Original Article

MOLECULAR METABOLISM

# IL-6 decodes sex and diet-dependent circadian and metabolic rhythms

Antía González-Vila[1,8], Ali Mohammad Ibrahim-Alasoufi[1,2,8], María Luengo-Mateos[1], Víctor Pardo-García[1], Alejandro Diaz-López[1], Belén Fernández-Rodríguez[1], Matti Poutanen[3,4], Claes Ohlsson[5], Manuel Tena-Sempere[6,7], Carlos Diéguez-González[1,7], María del Carmen García-García[1], Olga Barca-Mayo[1,*]

## ABSTRACT

**Objective:** Interleukin-6 (IL-6) is a pleiotropic cytokine involved in immune regulation and energy metabolism. Its diurnal secretion influences core circadian components, emphasizing its critical role in circadian biology. Despite known sex differences in immune, circadian, and metabolic processes, how IL-6 integrates these processes remains poorly understood.

**Methods:** IL6 knockout (KO) and control mice of both sexes were phenotyped for circadian and metabolic traits under standard (STD) and high-fat diet (HFD), fasting, and time-restricted feeding. Molecular analyses in muscle, liver, and hypothalamus assessed clock gene expression and IL-6 signaling pathway. Circulating sex steroid hormones were quantified to examine their contribution to the observed sex-specific phenotypes.

**Results:** IL-6 deficiency disrupts circadian locomotor and metabolic rhythms in a sex- and diet-dependent manner. Males exhibit impaired light-driven circadian rhythms under STD conditions and metabolic misalignment under HFD, whereas females display greater circadian resilience under STD conditions but increased vulnerability to circadian disruption during HFD. Additionally, IL-6 emerges as a novel regulator of the food-entrainable oscillator (FEO), linking food anticipatory activity and metabolic cycles under both STD and HFD in a sex-dependent manner.

**Conclusions:** These findings identify IL-6 as a critical mediator of circadian-metabolic plasticity, shaping sex- and diet-specific trade-offs between circadian stability and metabolic homeostasis. Our study highlights IL-6 as a potential therapeutic target for mitigating circadian misalignment-associated metabolic disorders, with implications for the timed modulation of IL-6 signaling.



**Keywords** Interleukin-6; Circadian rhythms; Metabolic cycles; Energy balance; Sexual dimorphism

## 1. INTRODUCTION

The mammalian circadian system consists of a network of central and peripheral clocks that synchronize physiological processes with environmental cues such as the light—dark cycle and food availability [1,2]. At its core, the hypothalamic suprachiasmatic nucleus (SCN) serves as the central pacemaker, orchestrating peripheral clocks in metabolic tissues, including the liver and muscle [1,2]. However, peripheral clocks can be entrained independently of the SCN through feeding schedules, such as time-restricted feeding (TRF), which induces food anticipatory activity (FAA), a pre-meal locomotor behavior that persists even in the absence of a functional SCN [3—5]. This suggests the presence of a food-entrainable oscillator (FEO) [6] that interacts with circadian and metabolic networks to regulate energy homeostasis. Understanding how feeding cues influence peripheral clocks is key to revealing how mistimed feeding and circadian disruption drive metabolic, inflammatory, and immune disorders [7—10]. Despite these associations, the molecular pathways mediating feeding-induced circadian entrainment, particularly under pathological conditions, remain incompletely characterized.

At the molecular level, circadian rhythms are regulated by transcriptional-translational feedback loops involving core clock genes. The BMAL1/CLOCK complex activates the transcription of clock-controlled genes, including *Period* (*Per1, Per2*) and *Cryptochrome* (*Cry1, Cry2*), which then feedback to inhibit BMAL1/CLOCK, generating a self-sustaining 24-hour cycle [1]. These rhythms regulate essential physiological processes, including immune function, metabolism, and behavior, yet the integration between circadian and metabolic signals remains poorly understood [11,12]. This intricate regulation not only governs systemic processes but also intersects with IL-6 signaling, a key regulator of immune function and metabolism [13—19]. IL-6 influences energy expenditure, glucose homeostasis, and lipid

[1]Physiology Department, Molecular Medicine and Chronic Diseases Research Centre (CiMUS), University of Santiago de Compostela, Santiago de Compostela, Spain [2]Biology Department, Al-Hussein Bin Talal University, Ma'an, Jordan [3]Research Center for Integrative Physiology and Pharmacology, Institute of Biomedicine, University of Turku, Turku, Finland [4]Turku Center for Disease Modeling, Turku, Finland [5]Sahlgrenska Osteoporosis Centre, Department of Internal Medicine and Clinical Nutrition, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden [6]Department of Cell Biology, Physiology and Immunology, University of Córdoba, Instituto Maimónides de Investigación Biomédica (IMIBIC)/Hospital Universitario Reina Sofía, Córdoba, Spain [7]CIBER Fisiopatología de la Obesidad y Nutrición, Instituto de Salud Carlos III, Madrid, Spain

[8] Antía González-Vila and Ali Mohammad Ibrahim-Alasoufi contributed equally to this work.

*Corresponding author. E-mail: olga.barca.mayo@usc.es (O. Barca-Mayo).

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metabolism, all of which exhibit circadian control [7]. Notably, IL-6 displays diurnal oscillations [20,21] and modulates core circadian components, including the *Period* genes [22,23], positioning it as a potential molecular link between circadian and metabolic pathways. Indeed, nutritional and metabolic challenges, such as high-fat diet (HFD), disrupt circadian regulation [24–26] and are associated with increased circulating IL-6 levels [27]. Under these conditions, IL-6 deficiency exacerbates insulin resistance, promotes inflammation, and disrupts metabolic homeostasis [28,29], underscoring its dual role in metabolism and immune function. Although IL-6 has been implicated in metabolic and immune regulation, its role as a direct modulator of circadian-metabolic alignment remains poorly understood, particularly in the context of metabolic stress.

Sex-specific differences in immune function, circadian regulation, and metabolism add another layer of complexity to IL-6's role [30–33]. Males and females exhibit distinct metabolic adaptations and differential responses to circadian and metabolic disruptions; however, the sex-specific role of IL-6 in these processes remains unclear. Given its dual role in circadian and metabolic homeostasis, we hypothesize that IL-6 acts as a key integrator of these rhythms in a sex-dependent manner, particularly under metabolic stress.

Here, we uncover a previously unrecognized role of IL-6 in integrating circadian and metabolic responses to dietary challenges, linking immune signaling to circadian homeostasis. Our findings show that IL-6 deficiency disrupts circadian and metabolic rhythms in a sex- and diet-specific manner, unveiling distinct adaptive strategies between males and females. Furthermore, we demonstrate that IL-6 modulates the FEO activity in a sex- and diet-specific manner. These findings establish IL-6 as a key regulator of sex-dependent resilience or susceptibility to circadian misalignment and metabolic stress, underscoring its potential as a therapeutic target for metabolic disorders associated with circadian dysfunction.

