decreased. IGF-1, an anabolic hormone produced by the liver, is also decreased in anorexia nervosa.^{122,123} Most IGF-1 is always bound to one of the six binding proteins (IGFBP). Several IGFBPs were also downregulated in deficient females (IGFBP5 and IGFALS) and males (IGFBP3, IGFBP4, and IGFBP5). Also, the IGF-1/BP-3 ratio was reduced in deficient

mice, which indicates reduced free IGF-1. Similar patterns have been observed in humans with low energy availability.²¹ Last, plasma TNMD levels were decreased, which aligns with reduced *Tnmd* transcript levels detected in the gastrocnemius tendon. TNMD has been associated with reduced running performance in mice.⁹⁹

We also used an untargeted plasma metabolomics approach to identify changes in the circulation in response to an energy deficiency. We identified a total of 990 (females) and 989 (males) unique biochemicals, of which 882 were named. We identified 276 changed metabolites in females (148 down- and 128 upregulated) and 387 changed metabolites in males (260 down- and 127 upregulated) (Figures 7F, S7A, and S7B; Table S7). Plasma corticosterone levels were increased in deficient mice (Figure 7G), which has been observed before³⁹ and is also seen in response to fasting in individuals with REDs.^{89–91} Moreover, various fatty acids and metabolites associated with fatty acid metabolism, such as margarate (17:0), linoleate (18:2n6), 12,13-DiHOME, isocaprolylglycine, docosatrienoate (22:3n6), and tetradecanedioate (C14), were changed in plasma upon energy deficiency (Figure S7C). The lipokine 12,13-DiHOME, known for its role as an exercise-induced mediator produced by BAT that enhances fatty acid uptake in skeletal muscle,¹²⁴ was increased in deficient mice (Figure S7D). Tetradecanedioate (C14), a metabolic byproduct of omega-oxidation in the synthesis of long-chain fatty acids, was reduced in deficient mice. Prior research has demonstrated a positive correlation between tetradecanedioate levels and BMD, suggesting protective implications for BMD.¹²⁵

Finally, plasma triglyceride levels were decreased in deficient female and male mice (Figure 7H), whereas plasma cholesterol levels were downregulated in deficient males only (Figure 7I).

DISCUSSION

Despite the significant pleiotropic impact of exercise-related negative energy balance on physiology and behavior, the lack of an animal model and a molecular atlas of multi-tissue response to energy deficiency has hindered a systematic understanding of the underlying molecular changes. In this study, we found that we can replicate multiple aspects of a REDs phenotype in mice through the implementation of the exercise-for-food paradigm (Figure 1A).^{38–40,126} Both female and male mice sustained high exercise loads while inadequately fueling over time, consequently inducing a state of negative energy balance. In response to this energy deficiency, we observed changes in behavior, physiology, and gene expression levels in a sex-, tissue-, and time-dependent manner.

Our findings highlight the impact of a negative energy balance on sleep-wake patterns, aligning with previous research that has demonstrated compromised sleep quality in individuals with

(E) Examples of other plasma proteins that changed in response to energy deficiency.

(F) Summary of metabolite changes in plasma. The number of changed metabolites between any two of the four different conditions is shown in the right panel.

(G) Plasma corticosterone levels.

(H and I) Plasma triglyceride (H) and cholesterol (I) levels. Statistics: unpaired t test (B and D–G) and two-way ANOVA (factors: group and ZT) and Tukey's multiple comparisons test (H and I).

Data are presented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

See also Figure S7 and Tables S6 and S7.

REDs or anorexia nervosa.^{6,46–53} Although the relationship between disturbed sleep/circadian misalignment and energy metabolism has been studied, the causal mechanisms of energy balance influencing sleep and circadian alignment remain less explored.¹²⁷ We also observed distinct behavioral patterns in the two sexes. Female mice exercised more (twice as active) to secure food. Consequently, they showed weight loss and altered activity patterns later in the experimental protocol compared to males. In contrast, male mice opted for a strategy of body mass reduction by reducing organ size, which can lower their basal metabolic rate (BMR).¹²⁸ Lower organ mass of key organs has previously been shown to be associated with reduced BMR.^{129–131} Interestingly, the size of the lungs did not change due to energy deficiency. Given the essential role of the lungs in facilitating running, it is possible that animals in an energy-deficient state prioritize allocating energy resources to maintain the lungs' capacity, a necessity for foraging/locomotion. In contrast, the differential expression of genes across various tissues pointed toward hypoxia, a condition characterized by reduced oxygen supply in the bloodstream. Prolonged exposure to hypoxia may lead to organ damage. This suggests that even though lung capacity remained the same, the available energy levels were insufficient to meet the demands to sustain optimal lung functioning during exercise.

By reducing their metabolism (via reduced organ mass, lowering core body temperature, and shift toward diurnal behavior), males effectively conserve energy but increase their vulnerability to predation by diurnal predators.^{38,39,87,132} In contrast, females opted for a different approach, preserving energy more cautiously while maintaining a lower risk of predation,⁸⁷ a strategy aligned with higher locomotor activity levels to secure adequate nourishment to maintain some fat deposition for later use during high energy demands of pregnancy and especially lactation, which is crucial for optimal reproductive success in females.¹³³ These findings show that females are more resilient with respect to organ preservation and behavior, while males are more flexible in their phenotype when the environment changes. Similarly, under a positive energy balance, female mice are more resistant to weight gain and metabolic disorders induced by a high-fat diet when compared to male mice.^{134,135} Such sex-dependent differences in temporal phenotypic plasticity under environmental change have been observed in various species.¹³⁶

The minimal overlap of transcriptomic changes between females and males indicates how energy deficiency may affect distinct molecular pathways and functions. This also highlights the importance of including both female and male subjects. Despite the energy deficiency, there was no significant alteration in the estrous cycle. Even among exercising women under energy deficiency, only a small fraction (5%–10%) experience an extreme form of menstrual dysfunction known as amenorrhea,^{137–139} which supports the hypothesis that severe energy deficiency can induce amenorrhea. Moreover, a dose-response relationship between the severity of daily energy deficit and the frequency of menstrual disturbances¹⁴⁰ or changes in LH pulsatility has been observed.²¹ Although we did observe an ~50% decrease in the mass of female reproductive organs, which has been previously observed in voles,¹⁴¹ we also observed changes in gene expression indicative of modified HPG axis ac-

tivity, a potential negative impact on successful implantation, and downregulation of *Oxtr*. It is therefore possible that females may have switched to a survival mode at the expense of reproduction.

Physical exercise contributes to increased bone density, promoting bone strength and overall bone health.¹⁴² However, when exercise is coupled with inadequate energy intake, it detrimentally affects bone health, resulting in diminished bone strength,¹⁴³ which we now also confirmed in our mouse model, having altered trabecular bone microarchitecture. Trabecular bone is indeed more susceptible to low BMD and impaired microarchitecture induced by low energy availability.³ The absence of changes in cortical bone among deficient mice could be attributed to the relatively brief duration of the protocol, which might not be extensive enough to trigger cortical bone alterations as observed in REDs patients.^{13–15} Human REDs patients often experience prolonged periods of reduced energy availability, contrasting our comparatively shorter experimental protocol. The synthesis and fibril formation of collagen, the main structural protein present throughout the body, is a metabolically demanding process not crucial for immediate survival. This process was reduced during energy deficiency, affecting the majority of organs. The collagen reduction could potentially result in diminished ECM content within connective tissues, reducing tissue strength and structure. This might explain both the reduced organ mass observed in REDs mice and the heightened susceptibility to injuries noted in REDs patients.

Interestingly, several transcripts activated by the GR or ER were changed in various tissues. When activated by glucocorticoids, GR can act as a TF to bind to GR response elements, thereby regulating the transcription of glucocorticoid-responsive genes.¹⁴⁴ Similarly, when activated by estrogen, ER can act as a TF to bind EREs, thereby regulating the transcription of estrogen-responsive genes (Figure S3B; Table S4). Given the mouse genome contains more than a thousand TFs, over-enrichment of GR and ER elements in the upstream regions of the DE genes is remarkable.

The pronounced upregulation of GR expression in the gonads suggests heightened sensitivity to corticosterone in reproductive tissues. This observation aligns with our and previous research confirming elevated plasma corticosterone levels in deficient mice and increased cortisol levels in individuals with REDs, potentially contributing to increased GR expression.^{39,89–91} Interestingly, glucocorticoids are catabolic to bone and muscle,^{145,146} a phenotype that was observed in our deficient mice. Furthermore, transcriptomic changes also pointed to reduced immune function, which could potentially be attributed to the elevated corticosterone levels, known for their immunosuppressive effects. Thus, it is possible that some of the phenotypic and transcriptomic changes we observed in response to energy deficiency can be attributed to changes in GR signaling. Future studies are needed to reveal the causal relationship between corticosterone and gene expression under energy-deficient conditions. Our findings are in line with the observed changes in gene expression patterns, indicative of the activation of the HPA axis and the suppression of the HPG axis, a pattern that is expected in REDs.⁵

A high prevalence of REDs is observed in both weight-restricted sports and endurance sports, where achieving a lower

weight can lead to enhanced performance outcomes.^{113,147–149} Our exercise-for-food model effectively mimics the physiological demands of endurance sports. However, it is important to note the potential for additional molecular responses in response to resistance exercise, a factor that remains unexplored in our study.

Exercising with insufficient energy intake reduces glucose levels, enhancing gluconeogenesis during fasting to elevate blood glucose levels for supply to vital organs. Glycogen and protein reserves from the liver and muscles are utilized, ultimately leading to skeletal muscle catabolism. Consequently, a prolonged negative energy balance can lead to a reduction in muscle mass, as observed in humans and in our mouse model.¹⁵⁰ Maintaining muscle mass is critical for athletes to sustain optimal performance levels. Furthermore, the extraction of triglycerides from adipocytes is a crucial source of energy under such circumstances, which was reflected in the reduction in fat mass in deficient mice. This is often accompanied by a decrease in muscle protein synthesis and an increase in muscle proteolysis, potentially contributing to the loss of muscle mass.

Our findings confirm that we can mimic a REDs condition in both female and male mice, distinct from existing caloric restriction^{59,77,151,152} and exercise paradigms.^{60,153,154} The transcriptome atlas of mouse metabolic/endocrine tissues under conditions of energy sufficiency and deficiency will be a valuable resource that will guide our future understanding of energy deficiency. Elucidating the molecular changes and organ interplay whereby metabolic feedback acts on the neuroendocrine system and endocrine/metabolic organs will have substantial implications for human health, including sleep regulation, metabolic functioning, and (athletic) performance, and will be essential for developing biomarkers and effective treatments for REDs.