## 2. RESULTS

### 2.1. Sex-specific impact of IL6 deficiency on circadian locomotor activity in mice

As SCN robustness declines with age [34], we investigated the role of IL-6 in circadian rhythmicity in older mice (6–7 months), addressing gaps in previous studies that focused on younger animals (2 months) and reported no alterations in free-running period [35]. Given the strong expression of IL-6 receptor (IL6R) in the hypothalamus, including the SCN (Supplemental Fig. 1A), we monitored wheel-running behavior in IL6KO and wild-type (WT) control mice of both sexes to assess SCN circadian function [36,37]. Mice were first entrained to a 12:12 h light–dark (LD) cycle, followed by constant darkness (DD), and subsequently re-entrained to a new LD cycle (rLD) [26,32,38,39]. Under LD and rLD conditions, IL6KO and WT mice showed similar total activity and circadian period, regardless of sex (Figure 1A–C, Supplemental Fig. 1B and C). However, under DD, IL6KO mice of both sexes exhibited a lengthened circadian period, indicating disrupted intrinsic rhythmicity (Figure 1B,C). Additionally, female mutants showed significantly reduced locomotor activity (Figure 1A, Supplemental Fig. 1C). Notably, under DD, we observed greater inter-individual variability in circadian activity patterns, including among WT controls (Figure 1A, Supplemental Fig. 1B), likely reflecting the absence of external entrainment cues that normally constrain variability. This effect was more pronounced in females and may partly reflect fluctuations in endogenous sex hormones across the estrous cycle [40,41], which become more apparent under prolonged constant conditions.

Both sexes displayed reduced periodogram amplitude, consistent with weakened circadian rhythm strength (Figure 1B, Supplemental Fig. 1D). However, while males showed reduced amplitude under LD, females exhibited greater reductions during DD and rLD. Monitoring vasoactive intestinal peptide (VIP), a key neuropeptide for SCN synchrony [42], revealed significantly lower levels in IL6KO males (Figure 1D), correlating with weakened rhythm strength. In contrast, females showed no VIP reduction, consistent with their preserved rhythm strength under LD (Supplemental Fig. 1E).

In summary, IL-6 deficiency disrupts circadian locomotor activity and rhythm strength in a sex-specific manner: males are more affected under light-driven conditions, likely due to reduced VIP levels, while females show greater vulnerability under intrinsic rhythmic challenges such as DD and rLD cycles.

### 2.2. Sex-specific disruptions in metabolic cycles in IL6KO mice under standard diet (STD)

Next, we assessed the impact of IL-6 deficiency on spontaneous locomotor activity (LA), energy expenditure (EE), respiratory quotient (RQ), and food intake (FI) in IL6KO and WT mice of both sexes under STD. Consistent with previous reports [15,19], IL6KO mice showed no differences in body weight or composition (fat and lean mass) compared to controls between 12 and 18 weeks of age (Supplemental Fig. 2A). Although IL6KO mice showed normal wheel-running activity under LD (Figure 1A), both sexes exhibited significantly reduced spontaneous LA compared to controls (Figure 2A). EE was also significantly lower in IL6KO mice, with a stronger reduction observed in males (Figure 2B, Supplemental Fig. 2B). Cumulative FI was unchanged in males, whereas female IL6KO mice consumed less food, with a trend toward reduced intake during the light phase (Figure 2C, Supplemental Fig. 2C). Although RQ tended to decrease in IL6KO mice, the change did not reach statistical significance (Supplemental Fig. 2D). These findings suggest that, in females, reduced caloric intake may partially compensate for lower EE to maintain body weight. In contrast, IL6KO males might rely on alternative compensatory mechanisms, such as increased lipid utilization, as suggested by a trend toward lower nocturnal RQ ($p = 0.066$).

Cosinor analysis revealed reduced mesor and amplitude of LA, a lower mesor of EE, and an lengthened FI period in both male and female IL6KO mice (Figure 2D, Supplemental Fig. 2E). However, only male IL6KO mice exhibited a lengthened period and an advanced acrophase of EE (Figure 2D), indicating circadian misalignment between LA and EE. This sex-specific decoupling suggests greater temporal desynchronization in IL6KO males, whereas females maintain tighter alignment between behavioral and metabolic rhythms, potentially via compensatory mechanisms such as reduced caloric intake.

To explore possible endocrine contributors to these differences, we measured plasma levels of key sex steroids in both genotypes and sexes under STD conditions (Figure 2E). IL6KO females exhibited a selective and significant increase in progesterone, while levels of estrogens and androgens (e.g., estradiol, testosterone) remained unchanged. In contrast to previous reports of elevated intratesticular androgens in IL6KO males [43], we observed only non-significant trends toward reduced plasma testosterone and dihydrotestosterone (DHT) (Figure 2E), suggesting possible tissue-specific regulation or systemic compensation.

Altogether, these findings indicate that IL-6 deficiency alters circadian and metabolic rhythms in a sex-specific manner. IL6KO females show coordinated reductions in EE and FI, along with increased circulating progesterone, which may contribute to the preservation of circadian-

 

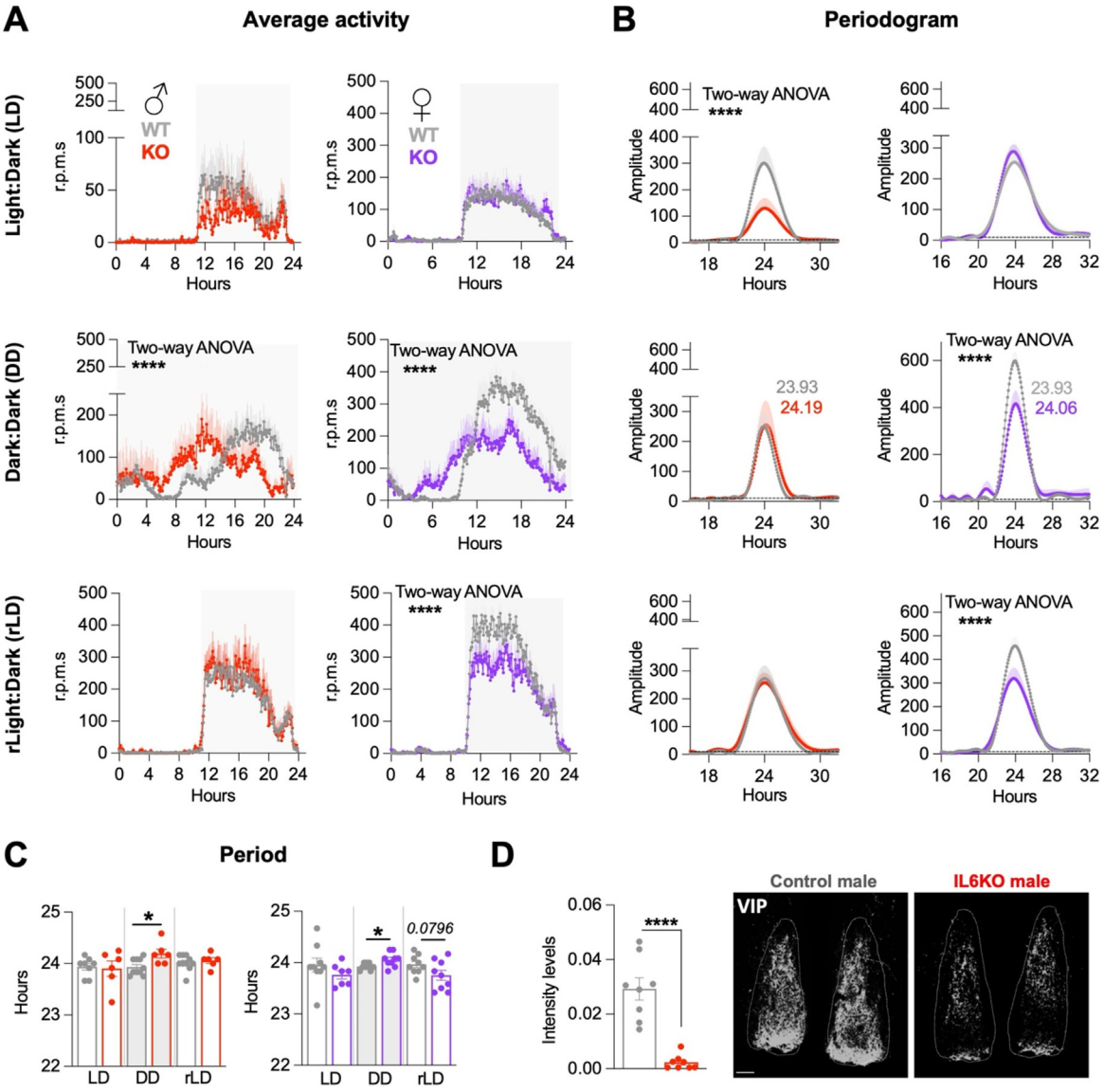


**Figure 1: Sex-specific impact of IL6 deficiency on circadian locomotor activity in mice. (A)** Activity waveforms under the LD, DD, and rLD for control and IL6KO males (left) and females (right). LD and rLD data use nighttime hours 8 to 20, presented in ZT. DD employs circadian time, with mean activity across each animal's circadian cycle. **(B)** Lomb-Scargle periodograms and **(C)** periodicity of control and IL6KO males (left) and females (right) in all lighting conditions. Data are presented as mean ± SEM; $n = 6–10$; two-way ANOVA or two-tailed, unpaired t-test. **(D)** VIP fluorescence intensity in the SCN of control and IL6KO males at ZT2 (mean ± SEM; $n = 8$; two-tailed, unpaired t-test). Representative SCN micrographs of VIP staining at ZT2 are shown. Scale bar, 50 μm $*p < 0.05$; $****p < 0.0001$.

metabolic alignment. In contrast, IL6KO males display greater rhythm disruption, accompanied by non-significant but consistent trends toward reduced circulating androgens.

### 2.3. Tissue-specific circadian and IL-6 signaling responses to fasting

Fasting imposes a strong metabolic challenge that requires both systemic and tissue-specific adaptations. To investigate IL-6's role in coordinating these responses, we analyzed clock proteins and IL-6 signaling components in muscle, liver, and hypothalamus under *ad libitum* (AL) feeding and 24-hour fasting conditions. All analyses were conducted in male mice, in line with prior studies examining IL-6 function during fasting [44–47], and to minimize biological variability in this initial mechanistic assessment. The tissues analyzed were chosen for their critical roles in energy balance, circadian regulation, and IL-6 signaling [48–51]: muscle (energy metabolism), liver (glucose and lipid homeostasis), and hypothalamus (systemic energy balance and circadian control).

In muscle (Figure 3A, C, Supplemental Fig. 3A), fasting induced IL-6 oscillations peaking at ZT14, concurrent with the loss of rhythmicity in IL6R, pSTAT3, and STAT3, suggesting reduced IL-6 signaling sensitivity. BMAL1 and CRY1 remained rhythmic but were delayed by ~7 and ~10 h, respectively, peaking at the day-to-night transition. CRY1 exhibited increased amplitude, while PER2 advanced by ~10 h but with reduced amplitude, reflecting circadian adjustments to meet fasting-induced metabolic demands.

In the hypothalamus (Figure 3B, C, Supplemental Fig. 3A), BMAL1 and STAT3 advanced by ~7 and ~6 h, respectively, while PER2 shifted ~3 h forward with increased amplitude. In contrast, CRY1, IL6R, and pSTAT3 lost rhythmicity. Despite these alterations, the sustained

 

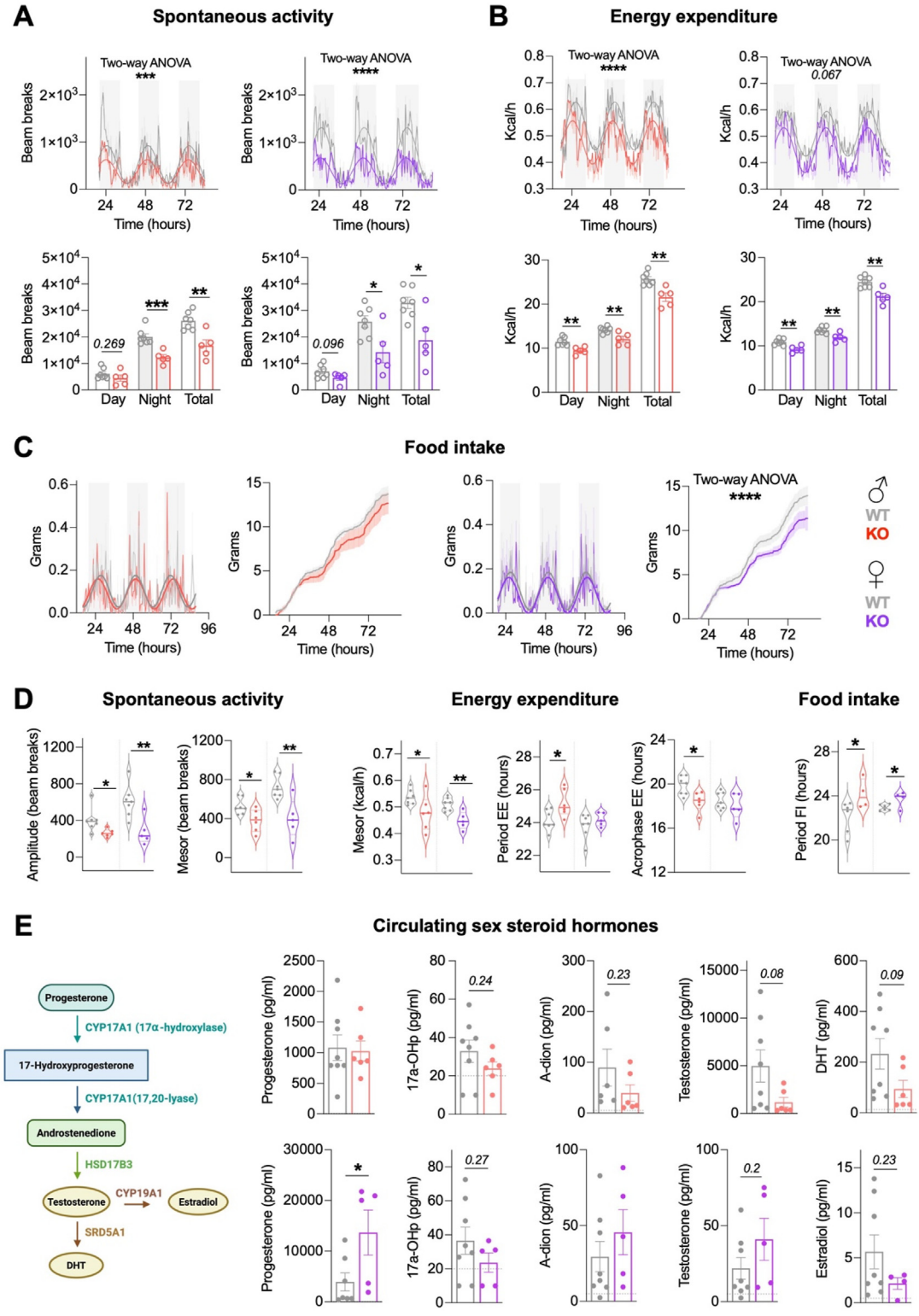


**Figure 2: Sex-specific disruptions in metabolic cycles in IL6KO mice.** **(A)** Spontaneous locomotor activity (LA) and **(B)** energy expenditure (EE) in control and IL6KO males (left pannels) and females (right pannels). Upper plots show hourly time courses; lower plots display day, night, and total averages. **(C)** Rhytmyc (left pannels) and cumulative (righr pannels) food intake (FI) in control and IL6KO mice. **(D)** Cosinor analysis of circadian parameters (amplitude, mesor, period, and acrophase) for spontaneous LA, EE, and FI in both sexes. **(E)** Circulating levels of sex steroid hormones under STD conditions. Top row: male mice; bottom row: female mice. Pathway schematic (left) shows key enzymes in sex steroid biosynthesis. Hormonal profiles include progesterone, 17$\alpha$-hydroxyprogesterone (17$\alpha$-OHP), androstenedione (A-dion), testosterone, dihydrotestosterone (DHT; males only), and estradiol (females only). Mean $\pm$ SEM mean $\pm$ SEM; $n = 4$—8; two-tailed, unpaired t-test and two-way ANOVA. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