Limitations of the study

Caution should be implemented for the translation of our findings from REDs mice to REDs humans since not all aspects of REDs are present in our model. In humans, REDs is a heterogeneous disease and may manifest either with or without an eating disorder, such as anorexia nervosa.^{4,24,155} The human REDs syndrome is more heterogeneous than our REDs mouse model. There is a higher prevalence of disordered eating among athletes compared to nonathletes.^{147,156} The desire to be lean, personality traits, and social pressures can all contribute to the development of REDs.^{155,157} The psychological component of the disease is lacking in our mouse model. In mice subjected to energy deficiency, a rapid increase in food intake and consequently weight gain upon food supplementation is observed, which is not the case in humans.³⁸ Despite the absence of the psychological component in our REDs mouse model, it still gives the opportunity to study the molecular changes induced by energy deficiency in organs that are difficult to access in human patients. To investigate the long-term effect of energy deficiency, future experiments are required to explore to what extent behavioral, physiological, and molecular changes can be reversible by supplementing food. Another limitation of our study is that although female mice were sacrificed across different estrous stages, Ctrl mice were predominantly sacrificed during estrus, whereas deficient mice were predominantly sacrificed during diestrus. While this discrepancy could potentially stem from

the energy deficit, we cannot rule out the possibility that part of the DE genes (primarily in the uterus and ovaries) might be explained by variations in estrous cycle stages. The time-of-day-dependent changes in DE genes may be due to change in phase of the circadian clock in different tissues. Studies integrating gene expression changes found at one time point representing day or night with time series gene expression changes with more frequent tissue sampling throughout the 24 h can better discern the contribution of change in clock phase and of change in energy deficit to overall transcriptome changes.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cmet.2024.08.001>.

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AUTHOR CONTRIBUTIONS

Conceptualization, L.v.R., R.A.H., and S.P.; investigation, L.v.R., J.Z., G.M., E.G.G., T.L., and S.R.; formal analysis, L.v.R., E.G.G., V.R., S.R., S.D., and R.K.R.; visualization, L.v.R.; supervision, R.A.H. and S.P.; funding acquisition, R.L.S., A.D.M., R.A.H., and S.P.; writing – original draft, L.v.R. and S.P.; writing – review & editing, L.v.R., J.Z., G.M., S.D., A.D.M., R.A.H., and S.P.; approved the final version of the manuscript, all authors.

DECLARATION OF INTERESTS

A.D.M. is a co-founder, equity holder, and advisor to Insilicomed and Vektor Medical, neither of which are related in any way to this study. R.A.H. is a scientific advisor for the GoodLightGroup and Chrono@Work; both companies were not involved in this study. S.P. is the author of the books *The Circadian Code* and *The Circadian Diabetes Code* and is a scientific advisor to The Hooke London. None of the interests were involved in this study.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work the author(s) used ChatGPT in order to improve readability and language. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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REFERENCES

- Nattiv, A., Loucks, A.B., Manore, M.M., Sanborn, C.F., Sundgot-Borgen, J., and Warren, M.P.; American College of Sports Medicine (2007). The female athlete triad. *Med. Sci. Sports Exerc.* 39, 1867–1882. <https://doi.org/10.1249/mss.0b013e318149f111>.
- Mountjoy, M., Sundgot-Borgen, J., Burke, L., Carter, S., Constantini, N., Lebrun, C., Meyer, N., Sherman, R., Steffen, K., Budgett, R., and Ljungqvist, A. (2014). The IOC consensus statement: Beyond the female athlete triad—relative energy deficiency in sport (RED-S). *Br. J. Sports Med.* 48, 491–497. <https://doi.org/10.1136/bjsports-2014-093502>.
- Mountjoy, M., Sundgot-Borgen, J., Burke, L., Ackerman, K.E., Blauwet, C., Constantini, N., Lebrun, C., Lundy, B., Melin, A., Meyer, N., et al. (2018). International Olympic committee (IOC) consensus statement on relative energy deficiency in sport (RED-S): 2018 update. *Int. J. Sport Nutr. Exerc. Metab.* 28, 316–331. <https://doi.org/10.1123/ijnsnem.2018-0136>.
- Mountjoy, M., Sundgot-Borgen, J., Burke, L., Ackerman, K.E., Blauwet, C., Constantini, N., Lebrun, C., Lundy, B., Melin, A., Torstveit, M.K., et al. (2023). 2023 International Olympic committee (IOC) consensus statement on relative energy deficiency in sport (REDs). *Br. J. Sports Med.* 57, 1073–1097. <https://doi.org/10.1123/ijnsnem.2018-0136>.
- Elliott-Sale, K.J., Tenforde, A.S., Parziale, A.L., Holtzman, B., and Ackerman, K.E. (2018). Endocrine effects of relative energy deficiency in sport. *Int. J. Sport Nutr. Exerc. Metab.* 28, 335–349. <https://doi.org/10.1123/ijnsnem.2018-0127>.
- Drew, M., Vlahovich, N., Hughes, D., Appaneal, R., Burke, L.M., Lundy, B., Rogers, M., Toomey, M., Watts, D., Lovell, G., et al. (2018). Prevalence of illness, poor mental health and sleep quality and low energy availability prior to the 2016 summer Olympic games. *Br. J. Sports Med.* 52, 47–53. <https://doi.org/10.1136/bjsports-2017-098208>.
- Ackerman, K.E., Holtzman, B., Cooper, K.M., Flynn, E.F., Bruinvels, G., Tenforde, A.S., Popp, K.L., Simpkin, A.J., and Parziale, A.L. (2019). Low energy availability surrogates correlate with health and performance consequences of relative energy deficiency in sport. *Br. J. Sports Med.* 53, 628–633. <https://doi.org/10.1136/bjsports-2017-098958>.
- Keay, N., Francis, G., and Hind, K. (2018). Low energy availability assessed by a sport-specific questionnaire and clinical interview indicative of bone health, endocrine profile and cycling performance in competitive male cyclists. *BMJ Open Sport Exerc. Med.* 4, e000424. <https://doi.org/10.1136/bmjsem-2018-000424>.
- Melin, A.K., Areta, J.L., Heikura, I.A., Stellingwerff, T., Torstveit, M.K., and Hackney, A.C. (2024). Direct and indirect impact of low energy availability on sports performance. *Scand. J. Med. Sci. Sports* 34, e14327. <https://doi.org/10.1111/sms.14327>.
- Burke, L.M., Ackerman, K.E., Heikura, I.A., Hackney, A.C., and Stellingwerff, T. (2023). Mapping the complexities of relative energy deficiency in sport (REDs): development of a physiological model by a subgroup of the International Olympic Committee (IOC) consensus on REDs. *Br. J. Sports Med.* 1, 1098–1108. <https://doi.org/10.1136/bjsports-2023-107335>.
- Rickenlund, A., Eriksson, M.J., Schenck-Gustafsson, K., and Hirschberg, A.L. (2005). Amenorrhea in female athletes is associated with endothelial dysfunction and unfavorable lipid profile. *J. Clin. Endocrinol. Metab.* 90, 1354–1359. <https://doi.org/10.1210/jc.2004-1286>.
- Grosman-Rimon, L., Wright, E., Freedman, D., Kachel, E., Hui, S., Epstein, I., Gutterman, D., and Ellat-Adar, S. (2019). Can improvement in hormonal and energy balance reverse cardiovascular risk factors in athletes with amenorrhea? *Am. J. Physiol. Heart Circ. Physiol.* 317, H487–H495. <https://doi.org/10.1152/AJPHEART.00242.2019>.
- Ackerman, K.E., Putman, M., Guereca, G., Taylor, A.P., Pierce, L., Herzog, D.B., Klibanski, A., Buxsein, M., and Misra, M. (2012). Cortical microstructure and estimated bone strength in young amenorrheic athletes, eumenorrheic athletes and non-athletes. *Bone* 51, 680–687. <https://doi.org/10.1016/j.bone.2012.07.019>.
- Ackerman, K.E., Nazem, T., Chapko, D., Russell, M., Mendes, N., Taylor, A.P., Buxsein, M.L., and Misra, M. (2011). Bone microarchitecture is impaired in adolescent amenorrheic athletes compared with eumenorrheic athletes and nonathletic controls. *J. Clin. Endocrinol. Metab.* 96, 3123–3133. <https://doi.org/10.1210/jc.2011-1614>.
- Hutson, M.J., O'Donnell, E., Brooke-Wavell, K., Sale, C., and Blagrove, R.C. (2021). Effects of low energy availability on bone health in endurance athletes and high-impact exercise as a potential countermeasure: A narrative review. *Sports Med.* 51, 391–403. <https://doi.org/10.1007/s40279-020-01396-4>.
- Marx, R.G., Saint-Phard, D., Callahan, L.R., Chu, J., and Hannafin, J.A. (2001). Stress fracture sites related to underlying bone health in athletic females. *Clin. J. Sport Med.* 11, 73–76. <https://doi.org/10.1097/00042752-200104000-00002>.
- Cabre, H.E., Moore, S.R., Smith-Ryan, A.E., and Hackney, A.C. (2022). Relative energy deficiency in sport (RED-S): scientific, clinical, and practical implications for the female athlete. *Dtsch. Z. Sportmed.* 73, 225–234. <https://doi.org/10.5960/dzsm.2022.546>.
- Bouqueneau, A., Dubois, B.E., Krzesinski, J.M., and Delanaye, P. (2012). Anorexia nervosa and the kidney. *Am. J. Kidney Dis.* 60, 299–307. <https://doi.org/10.1053/j.ajkd.2012.03.019>.
- Gibbs, J.C., Williams, N.I., Mallinson, R.J., Reed, J.L., Rickard, A.D., and De Souza, M.J. (2013). Effect of high dietary restraint on energy availability and menstrual status. *Med. Sci. Sports Exerc.* 45, 1790–1797. <https://doi.org/10.1249/MSS.0b013e3182910e11>.
- Gibbs, J.C., Williams, N.I., Scheid, J.L., Toombs, R.J., and De Souza, M.J. (2011). The association of a high drive for thinness with energy deficiency and severe menstrual disturbances: Confirmation in a large population of exercising women. *Int. J. Sport Nutr. Exerc. Metab.* 21, 280–290. <https://doi.org/10.1123/ijnsnem.21.4.280>.
- Loucks, A.B., and Thuma, J.R. (2003). Luteinizing hormone pulsatility is disrupted at a threshold of energy availability in regularly menstruating