 

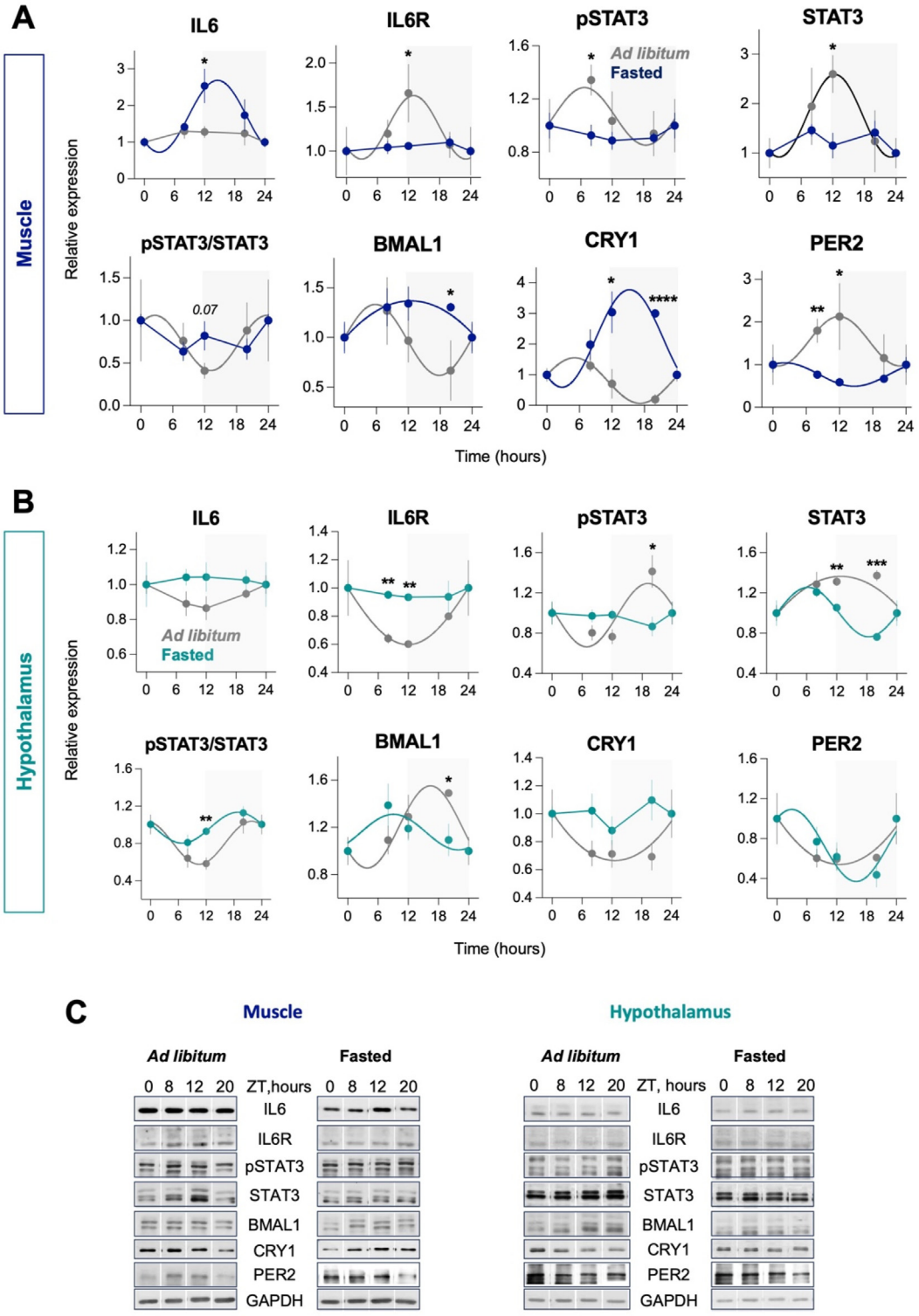


**Figure 3: Tissue-specific circadian and IL-6 signaling responses to fasting.** Expression levels of IL-6, IL6R, pSTAT3, STAT3, pSTAT3/STAT3 ratio, BMAL1, CRY1 and PER2 in muscle **(A)** and hypothalamus **(B)** across different times of the day in male mice under *ad libitum* feeding or following 24-hour fasting. Data are presented as mean ± SEM and were fitted using cosine analysis ($n = 3–4$). **(C)** Representative western blots corresponding to the data shown in panels **(A)** and **(B).** $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

 

oscillations of BMAL1, STAT3, and PER2 underscore the hypothalamus's resilience and its role in preserving systemic circadian coherence.

In the liver (Supplemental Fig. 3A and 3B), fasting induced IL-6 oscillations peaking at ZT6, which were absent under AL conditions. IL6R and pSTAT3 maintained rhythmicity but were delayed by ~5 h. BMAL1 and CRY1 advanced by ~4 and ~9 h, respectively, with enhanced CRY1 amplitude. PER2 showed a ~6-hour delay, highlighting hepatic circadian reorganization. The IL-6 peak likely reflects its role in promoting gluconeogenesis and enhancing hepatic insulin sensitivity [51], underscoring the integration of circadian and metabolic signals during fasting.

These findings highlight tissue-specific circadian adaptations to fasting, where phase shifts and amplitude changes in clock proteins in muscle, liver, and hypothalamus align with the body's metabolic demands. The increased amplitude of CRY1 in the liver and muscle, alongside the maintained PER2 oscillations in the hypothalamus, suggests that these tissues play critical roles in maintaining circadian coherence and prioritizing energy balance during fasting-induced stress. Our results suggest a hierarchical response to fasting, where the hypothalamus stabilizes systemic circadian rhythms, while peripheral tissues adjust their circadian and metabolic machinery to meet energy demands. IL-6 may contribute to this coordination by supporting hypothalamic stability and promoting metabolic flexibility in peripheral tissues, which is crucial for proper circadian and metabolic homeostasis during fasting.

### 2.4. IL-6 deficiency drive sex-dependent behavioral and metabolic responses to time-restricted feeding (TRF)

TRF is a potent zeitgeber that aligns circadian rhythms with feeding schedules, even in the absence of light cues [52]. Given IL-6's roles in energy homeostasis and circadian regulation, we investigated whether IL-6 deficiency alters food anticipatory activity (FAA) and metabolic adaptation to TRF. Male and female mutants were entrained to an LD cycle with AL feeding before transitioning to TRF. Food access was restricted to a 6-hour window (ZT4—ZT10, RF6h) for six days, followed by a 4-hour window (ZT4—ZT8, RF4h) for four days [26,32] (Figure 4A). Both IL6KO and WT mice exhibited FAA starting 1—4 h before food availability, but IL6KO mice showed significantly enhanced FAA. Males displayed pronounced FAA increases during both RF6h and RF4h, whereas females exhibited elevations mainly during RF6h (Figure 4B—D). Nocturnal activity decreased in both sexes during TRF, aligning with FAA acquisition (Supplemental Fig. 4A). These findings suggest that IL-6 suppresses food-entrainable oscillator activity in a sex-dependent manner. Total activity, cumulative food intake, and body weight remained comparable between IL6KO and WT mice (Supplemental Fig. 4A and 4B).