- women. *J. Clin. Endocrinol. Metab.* **88**, 297–311. <https://doi.org/10.1210/jc.2002-020369>.
22. Schneider, J.E. (2004). Energy balance and reproduction. *Physiol. Behav.* **81**, 289–317. <https://doi.org/10.1016/j.physbeh.2004.02.007>.
23. Logue, D.M., Madigan, S.M., Melin, A., Delahunt, E., Heinen, M., Donnell, S.J.M., and Corish, C.A. (2020). Low energy availability in athletes 2020: an updated narrative review of prevalence, risk, within-day energy balance, knowledge, and impact on sports performance. *Nutrients* **12**, 835. <https://doi.org/10.3390/nu12030835>.
24. Reece, L., Farnsworth, N., Whitney, K.E., and Ackerman, K.E. (2023). Eating disorders and relative energy deficiency in sport (RED-S). In *Psychological Considerations in the Young Athlete*, M.A. Christino, L.J. Micheli, and E.I. Pluhar, eds. (Springer).
25. Mountjoy, M., Sundgot-Borgen, J., Burke, L., Carter, S., Constantini, N., Lebrun, C., Meyer, N., Sherman, R., Steffen, K., Budgett, R., et al. (2015). Relative energy deficiency in sport (RED-S) clinical assessment tool (CAT). *Br. J. Sports Med.* **49**, 421–423. <https://doi.org/10.1136/bjsports-2014-094559>.
26. Heikura, I.A., Mountjoy Margo, L., Ackerman, K.E., Bailey, D.M., Burke, L.M., Constantini, N., Hackney, A.C., McCloskey, P., Melin, A.K., Pensgaard, A.M., et al. (2023). IOC REDs CAT2. *Br. J. Sports Med.* **57**, 1068–1072.
27. Kuikman, M.A., Mountjoy, M., Stellingwerff, T., and Burr, J.F. (2021). A review of nonpharmacological strategies in the treatment of relative energy deficiency in sport. *Int. J. Sport Nutr. Exerc. Metab.* **31**, 268–275. <https://doi.org/10.1123/IJSNEM.2020-0211>.
28. Berga, S.L., and Loucks, T.L. (2006). Use of cognitive behavior therapy for functional hypothalamic amenorrhea. *Ann. N. Y. Acad. Sci.* **1092**, 114–129. <https://doi.org/10.1196/annals.1365.010>.
29. Guebels, C.P., Kam, L.C., Maddalozzo, G.F., and Manore, M.M. (2014). Active women before/after an intervention designed to restore menstrual function: Resting metabolic rate and comparison of four methods to quantify energy expenditure and energy availability. *Int. J. Sport Nutr. Exerc. Metab.* **24**, 37–46. <https://doi.org/10.1123/ijnsnem.2012-0165>.
30. Cialdella-Kam, L., Guebels, C.P., Maddalozzo, G.F., and Manore, M.M. (2014). Dietary intervention restored menses in female athletes with exercise-associated menstrual dysfunction with limited impact on bone and muscle health. *Nutrients* **6**, 3018–3039. <https://doi.org/10.3390/nu6083018>.
31. Langbein, R.K., Martin, D., Allen-Collinson, J., and Jackman, P.C. (2022). “It’s hard to find balance when you’re broken”: Exploring female endurance athletes’ psychological experience of recovery from relative energy deficiency in sport (RED-S). *Perform. Enhanc. Health* **10**, 100214. <https://doi.org/10.1016/j.peh.2021.100214>.
32. De Souza, M.J., Mallinson, R.J., Strock, N.C.A., Koltun, K.J., Olmsted, M.P., Ricker, E.A., Scheid, J.L., Allaway, H.C., Mallinson, D.J., Kuruppmullage Don, P., and Williams, N.I. (2021). Randomised controlled trial of the effects of increased energy intake on menstrual recovery in exercising women with menstrual disturbances: the ‘REFUEL’ study. *Hum. Reprod.* **36**, 2285–2297. <https://doi.org/10.1093/humrep/deab149>.
33. De Souza, M.J., Ricker, E.A., Mallinson, R.J., Allaway, H.C.M., Koltun, K.J., Strock, N.C.A., Gibbs, J.C., Kuruppmullage Don, P., and Williams, N.I. (2022). Bone mineral density in response to increased energy intake in exercising women with oligomenorrhea/amenorrhea: The REFUEL randomized controlled trial. *Am. J. Clin. Nutr.* **115**, 1457–1472. <https://doi.org/10.1093/ajcn/nqac044>.
34. Zhang, J., and Dulawa, S.C. (2021). The utility of animal models for studying the metabo-psychiatric origins of anorexia nervosa. *Front. Psychiatry* **12**, 711181. <https://doi.org/10.3389/fpsy.2021.711181>.
35. Lambert, K.G. (1993). The activity-stress paradigm: Possible mechanisms and applications. *J. Gen. Psychol.* **120**, 21–32. <https://doi.org/10.1080/00221309.1993.9917859>.
36. DiMarco, N.M., Dart, L., and Sanborn, C.B. (2007). Modified activity-stress paradigm in an animal model of the female athlete triad. *J. Appl. Physiol.* **103**, 1469–1478. <https://doi.org/10.1152/jappphysiol.01137.2005>.
37. Metzger, C.E., Baek, K., Swift, S.N., De Souza, M.J., and Bloomfield, S.A. (2016). Exercise during energy restriction mitigates bone loss but not alterations in estrogen status or metabolic hormones. *Osteoporos. Int.* **27**, 2755–2764. <https://doi.org/10.1007/s00198-016-3590-y>.
38. Hut, R.A., Pilorz, V., Boerema, A.S., Strijkstra, A.M., and Daan, S. (2011). Working for food shifts nocturnal mouse activity into the day. *PLoS One* **6**, e17527. <https://doi.org/10.1371/journal.pone.0017527>.
39. van der Vinne, V., Riede, S.J., Gorter, J.A., Eijer, W.G., Sellix, M.T., Menaker, M., Daan, S., Pilorz, V., and Hut, R.A. (2014). Cold and hunger induce diurnality in a nocturnal mammal. *Proc. Natl. Acad. Sci. USA* **111**, 15256–15260. <https://doi.org/10.1073/pnas.1413135111>.
40. van Rosmalen, L., Riede, S.J., Pilorz, V., Adage, T., Scheurink, A.J.W., van der Vinne, V., and Hut, R.A. (2022). Nocturnal and diurnal behavior assessed by the “work-for-food” protocol in small rodents. In *Circadian Clocks* (Springer), pp. 187–216. https://doi.org/10.1007/978-1-0716-2577-4_9.
41. van Rosmalen, L., Deota, S., Maier, G., Le, H.D., Lin, T., Ramasamy, R.K., Hut, R.A., and Panda, S. (2024). Energy balance drives diurnal and nocturnal brain transcriptome rhythms. *Cell Rep.* **43**, 113951. <https://doi.org/10.1016/j.celrep.2024.113951>.
42. Flurkey, K., Curren, J.M., and Harrison, D.E. (2007). *The Mouse in Biomedical Research* (Elsevier).
43. Hawkes, K., and Smith, K.R. (2010). Do women stop early? Similarities in fertility decline in humans and chimpanzees. *Ann. N. Y. Acad. Sci.* **1204**, 43–53. <https://doi.org/10.1111/j.1749-6632.2010.05527.x>.
44. Xue, S., Kemal, O., Lu, M., Lix, L.M., Leslie, W.D., and Yang, S. (2020). Age at attainment of peak bone mineral density and its associated factors: The national health and nutrition examination survey 2005–2014. *Bone* **131**, 115163. <https://doi.org/10.1016/j.bone.2019.115163>.
45. Toth, L.A., and Bhargava, P. (2013). Animal models of sleep disorders. *Comp. Med.* **63**, 91–104.
46. Keay, N., Overseas, A., and Francis, G. (2020). Indicators and correlates of low energy availability in male and female dancers. *BMJ Open Sport Exerc. Med.* **6**, e000906. <https://doi.org/10.1136/bmjsem-2020-000906>.
47. Swinbourne, R., Gill, N., Vaile, J., and Smart, D. (2016). Prevalence of poor sleep quality, sleepiness and obstructive sleep apnoea risk factors in athletes. *Eur. J. Sport Sci.* **16**, 850–858. <https://doi.org/10.1080/17461391.2015.1120781>.
48. Groom, J.M. (2019). *Associations between low energy availability, stress and anxiety profiles and sleep patterns*. Master’s thesis (University of Otago).
49. Scheurink, A.J.W., Boersma, G.J., Nergårdh, R., and Södersten, P. (2010). Neurobiology of hyperactivity and reward: Agreeable restlessness in anorexia nervosa. *Physiol. Behav.* **100**, 490–495. <https://doi.org/10.1016/j.physbeh.2010.03.016>.
50. Saidi, O., Souabni, M., Del Sordo, G.C., Maviel, C., Peyrel, P., Maso, F., Vercruyssen, F., and Duché, P. (2024). Association between low energy availability (LEA) and impaired sleep quality in young rugby players. *Nutrients* **16**, 609. <https://doi.org/10.3390/nu16050609>.
51. Lauer, C.J., and Krieg, J.C. (2004). Sleep in eating disorders. *Sleep Med. Rev.* **8**, 109–118. [https://doi.org/10.1016/S1087-0792\(02\)00122-3](https://doi.org/10.1016/S1087-0792(02)00122-3).
52. Crisp, A.H., Stonehill, E., and Fenton, G.W. (1971). The relationship between sleep, nutrition and mood: a study of patients with anorexia nervosa. *Postgrad. Med. J.* **47**, 207–213. <https://doi.org/10.1136/pgmj.47.546.207>.
53. Crisp, A.H., and Stonehill, E. (1973). Aspects of the relationship between sleep and nutrition: a study of 375 psychiatric out-patients. *Br. J. Psychiatry* **122**, 379–394. <https://doi.org/10.1192/bjp.122.4.379>.
54. Matheson, G.O., Clement, D.B., McKenzie, D.C., Taunton, J.E., Lloyd-Smith, D.R., and Macintyre, J.G. (1987). Stress fractures in athletes: A study of 320 cases. *Am. J. Sports Med.* **15**, 46–58. <https://doi.org/10.1177/036354658701500107>.

55. Tenforde, A.S., Kraus, E., and Fredericson, M. (2016). Bone stress injuries in runners. *Phys. Med. Rehabil. Clin. N. Am.* 27, 139–149. <https://doi.org/10.1016/j.pmr.2015.08.008>.
56. Wentz, L., Liu, P.Y., Haymes, E., and Ilich, J.Z. (2011). Females have a greater incidence of stress fractures than males in both military and athletic populations: A systemic review. *Mil. Med.* 176, 420–430. <https://doi.org/10.7205/MILMED-D-10-00322>.
57. Ott, S.M. (2018). Cortical or trabecular bone: What's the difference? *Am. J. Nephrol.* 47, 373–375. <https://doi.org/10.1159/000489672>.
58. Marshall, D., Johnell, O., and Wedel, H. (1996). Meta-analysis of how well measures of bone mineral density predict occurrence of osteoporotic fractures. *Br. Med. J.* 312, 1254–1259. <https://doi.org/10.1136/bmj.312.7041.1254>.
59. Aon, M.A., Bernier, M., Mitchell, S.J., Di Germanio, C., Mattison, J.A., Ehrlich, M.R., Colman, R.J., Anderson, R.M., and de Cabo, R. (2020). Untangling determinants of enhanced health and lifespan through a multi-omics approach in mice. *Cell Metab.* 32, 100–116.e4. <https://doi.org/10.1016/j.cmet.2020.04.018>.
60. Furrer, R., Heim, B., Schmid, S., Dilbaz, S., Adak, V., Nordström, K.J.V., Ritz, D., Steurer, S.A., Walter, J., and Handschin, C. (2023). Molecular control of endurance training adaptation in male mouse skeletal muscle. *Nat. Metab.* 5, 2020–2035. <https://doi.org/10.1038/s42255-023-00891-y>.
61. Bourdeau, V., Deschênes, J., Métivier, R., Nagai, Y., Nguyen, D., Bretschneider, N., Gannon, F., White, J.H., and Mader, S. (2004). Genome-wide identification of high-affinity estrogen response elements in human and mouse. *Mol. Endocrinol.* 18, 1411–1427. <https://doi.org/10.1210/me.2003-0441>.
62. Lawson, E.A. (2017). The effects of oxytocin on eating behaviour and metabolism in humans. *Nat. Rev. Endocrinol.* 13, 700–709. <https://doi.org/10.1038/nrendo.2017.115>.
63. Acevedo-Rodriguez, A., Mani, S.K., and Handa, R.J. (2015). Oxytocin and estrogen receptor β in the brain: an overview. *Front. Endocrinol.* 6, 160. <https://doi.org/10.3389/fendo.2015.00160>.
64. Anacker, A.M.J., and Beery, A.K. (2013). Life in groups: the roles of oxytocin in mammalian sociality. *Front. Behav. Neurosci.* 7, 185. <https://doi.org/10.3389/fnbeh.2013.00185>.
65. Lawson, E.A., Donoho, D.A., Blum, J.I., Meenaghan, E.M., Misra, M., Herzog, D.B., Sluss, P.M., Miller, K.K., and Klibanski, A. (2011). Decreased nocturnal oxytocin levels in anorexia nervosa are associated with low bone mineral density and fat mass. *J. Clin. Psychiatry* 72, 1546–1551. <https://doi.org/10.4088/JCP.10m06617>.
66. Lawson, E.A., Ackerman, K.E., Estella, N.M., Guereca, G., Pierce, L., Sluss, P.M., Bouxsein, M.L., Klibanski, A., and Misra, M. (2013). Nocturnal oxytocin secretion is lower in amenorrheic athletes than non-athletes and associated with bone microarchitecture and finite element analysis parameters. *Eur. J. Endocrinol.* 168, 457–464. <https://doi.org/10.1530/EJE-12-0869>.
67. Tamma, R., Colaïanni, G., Zhu, L.I., Dibeneditto, A., Greco, G., Montemurro, G., Patano, N., Strippoli, M., Vergari, R., Mancini, L., et al. (2009). Oxytocin is an anabolic bone hormone. *Proc. Natl. Acad. Sci. USA* 106, 7149–7154. <https://doi.org/10.1073/pnas.0901890106>.
68. Chicharro, J.L., Hoyos, J., Bandrés, F., Gómez Gallego, F., Pérez, M., and Lucía, A. (2001). Plasma oxytocin during intense exercise in professional cyclists. *Horm. Res.* 55, 155–159. <https://doi.org/10.1159/000049988>.
69. Arseni, L., Lombardi, A., and Orioli, D. (2018). From structure to phenotype: Impact of collagen alterations on human health. *Int. J. Mol. Sci.* 19, 1407. <https://doi.org/10.3390/ijms19051407>.
70. Shimizu, K., Suzuki, N., Nakamura, M., Aizawa, K., Imai, T., Suzuki, S., Eda, N., Hanaoka, Y., Nakao, K., Suzuki, N., et al. (2012). Mucosal immune function comparison between amenorrheic and eumenorrheic distance runners. *J. Strength Cond. Res.* 26, 1402–1406. <https://doi.org/10.1519/JSC.0b013e31822e7a6c>.
71. Drew, M.K., Vlahovich, N., Hughes, D., Appaneal, R., Peterson, K., Burke, L., Lundy, B., Toomey, M., Watts, D., Lovell, G., et al. (2017). A multifactorial evaluation of illness risk factors in athletes preparing for the summer olympic games. *J. Sci. Med. Sport* 20, 745–750. <https://doi.org/10.1016/j.jsams.2017.02.010>.
72. Bertrand, C., Valet, P., and Castan-Laurell, I. (2015). Apelin and energy metabolism. *Front. Physiol.* 6, 115. <https://doi.org/10.3389/fphys.2015.00115>.
73. Sendinc, E., Valle-Garcia, D., Dhall, A., Chen, H., Henriques, T., Navarrete-Perea, J., Sheng, W., Gygi, S.P., Adelman, K., and Shi, Y. (2019). PCIF1 catalyzes m6Am mRNA methylation to regulate gene expression. *Mol. Cell* 75, 620–630.e9. <https://doi.org/10.1016/j.molcel.2019.05.030>.
74. Friedl, K.E., Moore, R.J., Hoyt, R.W., Marchitelli, L.J., Martinez-Lopez, L.E., and Askew, E.W. (2000). Endocrine markers of semistarvation in healthy lean men in a multistressor environment. *J. Appl. Physiol.* 88, 1820–1830. <https://doi.org/10.1152/jappl.2000.88.5.1820>.
75. Weindrich, R.H., Kristie, J.A., Cheney, K.E., and Walford, R.L. (1979). Influence of controlled dietary restriction on immunologic function and aging. *Fed. Proc.* 38, 2007–2016.
76. Rikke, B.A., Yerg, J.E., Battaglia, M.E., Nagy, T.R., Allison, D.B., and Johnson, T.E. (2003). Strain variation in the response of body temperature to dietary restriction. *Mech. Ageing Dev.* 124, 663–678. [https://doi.org/10.1016/S0047-6374\(03\)00003-4](https://doi.org/10.1016/S0047-6374(03)00003-4).
77. Speakman, J.R., and Mitchell, S.E. (2011). Caloric restriction. *Mol. Aspects Med.* 32, 159–221. <https://doi.org/10.1016/j.mam.2011.07.001>.
78. Wakeling, A., and Russell, G.F. (1970). Disturbances in the regulation of body temperature in anorexia nervosa. *Psychol. Med.* 1, 30–39. <https://doi.org/10.1017/S0033291700039994>.
79. Yang, S.L., Wu, C., Xiong, Z.F., and Fang, X. (2015). Progress on hypoxia-inducible factor-3: Its structure, gene regulation and biological function (review). *Mol. Med. Rep.* 12, 2411–2416. <https://doi.org/10.3892/mmr.2015.3689>.
80. Arnett, T.R. (2010). Acidosis, hypoxia and bone. *Arch. Biochem. Biophys.* 503, 103–109. <https://doi.org/10.1016/j.abb.2010.07.021>.
81. Petkus, D.L., Murray-Kolb, L.E., and De Souza, M.J. (2017). The unexplored crossroads of the female athlete triad and iron deficiency: a narrative review. *Sports Med.* 47, 1721–1737. <https://doi.org/10.1007/s40279-017-0706-2>.
82. Mayo, K.E., and Palmiter, R.D. (1982). Glucocorticoid regulation of the mouse metallothionein I gene is selectively lost following amplification of the gene. *J. Biol. Chem.* 257, 3061–3067. [https://doi.org/10.1016/s0021-9258\(19\)81073-6](https://doi.org/10.1016/s0021-9258(19)81073-6).
83. Watson, H.J., Yilmaz, Z., Thornton, L.M., Hübel, C., Coleman, J.R.I., Gaspar, H.A., Bryois, J., Hinney, A., Leppä, V.M., Mattheisen, M., et al. (2019). Genome-wide association study identifies eight risk loci and implicates metabo-psychiatric origins for anorexia nervosa. *Nat. Genet.* 51, 1207–1214. <https://doi.org/10.1038/s41588-019-0439-2>.
84. Zheng, Y., Rajcsanyi, L.S., Herpertz-Dahlmann, B., Seitz, J., de Zwaan, M., Herzog, W., Ehrlich, S., Zipfel, S., Giel, K., Egberts, K., et al. (2022). PTBP2 – a gene with relevance for both anorexia nervosa and body weight regulation. *Transl. Psychiatry* 12, 241. <https://doi.org/10.1038/s41398-022-02018-5>.
85. Vosselman, M.J., Hoeks, J., Brans, B., Pallubinsky, H., Nascimento, E.B.M., Van Der Lans, A.A.J.J., Broeders, E.P.M., Mottaghy, F.M., Schrauwen, P., and Van Marken Lichtenbelt, W.D. (2015). Low brown adipose tissue activity in endurance-trained compared with lean sedentary men. *Int. J. Obes.* 39, 1696–1702. <https://doi.org/10.1038/ijo.2015.130>.
86. Cannon, B., and Nedergaard, J. (2004). Brown adipose tissue: function and physiological significance. *Physiol. Rev.* 84, 277–359. <https://doi.org/10.1152/physrev.00015.2003>.
87. van der Vinne, V., Tachinardi, P., Riede, S.J., Akkerman, J., Scheepe, J., Daan, S., and Hut, R.A. (2019). Maximising survival by shifting the daily