TRF induced a diurnal shift in metabolic patterns in both IL6KO and WT mice, aligning with their feeding schedules [53] (Figure 4E, F; Supplemental Fig. 4C). However, IL6KO mice exhibited sex-specific differences in metabolic adaptation. Males showed attenuated nocturnal EE reductions during TRF compared to AL conditions, whereas females maintained consistently lower EE throughout the day and night, mirroring their AL patterns (Figures 2B and 4E). ANCOVA revealed disrupted EE-body weight scaling in IL6KO females, a pattern not observed in WT controls (Supplemental Fig. 4C). RQ analysis further revealed sex-specific differences: males showed a trend toward increased nighttime RQ, whereas females exhibited a significant daytime increase, suggesting enhanced carbohydrate utilization under TRF (Figure 4F). Cosinor analysis underscored these sex-specific differences in metabolic rhythms during TRF (Supplemental Fig. 4D). Male IL6KO mice exhibited increased EE amplitude but reduced mesor, suggesting more pronounced but overall lower metabolic rhythms compared to controls. Female IL6KO mice showed decreased EE mesor alongside significantly increased RQ amplitude, with a trend toward higher RQ mesor ($p = 0.08$), suggesting a shift in substrate utilization rhythms toward carbohydrate metabolism.

These findings reveal sex-specific metabolic adaptations to TRF in IL6KO mice. Males compensated for reduced metabolic efficiency by enhancing FAA and restricting EE reductions to the nocturnal phase. In contrast, females prioritized carbohydrate metabolism and maintained stable EE reductions throughout the day, achieving tighter alignment between substrate utilization and EE rhythms to sustain energy balance under TRF.

### 2.5. Dynamic IL-6 signaling in circadian and metabolic adaptations to TRF

To investigate how IL-6 signaling contributes to circadian and metabolic adaptations, we analyzed RNA sequencing data from mice under nocturnal (*ad libitum*, AL) and diurnal feeding schedules [53], focusing on key IL-6 signaling components including *Il6*, *Il6r*, Janus kinase 1 and 2 (*Jak1* and *Jak2*), Tyrosine kinase 2 (*Tyk2*), Suppressor of cytokine signaling 1 and 3 (*Socs1* and *Socs3*), and Signal transducer and activator of transcription 3 (*Stat3*). We focused on STAT3 as the primary JAK/STAT effector due to its central role in IL-6—dependent transcription. Although IL-6 can also signal via the SHP2-mediated MAPK/ERK cascade, we prioritized STAT3 for its well-established function and robust expression across central and metabolic tissues [51,54—56]. This approach enabled us to assess how feeding schedules modulate IL-6 sensitivity and downstream signaling across different tissues (Figure 5A). Under AL feeding, *Il6st* and *Jak2* peaked at the light-to-dark transition, aligning with increased energy demands, while *Jak1*, *Socs3,* and *Stat1* remained stable, acting as cytokine modulators. Diurnal feeding dampened the amplitude of oscillatory transcripts and shifted their phases. Notably, *Il6r* remained rhythmic in muscle (QUA) and acquired oscillations in the ventromedial hypothalamus (VMH) and brown adipose tissue (BAT), indicating enhanced IL-6 sensitivity. *Stat3*, which was oscillatory in the dorsomedial hypothalamus (DMH) and cerebellum (CER) under nocturnal feeding, lost rhythmicity with diurnal feeding. Meanwhile, *Socs1* began oscillating in the SCN, peaking at day onset, suggesting finely tuned IL-6 signaling for circadian-metabolic coordination under TRF. In contrast, none of the IL-6 signaling transcripts showed rhythmic expression in the arcuate nucleus (ARC) under either feeding condition, suggesting a limited role for this region in IL-6—mediated circadian regulation.

Muscle Il6r expression consistently peaked at the day—night transition under both feeding paradigms, indicating a prioritization of circadian cues over feeding demands (Figure 5A). To assess feeding-induced changes in IL-6 signaling and clock protein dynamics, muscle samples were collected at ZT4 (20-hour fasting) and ZT6 (2-hour post-feeding) from male and female IL6KO and WT mice subjected to TRF (Figure 5B). In males, muscle IL-6 showed a modest post-feeding reduction ($p = 0.06$), accompanied by significant IL6R decreases (Figure 5B; Supplemental Fig. 5). Despite this, pSTAT3 and STAT3 levels remained stable (Supplemental Fig. 5), indicating a lack of JAK-STAT activation. IL6KO males showed no significant changes in IL6R, confirming the absence of IL-6-dependent regulation (Supplemental Fig. 5). In females, muscle IL-6 levels significantly decreased post-feeding, with a greater reduction than in males, accompanied by significant IL6R declines (Figure 5B). Interestingly, pSTAT3 increased post-feeding, suggesting compensatory activation through metabolic signals (e.g., insulin or free fatty acids) independent of IL-6 [57]. In

 