- timing of activity. *Ecol. Lett.* 22, 2097–2102. <https://doi.org/10.1111/ele.13404>.
88. Ketchesin, K.D., Stinnett, G.S., and Seasholtz, A.F. (2017). Corticotropin-releasing hormone-binding protein and stress: from invertebrates to humans. *Stress* 20, 449–464. <https://doi.org/10.1080/10253890.2017.1322575>.
89. Loucks, A.B., Mortola, J.F., Gorton, L., and Yen, S.S. (1989). Alterations in the hypothalamic-pituitary-ovarian and the hypothalamic-pituitary-adrenal axes in athletic women. *J. Clin. Endocrinol. Metab.* 68, 402–411. <https://doi.org/10.1210/jcem-68-2-402>.
90. Ackerman, K.E., Patel, K.T., Guereca, G., Pierce, L., Herzog, D.B., and Misra, M. (2013). Cortisol secretory parameters in young exercisers in relation to LH secretion and bone parameters. *Clin. Endocrinol.* 78, 114–119. <https://doi.org/10.1111/j.1365-2265.2012.04458.x>.
91. Bergendahl, M., Aloï, J.A., Iranmanesh, A., Mulligan, T.M., and Veldhuis, J.D. (1998). Fasting suppresses pulsatile luteinizing hormone (LH) secretion and enhances orderliness of LH release in young but not older men. *J. Clin. Endocrinol. Metab.* 83, 1967–1975. <https://doi.org/10.1210/jc.83.6.1967>.
92. Kato, Y., Iwasaki, Y., Iwasaki, J., Abe, H., Yanaiharu, N., and Imura, H. (1978). Prolactin release by vasoactive intestinal polypeptide in rats. *Endocrinology* 103, 554–558. <https://doi.org/10.1210/endo-103-2-554>.
93. Thein-Nissenbaum, J.M., Rauh, M.J., Carr, K.E., Loud, K.J., and McGuire, T.A. (2011). Associations between disordered eating, menstrual dysfunction, and musculoskeletal injury among high school athletes. *J. Orthop. Sports Phys. Ther.* 41, 60–69. <https://doi.org/10.2519/jospt.2011.3312>.
94. Gillies, A.R., and Lieber, R.L. (2011). Structure and function of the skeletal muscle extracellular matrix. *Muscle Nerve* 44, 318–331. <https://doi.org/10.1002/mus.22094>.
95. Kragstrup, T.W., Kjaer, M., and Mackey, A.L. (2011). Structural, biochemical, cellular, and functional changes in skeletal muscle extracellular matrix with aging. *Scand. J. Med. Sci. Sports* 21, 749–757. <https://doi.org/10.1111/j.1600-0838.2011.01377.x>.
96. Spanheimer, R., Zlatev, T., Umpierrez, G., and DiGirolamo, M. (1991). Collagen production in fasted and food-restricted rats: Response to duration and severity of food deprivation. *J. Nutr.* 121, 518–524. <https://doi.org/10.1093/jn/121.4.518>.
97. Stefanetti, R.J., Voisin, S., Russell, A., and Lamon, S. (2018). Recent advances in understanding the role of FOXO3. *F1000Res.* 7, 1372. <https://doi.org/10.12688/f1000research.15258.1>.
98. Balducci, A., Bianco, A.M., Mangogna, A., Zicari, A.M., Leonardi, L., Cinicola, B.L., Capponi, M., Tommasini, A., Agostinis, C., D'Adamo, A.P., and Bulla, R. (2023). Genetic bases of C7 deficiency: systematic review and report of a novel deletion determining functional hemizygosity. *Front. Immunol.* 14, 1192690. <https://doi.org/10.3389/fimmu.2023.1192690>.
99. Dex, S., Alberton, P., Willkomm, L., Söller, T., Bago, S., Milz, S., Shakibaei, M., Ignatius, A., Bloch, W., Clausen-Schaumann, H., et al. (2017). Tenomodulin is required for tendon endurance running and collagen I fibril adaptation to mechanical load. *EBioMedicine* 20, 240–254. <https://doi.org/10.1016/j.ebiom.2017.05.003>.
100. Monin, L., Whettlock, E.M., and Male, V. (2020). Immune responses in the human female reproductive tract. *Immunology* 160, 106–115. <https://doi.org/10.1111/imm.13136>.
101. Erol, A., and Ozten, M. (2021). The relationship between weight change and C3 complement levels in patients with anorexia nervosa. *J. Psychiatry Neurol. Sci.* 34, 97–101. <https://doi.org/10.14744/DAJPNS.2021.00125>.
102. Straub, R.H., Cutolo, M., Buttgerit, F., and Pongratz, G. (2010). Energy regulation and neuroendocrine-immune control in chronic inflammatory diseases. *J. Intern. Med.* 267, 543–560. <https://doi.org/10.1111/j.1365-2796.2010.02218.x>.
103. Johnson, G.A., Burghardt, R.C., Bazer, F.W., and Spencer, T.E. (2003). Osteopontin: Roles in implantation and placentation. *Biol. Reprod.* 69, 1458–1471. <https://doi.org/10.1095/biolreprod.103.020651>.
104. White, F.J., Burghardt, R.C., Hu, J., Joyce, M.M., Spencer, T.E., and Johnson, G.A. (2006). Secreted phosphoprotein 1 (osteopontin) is expressed by stromal macrophages in cyclic and pregnant endometrium of mice, but is induced by estrogen in luminal epithelium during conceptus attachment for implantation. *Reproduction* 132, 919–929. <https://doi.org/10.1530/REP-06-0068>.
105. Zhang, H., Zhao, H., Wang, X., Cui, X., and Jin, L. (2020). Keratin 86 is up-regulated in the uterus during implantation, induced by oestradiol. *BMC Dev. Biol.* 20, 3–10. <https://doi.org/10.1186/s12861-020-0208-6>.
106. Velicky, P., Windsperger, K., Petroczi, K., Pils, S., Reiter, B., Weiss, T., Vondra, S., Ristl, R., Dekan, S., Fiala, C., et al. (2018). Pregnancy-associated diamine oxidase originates from extravillous trophoblasts and is decreased in early-onset preeclampsia. *Sci. Rep.* 8, 6342. <https://doi.org/10.1038/s41598-018-24652-0>.
107. Zhu, J., Wang, R., Cao, H., Zhang, H., Xu, S., Wang, A., Liu, B., Wang, Y., and Wang, R. (2015). Expression of claudin-5, -7, -8 and -9 in cervical carcinoma tissues and adjacent non-neoplastic tissues. *Int. J. Clin. Exp. Pathol.* 8, 9479–9486.
108. Yang, B. (2023). *Aquaporins Second*, B. Yang, ed. (Springer).
109. Gao, Y.Y., Chen, L., Wang, T., Nie, Z.W., Zhang, X., and Miao, Y.L. (2016). Oocyte aging-induced neuronatin (NNAT) hypermethylation affects oocyte quality by impairing glucose transport in porcine. *Sci. Rep.* 6, 36008. <https://doi.org/10.1038/srep36008>.
110. Huang, R., Xue, X., Li, S., Wang, Y., Sun, Y., Liu, W., Yin, H., and Tao, T. (2018). Alterations of polyunsaturated fatty acid metabolism in ovarian tissues of polycystic ovary syndrome rats. *J. Cell Mol. Med.* 22, 3388–3396. <https://doi.org/10.1111/jcmm.13614>.
111. Wojciechowski, T., Billert, M., Jasaszwili, M., Strowski, M.Z., Nowak, K.W., and Skrzypski, M. (2021). The role of neuropeptide B and its receptors in controlling appetite, metabolism and energy homeostasis. *Int. J. Mol. Sci.* 22, 6632. <https://doi.org/10.3390/ijms22126632>.
112. Al-Farsi, H., Al-Azwani, I., Malek, J.A., Chouchane, L., Rafii, A., and Halabi, N.M. (2022). Discovery of new therapeutic targets in ovarian cancer through identifying significantly non-mutated genes. *J. Transl. Med.* 20, 244. <https://doi.org/10.1186/s12967-022-03440-5>.
113. Melin, A., Tornberg, Å.B., Skouby, S., Möller, S.S., Sundgot-Borgen, J., Faber, J., Sidelmann, J.J., Aziz, M., and Sjödin, A. (2015). Energy availability and the female athlete triad in elite endurance athletes. *Scand. J. Med. Sci. Sports* 25, 610–622. <https://doi.org/10.1111/sms.12261>.
114. Hunt, L.M., Hogeland, E.W., Henry, M.K., and Swoap, S.J. (2004). Hypotension and bradycardia during caloric restriction in mice are independent of salt balance and do not require ANP receptor. *Am. J. Physiol. Heart Circ. Physiol.* 287, 1446–1451. <https://doi.org/10.1152/ajpheart.00353.2004>.
115. Nicoll, R., and Henein, M.Y. (2018). Caloric restriction and its effect on blood pressure, heart rate variability and arterial stiffness and dilatation: A review of the evidence. *Int. J. Mol. Sci.* 19, 751. <https://doi.org/10.3390/ijms19030751>.
116. Ibarra, F., Crambert, S., Eklöf, A.C., Lundquist, A., Hansell, P., and Holtbäck, U. (2005). Prolactin, a natriuretic hormone, interacting with the renal dopamine system. *Kidney Int.* 68, 1700–1707. <https://doi.org/10.1111/j.1523-1755.2005.00586.x>.
117. Mirković, K., Doorenbos, C.R.C., Dam, W.A., Lambers Heerspink, H.J., Slagman, M.C.J., Nauta, F.L., Kramer, A.B., Gansevoort, R.T., van den Born, J., Navis, G., et al. (2013). Urinary vitamin D binding protein: A potential novel marker of renal interstitial inflammation and fibrosis. *PLoS One* 8, 1–9. <https://doi.org/10.1371/journal.pone.0055887>.
118. Matsuo, K., Sato, K., Suemoto, K., Miyamoto-Mikami, E., Fuku, N., Higashida, K., Tsuji, K., Xu, Y., Liu, X., Iemitsu, M., et al. (2017). A mechanism underlying preventive effect of high-intensity training on colon cancer. *Med. Sci. Sports Exerc.* 49, 1805–1816. <https://doi.org/10.1249/MSS.0000000000001312>.

119. Bradshaw, A.D. (2009). The role of SPARC in extracellular matrix assembly. *J. Cell Commun. Signal.* 3, 239–246. <https://doi.org/10.1007/s12079-009-0062-6>.
120. O'Donnell, E., and De Souza, M.J. (2011). Increased serum adiponectin concentrations in amenorrheic physically active women are associated with impaired bone health but not with estrogen exposure. *Bone* 48, 760–767. <https://doi.org/10.1016/j.bone.2010.12.018>.
121. Donoso, M.A., Muñoz-Calvo, M.T., Barrios, V., Garrido, G., Hawkins, F., and Argente, J. (2010). Increased circulating adiponectin levels and decreased leptin/soluble leptin receptor ratio throughout puberty in female ballet dancers: association with body composition and the delay in puberty. *Eur. J. Endocrinol.* 162, 905–911. <https://doi.org/10.1530/EJE-09-0874>.
122. Counts, D.R., Gwirtsman, H., Carlsson, L.M., Lesem, M., and Cutler, G.B. (1992). The effect of anorexia nervosa and refeeding on growth hormone-binding protein, the insulin-like growth factors (IGFs), and the IGF-binding proteins. *J. Clin. Endocrinol. Metab.* 75, 762–767. <https://doi.org/10.1210/jc.75.3.762>.
123. Loucks, A.B., Verdun, M., and Heath, E.M. (1998). Low energy availability, not stress of exercise, alters LH pulsatility in exercising women. *J. Appl. Physiol.* 84, 37–46. <https://doi.org/10.1152/jappl.1998.84.1.37>.
124. Stanford, K.I., Lynes, M.D., Takahashi, H., Baer, L.A., Arts, P.J., May, F.J., Lehnig, A.C., Middelbeek, R.J.W., Richard, J.J., So, K., et al. (2018). 12,13-diHOME: An exercise-induced lipokine that increases skeletal muscle fatty acid uptake. *Cell Metab.* 27, 1111–1120.e3. <https://doi.org/10.1016/j.cmet.2018.03.020>.
125. Wu, M., Du, Y., Zhang, C., Li, Z., Li, Q., Qi, E., Ruan, W., Feng, S., and Zhou, H. (2023). Mendelian randomization study of lipid metabolites reveals causal associations with heel bone mineral density. *Nutrients* 15, 4160. <https://doi.org/10.3390/nu15194160>.
126. Perrigo, G., and Bronson, F.H. (1983). Foraging effort, food intake, fat deposition and puberty in female mice. *Biol. Reprod.* 29, 455–463. <https://doi.org/10.1095/biolreprod29.2.455>.
127. Chaput, J.P., Mchill, A.W., Cox, R.C., Broussard, J.L., Dutil, C., da Costa, B.G.G., Sampasa-Kanyinga, H., and Wright, K.P., Jr. (2023). The role of insufficient sleep and circadian misalignment in obesity. *Nat. Rev. Endocrinol.* 19, 82–97. <https://doi.org/10.1038/s41574-022-00747-7>.
128. Speakman, J.R. (2005). Body size, energy metabolism and lifespan. *J. Exp. Biol.* 208, 1717–1730. <https://doi.org/10.1242/jeb.01556>.
129. Konarzewski, M., and Diamond, J. (1995). Evolution of basal metabolic rate and organ masses in laboratory mice. *Evolution* 49, 1239–1248. <https://doi.org/10.1111/j.1558-5646.1995.tb04450.x>.
130. Selman, C., Lumsden, S., Bünger, L., Hill, W.G., and Speakman, J.R. (2001). Resting metabolic rate and morphology in mice (*Mus musculus*) selected for high and low food intake. *J. Exp. Biol.* 204, 777–784. <https://doi.org/10.1242/jeb.204.4.777>.
131. Daan, S., Masman, D., Strijkstra, A., and Verhulst, S. (1989). Intraspecific allometry of basal metabolic rate: Relations with body size, temperature, composition, and circadian phase in the kestrel, *Falco tinnunculus*. *J. Biol. Rhythms* 4, 155–171. <https://doi.org/10.1177/074873048900400212>.
132. van der Vinne, V., Gorter, J.A., Riede, S.J., and Hut, R.A. (2015). Diurnality as an energy-saving strategy: energetic consequences of temporal niche switching in small mammals. *J. Exp. Biol.* 218, 2585–2593. <https://doi.org/10.1242/jeb.119354>.
133. Perrigo, G., and Bronson, F.H. (1985). Sex differences in the energy allocation strategies of house mice. *Behav. Ecol. Sociobiol.* 17, 297–302. <https://doi.org/10.1007/BF00300150>.
134. Pettersson, U.S., Waldén, T.B., Carlsson, P.O., Jansson, L., and Phillipson, M. (2012). Female mice are protected against high-fat diet induced metabolic syndrome and increase the regulatory T cell population in adipose tissue. *PLoS One* 7, e46057. <https://doi.org/10.1371/journal.pone.0046057>.
135. Grove, K.L., Fried, S.K., Greenberg, A.S., Xiao, X.Q., and Clegg, D.J. (2010). A microarray analysis of sexual dimorphism of adipose tissues in high-fat-diet-induced obese mice. *Int. J. Obes.* 34, 989–1000. <https://doi.org/10.1038/ijo.2010.12>.
136. Williams, C.T., Chmura, H.E., Deal, C.K., and Wilsterman, K. (2022). Sex-differences in phenology: a Tinbergian perspective. *Integr. Comp. Biol.* 62, 980–997. <https://doi.org/10.1093/icb/icac035>.
137. Chen, E.C., and Brzyski, R.G. (1999). Exercise and reproductive dysfunction. *Fertil. Steril.* 71, 1–6. [https://doi.org/10.1016/s0015-0282\(98\)00392-6](https://doi.org/10.1016/s0015-0282(98)00392-6).
138. De Souza, M.J., and Metzger, D.A. (1991). Reproductive dysfunction in amenorrheic athletes and anorexic patients: a review. *Med. Sci. Sports Exerc.* 23, 995–1007.
139. Gibbs, J.C., Williams, N.I., and De Souza, M.J. (2013). Prevalence of individual and combined components of the female athlete triad. *Med. Sci. Sports Exerc.* 45, 985–996. <https://doi.org/10.1249/MSS.0b013e31827e1bdc>.
140. Williams, N.I., Leidy, H.J., Hill, B.R., Lieberman, J.L., Legro, R.S., and De Souza, M.J. (2015). Magnitude of daily energy deficit predicts frequency but not severity of menstrual disturbances associated with exercise and caloric restriction. *Am. J. Physiol. Endocrinol. Metab.* 308, E29–E39. <https://doi.org/10.1152/ajpendo.00386.2013>.
141. van Rosmalen, L., and Hut, R.A. (2021). Food and temperature change photoperiodic responses in two vole species. *J. Exp. Biol.* 224, jeb243030. <https://doi.org/10.1242/jeb.243030>.
142. Chilibeck, P.D., Sale, D.G., and Webber, C.E. (1995). Exercise and bone mineral density. *Sports Med.* 19, 103–122. <https://doi.org/10.2165/00007256-199519020-00003>.
143. Hattori, S., Park, J.H., Agata, U., Akimoto, T., Oda, M., Higano, M., Aikawa, Y., Nabekura, Y., Yamato, H., Ezawa, I., and Omi, N. (2013). Influence of food restriction combined with voluntary running on bone morphology and strength in male rats. *Calcif. Tissue Int.* 93, 540–548. <https://doi.org/10.1007/s00223-013-9787-6>.
144. Weikum, E.R., Knuesel, M.T., Ortlund, E.A., and Yamamoto, K.R. (2017). Glucocorticoid receptor control of transcription: precision and plasticity via allostery. *Nat. Rev. Mol. Cell Biol.* 18, 159–174. <https://doi.org/10.1038/nrm.2016.152>.
145. Hardy, R.S., Zhou, H., Seibel, M.J., and Cooper, M.S. (2018). Glucocorticoids and bone: Consequences of endogenous and exogenous excess and replacement therapy. *Endocr. Rev.* 39, 519–548. <https://doi.org/10.1210/er.2018-00097>.
146. Hasselgren, P.O. (1999). Glucocorticoids and muscle catabolism. *Curr. Opin. Clin. Nutr. Metab. Care* 2, 201–205. <https://doi.org/10.1097/00075197-199905000-00002>.
147. Sundgot-Borgen, J., and Torstveit, M.K. (2004). Prevalence of eating disorders in elite athletes is higher than in the general population. *Clin. J. Sport Med.* 14, 25–32. <https://doi.org/10.1097/00042752-200401000-00005>.
148. Dipla, K., Kraemer, R.R., Constantini, N.W., and Hackney, A.C. (2021). Relative energy deficiency in sports (RED-S): elucidation of endocrine changes affecting the health of males and females. *Hormones (Basel)* 20, 35–47. <https://doi.org/10.1007/s42000-020-00214-w>.
149. Lane, A.R., Hackney, A.C., Smith-Ryan, A., Kucera, K., Registrar-Mihalik, J., and Ondrak, K. (2019). Prevalence of low energy availability in competitively trained male endurance athletes. *Med* 55, 665. <https://doi.org/10.3390/medicina55100665>.
150. Carbone, J.W., McClung, J.P., and Pasiakos, S.M. (2012). Skeletal muscle responses to negative energy balance: Effects of dietary protein. *Adv. Nutr.* 3, 119–126. <https://doi.org/10.3945/an.111.001792>.
151. Acosta-rodríguez, V., Rijo-Ferreira, F., Izumo, M., Xu, P., Wight-Carter, M., Green, C.B., and Takahashi, J.S. (2022). Circadian alignment of early onset caloric restriction promotes longevity in male C57BL/6J mice. *Science* 376, 1192–1202. <https://doi.org/10.1126/science.abk0297>.
152. Mitchell, S.E., Delville, C., Konstantopodou, P., Derous, D., Green, C.L., Wang, Y., Han, J.-D.J., Promislow, D.E.L., Douglas, A., Chen, L., et al. (2016). The effects of graded levels of caloric restriction: V. Impact of short term caloric and protein restriction on physical activity in the