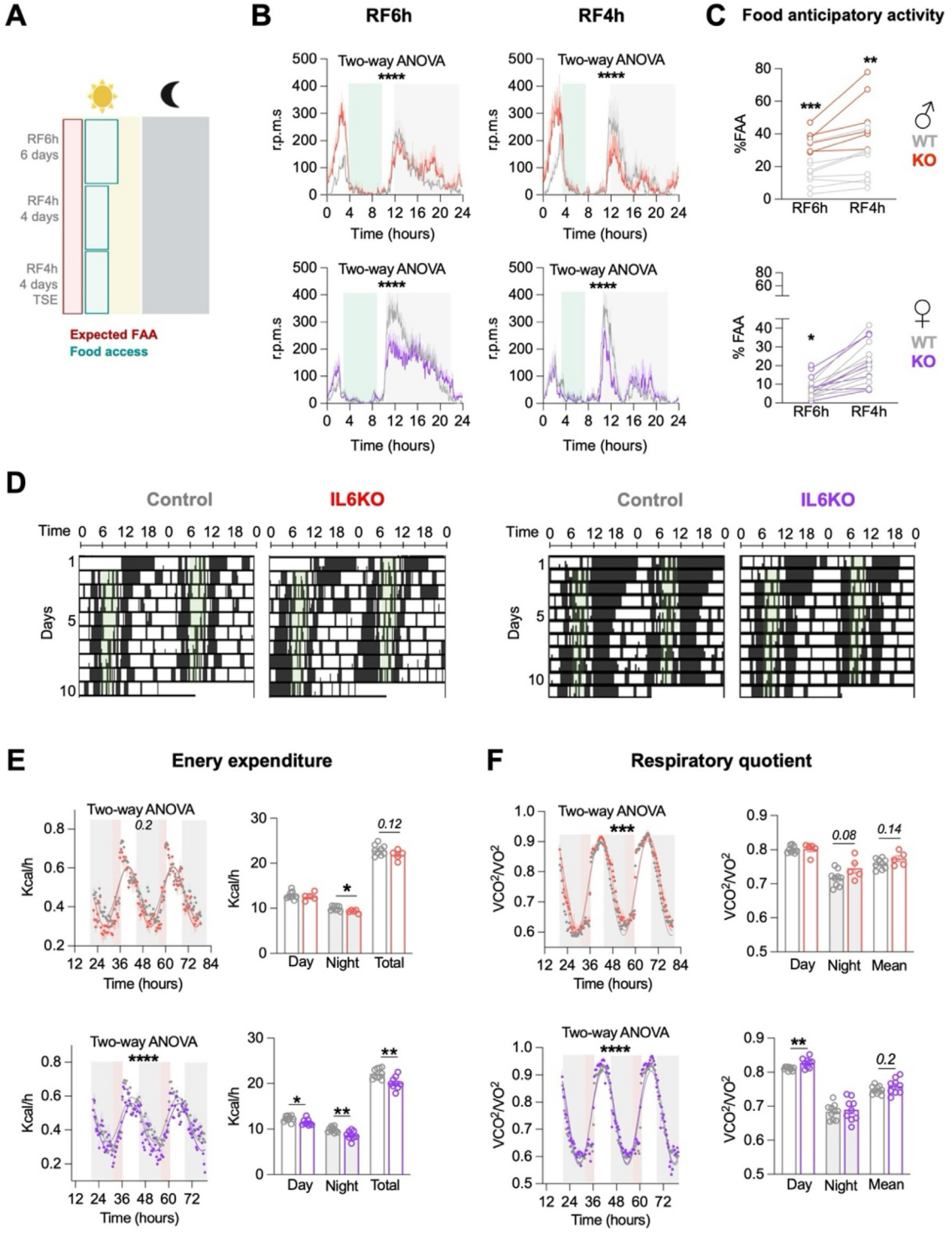


**Figure 4: IL-6 deficiency drive sex-dependent behavioral and metabolic responses to TRF. (A)** Schematic of the TRF paradigm. **(B)** Average activity waveforms of control and IL6KO males (upper) and females (lower) during RF6h (ZT 4—10) and RF4h (ZT 4—8). Green areas indicate feeding time. **(C)** FAA percentage in control and IL6KO males (upper) and females (lower). **(D)** Representative actograms in control and IL6KO males (left) and females (right). Green areas indicate feeding time. **(E)** EE and **(F)** RQ in control and IL6KO males and females during RF4h. Left panels show hourly plots, and right panels display total values. Gray areas indicate the dark phase (nighttime), and red shading marks the FAA window. Data are presented as mean ± SEM; $n = 5—9$; two-way ANOVA or two-tailed t-tests. $^{*}p < 0.05$, $^{**}p < 0.01$ $^{***}p < 0.001$, $^{****}p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

 

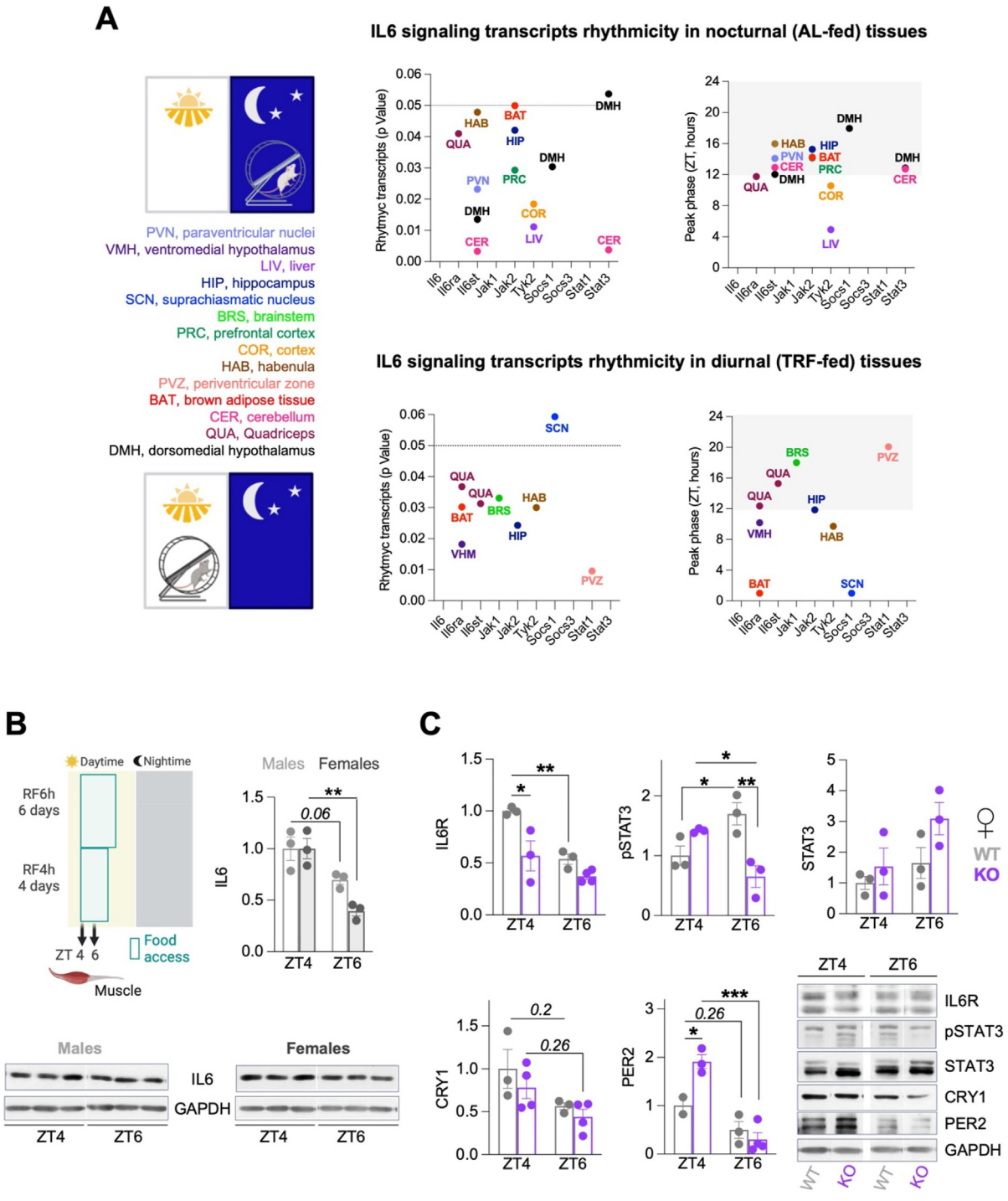


**Figure 5: Dynamic IL-6 signaling in circadian and metabolic adaptations to TRF. (A)** Circadian expression of IL-6 signaling components in nocturnal (AL-fed) and diurnal (TRF-fed) animals across tissues. Left panels show the p-value of circadian expression; right panels display the phase of rhythmic components. **(B)** Schematic of the TRF paradigm and muscle sample collection at ZT4 (20 h of fasting) and ZT6 (2 h of feeding). IL-6 expression in control animals is shown for both time points. **(C)** Muscle expression of IL6R, pSTAT3, STAT3, CRY1, and PER2 in control and IL6KO females during the TRF paradigm at ZT4 and ZT6. Data are presented as mean ± SEM; $n = 3–4$ per group. Two-way ANOVA. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

IL6KO females, IL6R levels were significantly lower at ZT4, with markedly reduced pSTAT3 levels post-feeding (Figure 5C), demonstrating disrupted JAK-STAT activation in the absence of IL-6.

Clock proteins showed sex-specific dynamics under TRF. In WT males, CRY1 and PER2 levels remained stable between ZT4 and ZT6 (Supplemental Fig. 5), indicating that IL6R modulation does not directly influence circadian components in muscle. In contrast, WT females exhibited stable CRY1 levels, but PER2, elevated at ZT4, normalized post-feeding in IL6KO females (Figure 5C), suggesting compensatory mechanisms for circadian-metabolic coherence in the absence of IL-6.

 

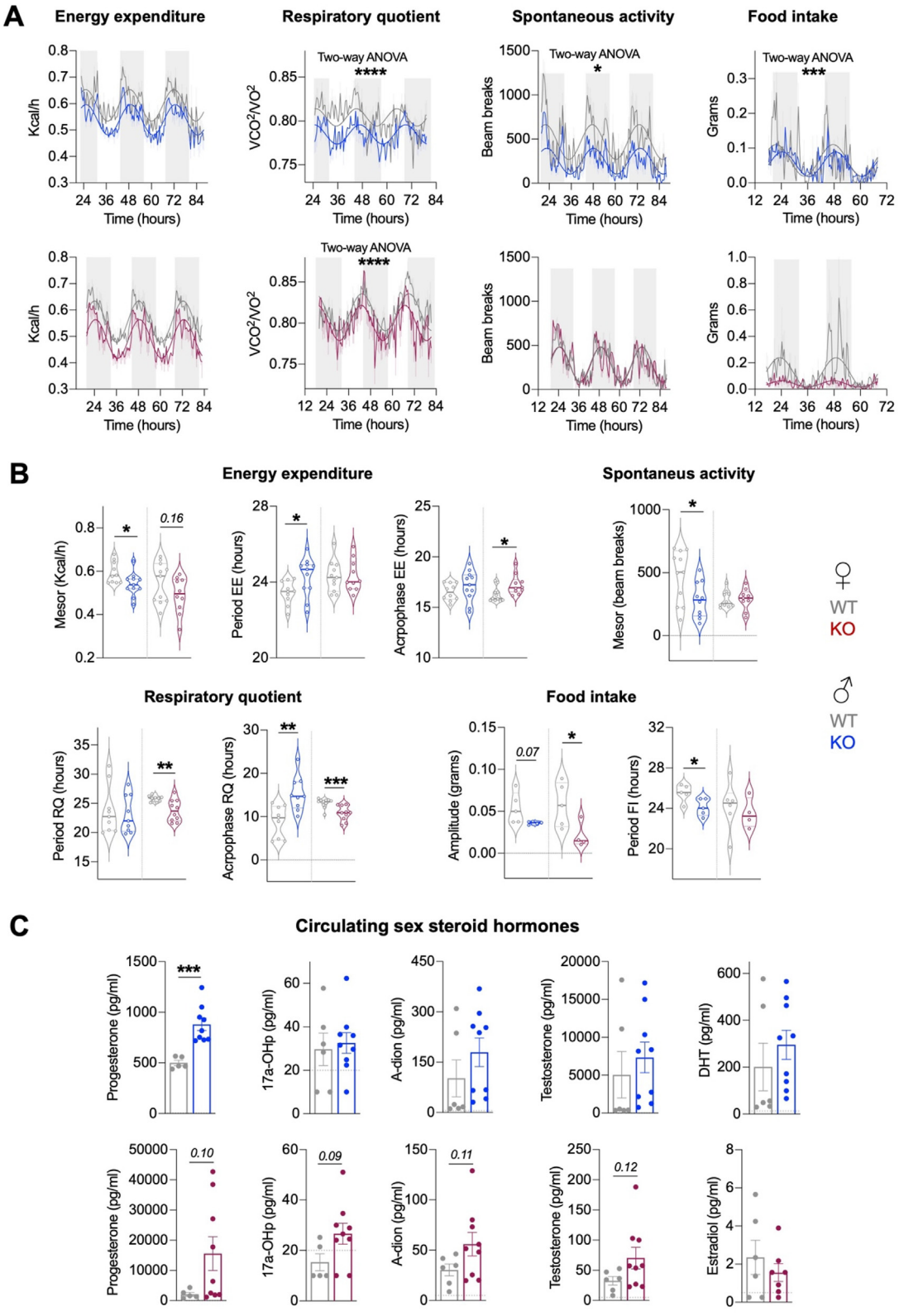


**Figure 6: Sex-specific disruptions in metabolic cycles of IL6KO mice on a HFD. (A)** Hourly profiles of energy expenditure (EE), respiratory quotient (RQ), spontaneous locomotor activity (LA), and food intake (FI) in male (top, blue) and female (bottom, red) IL6KO and control mice under HFD. **(B)** Cosinor analysis of mesor, period, amplitude, and acrophase of EE, RQ, LA, and FI. **(C)** Circulating sex steroid hormone levels under HFD conditions. Males (top row, blue), females (bottom row, red). Measured hormones include progesterone, 17$\alpha$-hydroxyprogesterone (17$\alpha$-OHP), androstenedione (A-dion), testosterone, dihydrotestosterone (DHT; males only), and estradiol (females only). Data are presented as mean $\pm$ SEM; $n = 5$–11; two-way ANOVA and two-tailed t-tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

 

These findings highlight IL-6's role in sex-specific circadian and metabolic adaptations during TRF. WT females exhibit compensatory pSTAT3 activation post-feeding, a mechanism absent in IL6KO females, suggesting that IL-6 is essential for maintaining metabolic flexibility. The distinct responses in males and females reveal an intricate interplay between IL-6 signaling and feeding schedules, shaping circadian and metabolic outcomes.

### 2.6. Sex-specific disruptions in metabolic cycles of IL6KO mice on a high-fat diet (HFD)

Nutritional challenges like a HFD disrupt metabolic and circadian regulation in a sex-specific manner [24—26] and are associated with increased circulating IL-6 levels [27]. To investigate how IL-6 influences the interplay between metabolic and circadian processes under HFD, we analyzed metabolic cycles in male and female IL6KO and WT mice fed a 60% HFD for 12 weeks. Both male and female IL6KO mice exhibited significantly reduced body weight and food intake compared to WT controls under HFD conditions, consistent with previous reports [28,29] (Supplemental Fig. 6A and 6B).

Male mutants also showed reduced EE and LA during both day and night, along with lower daytime RQ, suggesting a metabolic shift toward increased fat oxidation as the primary energy source (Figure 6A; Supplemental Fig. 6C). Cosinor analysis revealed circadian disruptions in males, including a prolonged EE period, reduced mesor, decreased LA mesor, delayed RQ acrophase, and shortened FI period, indicating misalignement between energy balance, feeding behavior and substrate utilization relative to the LD cycle (Figure 6B; Supplemental Fig. 6D). In contrast, IL6KO females exhibited relatively stable LA, with only a trend toward reduced EE and RQ (Figure 6A; Supplemental Fig. 6C). Circadian analysis revealed milder disruptions in females, including a delayed acrophase of EE, reduced amplitude of FI, and a shortened period and advanced acrophase of RQ, while other circadian parameters, such as LA and overall EE, remained largely preserved (Figure 6B, Supplemental Fig. 6D). These findings suggest that females engage adaptive mechanisms to buffer the impact of IL-6 deficiency under HFD.

Determination of circulating sex steroid levels in both sexes under HFD (Figure 6C) revealed a significant increase in progesterone in IL6KO males, with no significant changes in androgens or estrogens. This may reflect a compensatory endocrine response to disrupted IL-6 signaling. In contrast, IL6KO females showed non-significant trends toward higher levels of several steroids, including progesterone, 17$\alpha$-hydroxyprogesterone, androstenedione, and testosterone.

Overall, these results highlight distinct and sex-specific metabolic responses to HFD-induced stress in IL6KO mice. While males exhibited marked impairments in metabolic rhythms, females maintained greater temporal stability, with circadian disruptions mainly affecting substrate utilization patterns. These findings underscore the essential role of IL-6 in promoting circadian and metabolic resilience under dietary challenge.

### 2.7. Sex-specific circadian behavioral responses to HFD in IL6KO mice

Given the well-established link between HFD and circadian disruptions [24—26], we investigated whether IL-6 deficiency alters the ability to maintain circadian rhythmicity under dietary stress. Male IL6KO mice maintained stable circadian behavior under HFD, showing no significant disruptions under any lighting condition compared to controls (Figure 7A—C, Supplemental Fig. 7A—7C). This suggests that HFD-induced stress may activate compensatory mechanisms or alter circadian regulatory pathways. Conversely, female IL6KO mice exhibited reduced periodogram amplitude across all lighting conditions, indicating weakened rhythm strength (Figure 7B, C). The increase in activity during rLD (Figure 7A, Supplemental Fig. 7B) suggests an attempt to sustain rhythmicity. However, overall rhythm strength remained significantly reduced, highlighting an increased vulnerability to circadian misalignment under dietary stress. To investigate potential mechanisms underlying circadian responses to HFD, we analyzed VIP levels. In males, VIP levels remained unchanged compared to WT controls (Figure 7D). In females, VIP levels were significantly elevated (Figure 7D), suggesting a compensatory attempt to stabilize circadian rhythms. However, this increase was not sufficient to fully restore rhythm strength, as circadian coherence remained compromised.