- C57BL/6 mouse. *Oncotarget* 7, 19147–19170. <https://doi.org/10.18632/oncotarget.8158>.
153. MoTrPAC Study Group (2022). Temporal dynamics of the multi-omic response to endurance exercise training across tissues. Preprint at bioRxiv. <https://doi.org/10.1101/2022.09.21.508770>.
154. O'Neal, T.J., Friend, D.M., Guo, J., Hall, K.D., and Kravitz, A.V. (2017). Increases in physical activity result in diminishing increments in daily energy expenditure in mice. *Curr. Biol.* 27, 423–430. <https://doi.org/10.1016/j.cub.2016.12.009>.
155. Wasserfurth, P., Palmowski, J., Hahn, A., and Krüger, K. (2020). Reasons for and consequences of low energy availability in female and male athletes: Social environment, adaptations, and prevention. *Sports Med. Open* 6, 44. <https://doi.org/10.1186/s40798-020-00275-6>.
156. Wells, K.R., Jeacocke, N.A., Appaneal, R., Smith, H.D., Vlahovich, N., Burke, L.M., and Hughes, D. (2020). The Australian institute of sport (AIS) and national eating disorders collaboration (NEDC) position statement on disordered eating in high performance sport. *Br. J. Sports Med.* 54, 1247–1258. <https://doi.org/10.1136/bjsports-2019-101813>.
157. Joy, E., Kussman, A., and Nattiv, A. (2016). 2016 update on eating disorders in athletes: A comprehensive narrative review with a focus on clinical assessment and management. *Br. J. Sports Med.* 50, 154–162. <https://doi.org/10.1136/bjsports-2015-095735>.
158. Spoelstra, K. (2018). ChronoShop 1.1 for the analysis of circadian properties of behaviour. <https://doi.org/10.34894/RFXUQX>.
159. Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. J.* 17, 10–12. <https://doi.org/10.14806/ej.17.1.200>.
160. Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21. <https://doi.org/10.1093/bioinformatics/bts635>.
161. Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 550. <https://doi.org/10.1186/s13059-014-0550-8>.
162. Zhou, Y., Zhou, B., Pache, L., Chang, M., Khodabakhshi, A.H., Tanaseichuk, O., Benner, C., and Chanda, S.K. (2019). Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat. Commun.* 10, 1523. <https://doi.org/10.1038/s41467-019-09234-6>.
163. Wickham, H. (2016). ggplot2: Elegant Graphics for Data Analysis (Springer-Verlag). <https://doi.org/10.1007/978-0-387-98141-3>.
164. McLean, A.C., Valenzuela, N., Fai, S., and Bennett, S.A.L. (2012). Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. *J. Vis. Exp.* 67, e4389. <https://doi.org/10.3791/4389>.
165. Ajayi, A.F., and Akhigbe, R.E. (2020). Staging of the estrous cycle and induction of estrus in experimental rodents: an update. *Fertil. Res. Pract.* 6, 5–15. <https://doi.org/10.1186/s40738-020-00074-3>.
166. Lenert, M.E., Chaparro, M.M., and Burton, M.D. (2021). Homeostatic regulation of estrus cycle of young female mice on western diet. *J. Endocr. Soc.* 5, bvab010. <https://doi.org/10.1210/jendso/bvab010>.
167. Pantier, L.K., Li, J., and Christian, C.A. (2019). Estrous cycle monitoring in mice with rapid data visualization and analysis. *Bio. Protoc.* 9, e3354. <https://doi.org/10.21769/bioprotoc.3354>.
168. Keenan, A.B., Torre, D., Lachmann, A., Leong, A.K., Wojciechowicz, M.L., Utti, V., Jagodnik, K.M., Kropiwnicki, E., Wang, Z., and Ma'ayan, A. (2019). ChEA3: transcription factor enrichment analysis by orthogonal omics integration. *Nucleic Acids Res.* 47, W212–W224. <https://doi.org/10.1093/nar/gkz446>.
169. Blume, J.E., Manning, W.C., Troiano, G., Hornburg, D., Figa, M., Hesterberg, L., Platt, T.L., Zhao, X., Cuadras, R.A., Everley, P.A., et al. (2020). Rapid, deep and precise profiling of the plasma proteome with multi-nanoparticle protein corona. *Nat. Commun.* 11, 3662. <https://doi.org/10.1038/s41467-020-17033-7>.
170. Evans, A.M., Dehaven, C.D., Barrett, T., Mitchell, M., and Milgram, E. (2009). Integrated, nontargeted ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry platform for the identification and relative quantification of the small-molecule complement of biological systems. *Anal. Chem.* 81, 6656–6667. <https://doi.org/10.1021/ac901536h>.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
TRIzol Reagent	Invitrogen	15596026
Quant-IT DNA Assay Kit	Thermo Scientific	Q33120
TruSeq Stranded mRNA kit	Illumina	20020595
Triglycerides Reagent	Thermo Scientific	TR22421
Total Cholesterol Reagent	Thermo Scientific	TR13421
Glycogen Assay Kit	Abcam	ab65620
Deposited data		
Raw files for RNA-seq	This paper	GEO: GSE243060
Raw counts, normalized counts	This paper	GEO: GSE243060
Statistical analysis for differential gene expression	This paper	Tables S1, S2, S3, S4, S5, S6, and S7; Mendeley Data: https://dx.doi.org/10.17632/skbxc86s9b.2
RNA-seq dataset, Energy deficient mouse hypothalamus	van Rosmalen et al. ⁴¹	GEO: GSE228967
Experimental models: Organisms/strains		
Mouse CBA/CAJ	The Jackson Laboratory	JAX: 000654
Software and algorithms		
MedPC IV	Med Associates	https://med-associates.com/product/med-pc-v/
Circadian Activity Monitoring System (CAMS)	Developed by HM Cooper, INSERM, Bron, France	N/A
R, v4.1.2	R Development Core Team, 2022	https://www.r-project.org
RStudio, v2022.07.1 + 554	RStudio: Integrated Development for R, Boston, MA	https://www.rstudio.com
ActoView for MS Excel 2010	Developed by C. Mulder, University of Groningen	N/A
ChronoShop, v1.1	Spoelstra et al. ¹⁵⁸	https://dataverse.nl/dataset.xhtml?persistentId=hdl:10411/YHJEFV
Cutadapt, v4.1	Martin et al. ¹⁵⁹	https://cutadapt.readthedocs.io/en/stable/
FastQC, v0.11.9	Babraham Bioinformatics, Cambridge, UK	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
STAR, v2.7.10a	Dobin et al. ¹⁶⁰	https://github.com/alexdobin/STAR
DESeq2, v1.34.0	Love et al. ¹⁶¹	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
Metascape, v3.5.20230101	Zhou et al. ¹⁶²	https://metascape.org/gp/index.html#/main/step1
Ggplot2, v3.3.5	Wickham et al. ¹⁶³	https://ggplot2.tidyverse.org
LAS-X, v3.7.5.24914	Leica	N/A
NRecon	Bruker	https://www.microphotonic.com/micro-ct-systems/nrecon-reconstruction-software/
Other		
45-mg grain-based dustless precision food pellets	BioServ	F0165
Standard rodent chow	Altromin	141005
Food pellet dispenser	Med Associates	ENV-203-45

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Plastic running wheels	Savic	N/A
Spruce wood bedding material	Safe-lab	BK8-15
NovaMax Plus Glucose/Ketone monitor + strips	Nova Biomedical	N/A
EchoMRI-100	Echo Medical Systems	N/A
SkyScan 1076	SkyScan	N/A
Phase Lock Gel – Heavy tubes	QuantaBio	N/A
glycoblue coprecipitant	Invitrogen	AM9516
Leica DMI6000B microscope	Leica	DMI6000B
Tissue pulverizer	CellCrusher	N/A
NovaSeq 6000	Illumina	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author Satchidananda Panda (satchin@salk.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All raw (FASTQ files) and processed (raw counts and normalized counts) bulk RNA-seq data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are publicly available as of the date of publication through GEO series accession number GSE243060 and BioProject PRJNA1015634. Statistics and other parameters of gene expression can be found in [Tables S1, S2, S3, S4, S5, S6, and S7](#), which are deposited on Mendeley at <https://dx.doi.org/10.17632/skbc86s9b.2> and are publicly available as of the date of publication. This paper also analyzes existing, publicly available data. The accession number for the dataset is listed in the [key resources table](#).
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

A total of 40 female and 40 male CBA/CaJ mice (JAX strain 000654, a melatonin-proficient inbred mouse strain with intact retina), were individually housed in transparent macrolon cages, 32 cm (L) x 13 cm (W) x 15 cm (H). Each cage was equipped with a plastic running wheel (Savic, Kortrijk, Belgium) with a diameter of 14 cm and filled with spruce wood bedding material (BK8-15; Safe-lab, Rosenberg, Germany). Mice were entrained to a 12-h light: 12-h dark cycle (60–70 lux at cage level). The light spectrum consisted of peak wavelengths at 458 nm and 610 nm. Light levels during nighttime were <0.01 lux. The ambient conditions were maintained at an ambient temperature of $21 \pm 1^\circ\text{C}$ and relative humidity of $55 \pm 5\%$ by using climate-controlled chambers. Prior to baseline measurements, the mice were provided with standard rodent chow (no. 141005; Altromin, Lage, Germany) *ad libitum* for at least two weeks. Throughout the experiments, the mice had unrestricted access to water. All animal experiments were conducted in compliance with the guidelines set by the local animal welfare body (IvD), adhering to Directive 2010/63/EU, and approved by the CCD (Centrale Commissie Dierproeven) of the Netherlands (CCD, license number: AVD1050020186147).

'Exercise-for-food' paradigm

Twelve-week-old mice were individually housed in specialized experimental cages. Each cage was equipped with a food pellet dispenser (ENV-203-45; Med Associates Inc., St. Albans, VT, USA) that was controlled by the running wheel activity of the mouse. A 45-mg grain-based dustless precision food pellet (21% protein, 4% fat, 54% carbohydrate, 630 J per pellet; F0165, BioServ, Flemington, NJ, USA) was dispensed into the cage through a feeder tube after a predetermined number of wheel revolutions. Following a two-week habituation period, animals were randomly divided into two groups: a low-workload and a high-workload group. All animals (14-weeks old, females 22.08 ± 0.24 g, males 27.07 ± 0.40 g, $n = 80$) initially started with a low workload, defined as 100 wheel revolutions/food pellet, equivalent to a reward of 0.068 m/Joule (m/J). In the high-workload group, the workload was progressively increased by 20 revolutions per food pellet (0.014 m/J) on a daily basis until the animals started to lose weight. Once weight loss

occurred, the workload was increased by 10 revolutions/pellet (0.007 m/J), to induce a gradual energy deficiency over time. The workload remained stable once weight loss exceeded or equaled 0.5 g/day. The low-workload group maintained a workload of approximately 100 wheel revolutions/food pellet (0.068 m/J), resembling an *ad libitum* condition where food pellets were constantly available within the cage. Food intake and body weight were monitored daily between ZT6–ZT11, with ZT0 representing the time when the light turned on and ZT12 representing the time when the light turned off. In addition, female mice underwent daily vaginal lavage between ZT6–ZT11 to determine estrous stage. A more detailed description of the ‘exercise-for-food’ paradigm in small rodents can be found here.⁴⁰

METHOD DETAILS

Wheel-running behavior

The daily running wheel activity patterns of each individual mouse were recorded using a Circadian Activity Monitoring System (CAMS; developed by HM Cooper, INSERM, Bron, France). The system allowed for continuous recording of activity in 1-min intervals until the time of tissue collection. To visualize the activity patterns, actograms and activity profiles were generated using ActoView for MS Excel 2010 (programmed by C. Mulder, University of Groningen). ChronoShop (version 1.1) was used to calculate circadian parameters of wheel-running activity, including onset, center of gravity (COG), and offset.¹⁵⁸ To calculate the proportion of activity during daytime, the sum of wheel revolutions during the light phase was divided by the total wheel revolutions throughout the 24-h period. Activity profiles were generated by 3-h running averages of the last 5 days of the protocol. The number of activity bouts was calculated by ActoView and the fragmentation of activity profiles was determined by the following formula: (active bouts/active bins) * 100.

Estrous cycle staging

The estrous cycle stage was daily determined using the protocol established by McLean and colleagues.¹⁶⁴ Briefly, vaginal lavage was performed by flushing the vagina 3–5 times with 50 μ L autoclaved MilliQ daily between ZT6–ZT11. 1 μ L smears obtained from the lavage were permeabilized with 70% ethanol and subsequently stained with 0.1% crystal violet. High-resolution images were captured using a Leica DMI6000B microscope at 50X and 100X magnification, and software LAS-X version 3.7.5.24914. The estrous stage was determined based on analyzing the ratio of cell types: primarily nucleated epithelial cells (proestrus), primarily cornified epithelial cells (estrus), 50% cornified epithelial cells and leukocytes (metestrus), or primarily leukocytes (diestrus).^{165,166} Cycle stage data analysis and visualization were performed using the protocol and Python software developed by Pantier and colleagues.¹⁶⁷ Estrous cycle length was defined as the days between two days of proestrus, with the different stages occurring in between. Proestrus and metestrus stages are sometimes too brief to fully capture, therefore, if the smears indicated a transition from estrus to metestrus or from proestrus to estrus the cycle was identified as a full cycle. The cycle length was calculated based on the last complete cycle or if the ongoing cycle was longer than the previous cycle.

Glucose and ketone

Blood glucose and Ketone levels were measured for a subset of animals ($n = 48$) at four different time points throughout the protocol (day 0, 7, 14, and 20) using the NovaMax Plus Glucose/Ketone monitor (Nova Biomedical, Waltham, MA, USA). Tail clips were taken at either ZT5 $\pm$ 1 (light) or ZT17 $\pm$ 1 (dark) to obtain the blood samples and directly measure glucose and ketone levels.

Body composition

To distinguish between lean and fat mass, body composition was assessed in a subset of live mice ($n = 48$) at ZT5 $\pm$ 1 1–2 days before tissue collections using a Magnetic Resonance Imaging machine (EchoMRI-100, Echo Medical Systems, Houston, TX, USA). Mice were transferred from their home cage to the apparatus and underwent a 2-min scanning period.

Sample collection and processing

After 3 weeks in the ‘exercise-for-food’ paradigm, 40 mice per condition (80 mice in total) were sacrificed by decapitation with prior CO₂ exposure until unconscious. Of which 20 mice (10 males and 10 females) were sacrificed at ZT5 $\pm$ 30min (light) and 20 mice (10 males and 10 females) at ZT17 $\pm$ 30min (dark). Animals that were sacrificed during the dark phase, were killed under low-intensity red dim light (<0.1 lux). 20 tissues, pituitary (PIT), heart (HEA), lung, (LUN), brown adipose tissue (BAT), liver (LIV), adrenal glands (ADR), kidney (KID), spleen (SPL), ovaries (OVA), uterus (UTE), testis (TES), quadriceps (QUA), gastrocnemius (GAS), gastrocnemius tendon (GAS_t), extensor digitorum longus (EDL), extensor digitorum longus tendon (EDL_t), tibialis anterior (TA), tibialis anterior tendon (TAT), soleus (SOL), were removed and snap-frozen in liquid N₂ directly after dissection and stored at -80°C until RNA extraction. Soft tissue was carefully removed from the tibia bone (BON) and the bones were stored at -80°C until imaging.

Blood collection

Blood was taken by heart puncture, immediately transferred to EDTA tubes, and subsequently centrifuged at 1200g for 15 min at 4°C , allowing for the separation of plasma, which was immediately transferred to a clean tube and stored at -80°C until further procedure.

Plasma triglyceride and cholesterol quantification

Plasma triglyceride quantity was assessed with Thermo Scientific Triglycerides Reagent TR22421 according to the manufacturer's protocol (Thermo Scientific, Waltham, MA, USA). Plasma cholesterol quantity was assessed with Thermo Scientific Total Cholesterol Reagent TR13421 according to the manufacturer's protocol (Thermo Scientific). In short, 1.5 μ L plasma was diluted in 150 μ L reagent and incubated at 37°C for 5 min. Absorbance was measured at 505nm for 30 min.

Hepatic and muscle glycogen quantification

10–15 mg of frozen liver and muscle powder was used to measure glycogen levels using a colorimetric enzymatic kit according to the manufacturer's instructions (ab65620, Glycogen Assay Kit, Abcam, Cambridge, UK). The sensitivity was 0.04 μ g/mL, and levels between 0.04 and 2 μ g/well can be detected.

Microcomputed tomography (μ CT): Scanning and Reconstruction

The tibia bones of a subset of female mice ($n = 12$) were scanned by μ CT at (9 μ m)³ isotropic voxel resolution (SkyScan 1076; SkyScan, Kontich, Belgium; 50 kVp, 200 μ A, 0.5 mm aluminum filter, 180° scan, $\Delta = 0.8^\circ$). Specimens were supported and hydrated with PBS during scanning. Images were reconstructed using NRecon (Bruker, Belgium) using a smoothing factor of 1, ring artifact reduction factor of 6, beam hardening correction factor of 40%, and with a dynamic range from 0 to 0.11 attenuation units. Tibia aligned and registered to a representative control femur and cropped to a standard VOI size of (750 $\times$ 750 $\times$ 3200 px) using DataViewer (SkyScan, Kontich, Belgium). Bone structure parameters were analyzed using CTAn (SkyScan). Trabecular bone was analyzed by semi-automatic contouring of the medullary volume 0.35–0.85 mm distal to the proximal tibial metaphysis ($H = 0.05$ mm, 58 slices, 8.8 μ m slice thickness), using an adaptive threshold to select trabecular bone. Cortical bone was analyzed by selection of the cortical bone 1.5–2.5 mm distal to the proximal tibial metaphysis ($H = 1.05$ mm, 121 slices, 8.8 μ m slice thickness), using a global threshold (70–255). One outlier was removed from the analysis because its bone mineral density (BMD) deviated >2 SD of the mean.

RNA extraction

All frozen tissues, with the exception of the pituitary, adrenal glands, ovaries, and tendons, were cryopulverized into fine frozen powder using a tissue pulverizer (CellCrusher, Portland, OR, USA). Total RNA was extracted from ~ 30 mg of the powdered tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol for RNA extraction using half volumes. 100 μ L chloroform was replaced with 50 μ L 1-Bromo-3-chloropropane. Stainless steel screws were used to homogenize tissue using a vortex mixer. Phase lock gel heavy tubes (QuantaBio) were used to enhance phase separation. 2 μ L glycoblue coprecipitant (AM9516, Invitrogen) was added to the isopropanol step for small tissues (<20 mg; pituitary, adrenal glands, ovaries, tendons). Extracted RNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific).

Tissue	RNA extraction method	Sequencing platform	Sequencing depth
PIT	TRIzol, glycoblue	Illumina Novaseq 6000	PE100
HEA	TRIzol	Illumina Novaseq 6000	PE100
LUN	TRIzol	Illumina Novaseq 6000	PE100
BAT	TRIzol	Illumina Novaseq 6000	PE100
LIV	TRIzol	Illumina Novaseq 6000	PE100
ADR	TRIzol, glycoblue	Illumina Novaseq 6000	PE100
KID	TRIzol	Illumina Novaseq 6000	PE100
SPL	TRIzol	Illumina Novaseq 6000	PE100
OVA	TRIzol, glycoblue	Illumina Novaseq 6000	PE100
UTE	TRIzol	Illumina Novaseq 6000	PE100
TES	TRIzol	Illumina Novaseq 6000	PE100
QUA	TRIzol	Illumina Novaseq 6000	PE100
GAS	TRIzol	Illumina Novaseq 6000	PE100
GAS _t	TRIzol, glycoblue	Illumina Novaseq 6000	PE100
EDL	TRIzol	Illumina Novaseq 6000	PE100
EDL _t	TRIzol, glycoblue	Illumina Novaseq 6000	PE100
TA	TRIzol	Illumina Novaseq 6000	PE100
TAT	TRIzol, glycoblue	Illumina Novaseq 6000	PE100
SOL	TRIzol	Illumina Novaseq 6000	PE100

mRNA library preparation and sequencing

Libraries were prepared using Illumina's TruSeq Stranded mRNA (PolyA+) kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions, with the use of half volumes. 500 ng of total RNA was used to generate libraries. Unique dual (i5 and i7) 8 base pair sequencing indexes for Illumina-TruSeq DNA were used to enable multiplexing up to 96 library samples for next-generation sequencing (Illumina). Quality and quantity of libraries were inspected by using a nanodrop spectrophotometer (Thermo Scientific), Quant-IT dsDNA HS assay kit (ThermoFisher Scientific) and gel electrophoresis. Libraries were pooled and sequencing was performed using NovaSeq 6000 (Illumina) to obtain paired-end 100 base pair reads with a targeted sequencing depth of 25 million total reads per sample. Samples with <10 million reads were removed from the analysis. In total, 419 samples passed quality control.

Read mapping, annotation, quantification and normalization

The quality of the sequencing data was assessed using FastQC, version 0.11.9 (Babraham Bioinformatics, Cambridge, UK). Adapter sequences were removed using Cutadapt.¹⁵⁹ The sequence reads were aligned to the *Mus musculus* (house mouse) genome GRCh38 (mm10) and counted per gene using STAR aligner.¹⁶⁰ The average sequencing depth was 25M input reads and 20M uniquely mapped reads per sample (Table S1). Sequencing quality was assessed using FastQC, version 0.11.9 (Babraham Bioinformatics, Cambridge, UK). Gene counts were filtered for low counts and normalized per tissue (females and males together) using the DESeq2 workflow.¹⁶¹

Differential gene expression analysis

Differential gene expression analysis per tissue was carried out using DESeq2,¹⁶¹ with a design that accounted for energy deficiency. Subsequently, contrast analyses were performed (sex, energy deficiency, and zeitgeber time). Genes were considered expressed if normalized average gene counts were ≥ 10 . On average, 15,077 genes (protein coding and non-coding) were expressed per tissue (Table S1). Differentially expressed (DE) genes were identified based on significance threshold of Benjamini and Hochberg adjusted $p < 0.05$ (Tables S2, S3, and S5).

Gene ontology

Gene ontology (GO) analysis was performed on groups of differential expressed (DE) genes using the gene annotation tool Metascape.¹⁶² Top most significant clustered representative GO Biological Processes (GO:BP) were used as terms for subsequent analysis and can be found for each tissue in Table S2.

Transcription factor binding site enrichment analyses

Transcription factor (TF) enrichment analysis was performed on all DE genes for all tissues by using the ChEA3 software (Table S4).¹⁶⁸

Hypothalamus RNA-seq data

Bulk RNA-seq data from male mice (control and energy deficient) from three different hypothalamic brain tissues, paraventricular nucleus (PVN), paraventricular zone (PVZ), and preoptic area (POA), were used from GEO: GSE228967.⁴¹

Untargeted plasma proteomics

48 plasma samples (12 per group) were processed for untargeted proteomics analysis. 125 μ L of plasma was diluted 1:1 to 250 μ L and used for proteomics analysis by SeerBio (Redwood City, CA, USA). 250 μ L per sample was loaded onto the SP100 automation instrument, protein corona's were formed and processed to extract purified peptides to identify quantity by LC-MS.¹⁶⁹ LC-MS (Thermo Scientific UltiMate 3000 RSLCnano), nanoflow, 100 nL/min, 26 min gradient. Injected five nanoparticles, 1 by 1 acquisition, 30 min run, 20 min gradient. 2.5h total acquisition per sample. Data Independent Acquisition (DIA) protocol, 33 min total acquisition time. The data was analyzed using the Proteograph Analysis Suite (PAS) software (DIA-NN version 1.8.1). Data was filtered to 1% peptide and protein FDR for identification on peptide and protein group level. The uniprot database UP000005640_9606 was used as reference with a library free search. Raw MS intensity values were median normalized on a run-by-run basis, and used for subsequent analysis. Proteins were included in the analysis if they were detected in $\geq 50\%$ of samples (Table S6). Protein values are depicted as normalized values in all figures.

Untargeted plasma metabolomics

31 plasma samples (7–8 per group) were sent to Metabolon (Morrisville, NC, USA) for untargeted metabolite analysis using Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS).¹⁷⁰ Batch normalized (median-scaled) imputed metabolite counts were used for analysis. For each metabolite, the minimum value across all batches in the median scaled data is imputed for the missing values. Results are summarized in Table S7.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

One-way ANOVA was used to analyze the effect of an energy deficit on different physiological parameters between experimental groups. Two-way repeated measures ANOVA was used to analyze the effect of energy deficit on different behavioral and physiological parameters over multiple time points. Post hoc test (Tukey's HSD) was performed to detect statistical differences between groups at specific time points. Linear regression models were fit by using the `lm` function in RStudio to assess the correlation between different parameters. All analysis and statistical tests were performed in RStudio (version 2022.07.1; <http://www.rstudio.com/>). Unless otherwise described, all data are presented as mean $\pm$ SEM, with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All figures were generated using the R-package 'ggplot2'.¹⁶³