These findings reveal sex-specific strategies for coping with HFD-induced circadian stress. While males maintain circadian stability despite metabolic challenges, females exhibit weakened rhythms despite elevated VIP levels, failing to fully compensate for IL-6 deficiency. This underscores distinct regulatory mechanisms, with females being more vulnerable to circadian misalignment under dietary stress.

### 2.8. Sex-specific behavioral and metabolic adaptations to TRF on a HFD in IL6KO mice

HFD is known to impair the development of robust FAA [58]. Given the enhanced FAA observed in IL6KO mice under STD conditions (Figure 4B, C), we examined whether IL-6 deficiency differentially affects FAA and metabolic adaptations under HFD when subjected to TRF (Figure 4A). Male IL6KO mice exhibited reduced FAA (Figure 8A, B), while increased nocturnal activity, especially during RF4h, diminished the relative contribution of FAA to total activity (Figure 8B, Supplemental Fig. 8A and 8B). Notably, body weight and cumulative food intake remained comparable to controls (Figure 8C), suggesting that IL-6 deficiency does not protect against HFD-induced obesity under TRF in males. Conversely, IL6KO females showed enhanced FAA during RF6h (Figure 8A, B), consistent with their response under STD conditions (Figure 4B,C). This was accompanied by a reduced percentage of nocturnal activity during RF6h, while total activity remained unchanged compared to control animals (Supplemental Fig. 8A and 8B). WT females showed a trend toward weight loss during TRF, whereas IL6KO females maintained stable body weight despite increased food intake (Figure 8C).

Metabolic responses to HFD and RF4h were further assessed through EE and RQ analyses. Both male and female IL6KO mice showed trends toward reduced EE compared to WT controls, though differences were not statistically significant (Figure 8D, Supplemental Fig. 8C). Cosinor analysis revealed trends toward a reduced RQ mesor and delayed acrophase in IL6KO males, suggesting preserved metabolic adjustments despite elevated activity levels. In contrast, female IL6KO mice exhibited a significant delay in RQ acrophase (Supplemental Fig. 8D), reflecting a greater reliance on substrate utilization adaptations to sustain energy balance under HFD.

These findings reveal that IL-6 deficiency drives sex-specific adaptations to TRF under HFD. IL6KO males maintained energy balance despite altered activity patterns and reduced FAA, likely through preserved metabolic adjustments. In contrast, IL6KO females relied on increased food intake and metabolic flexibility, as reflected in altered RQ rhythms and preserved EE, to buffer against HFD-induced stress and prevent weight gain. These results underscore IL-6's critical role in orchestrating sex-dependent resilience to circadian and metabolic challenges.

 

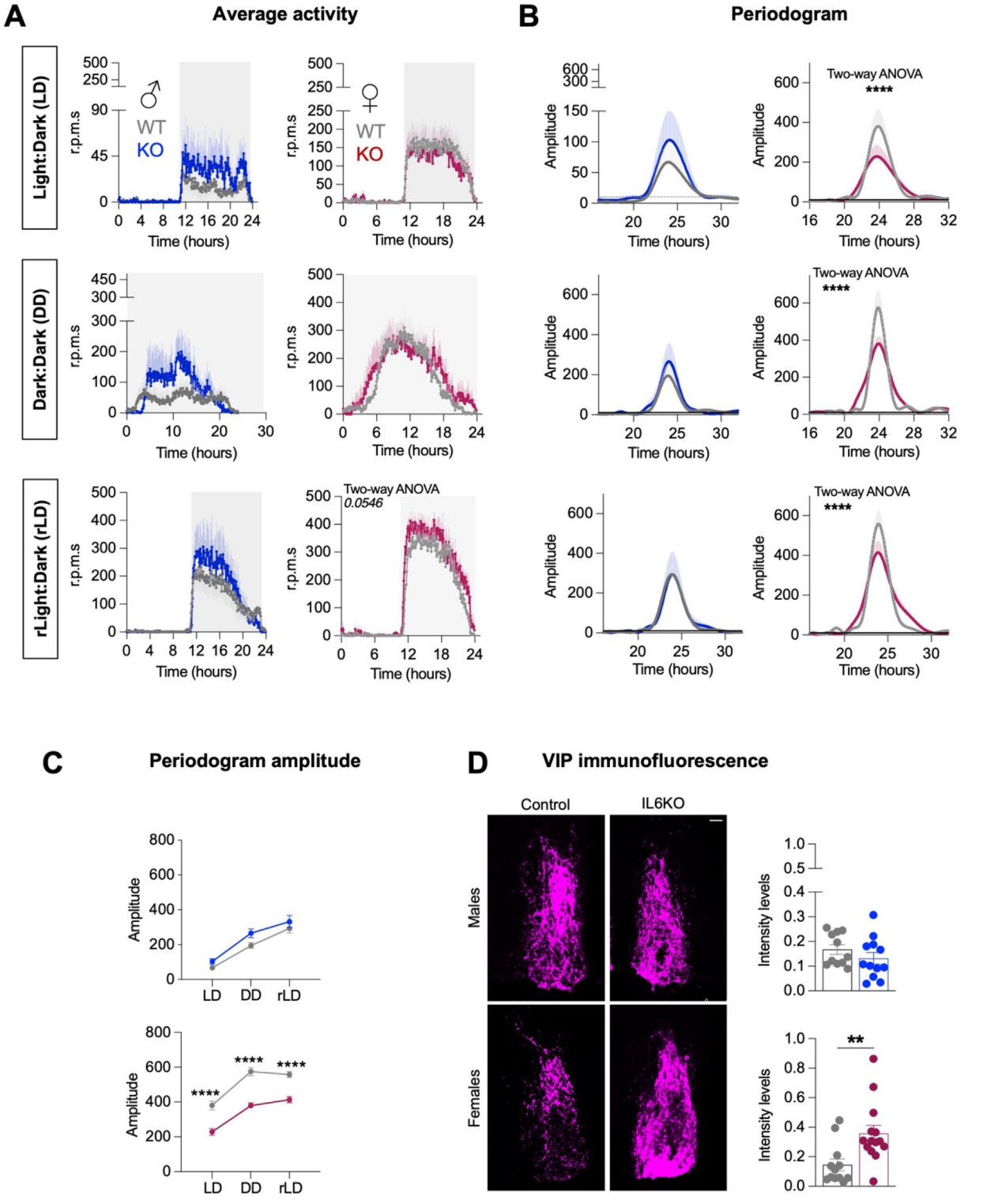


**Figure 7: Sex-specific circadian behavioral responses to HFD in IL6KO mice. (A)** Activity waveforms under LD, DD, and rLD in control and IL6KO males (left) and females (right) after 12 weeks on a HFD. **(B)** Lomb-Scargle periodograms and **(C)** periodogram amplitude of control and IL6KO males and females in all lighting conditions. **(D)** VIP fluorescence intensity in the SCN of control and IL6KO males and females at ZT2, with representative SCN micrographs (scale bar: 50 μm). Data are presented as mean ± SEM; $n = 7–14$; two-way ANOVA and two-tailed t-tests. $^{**}p < 0.01$, $^{****}p < 0.0001$.

 

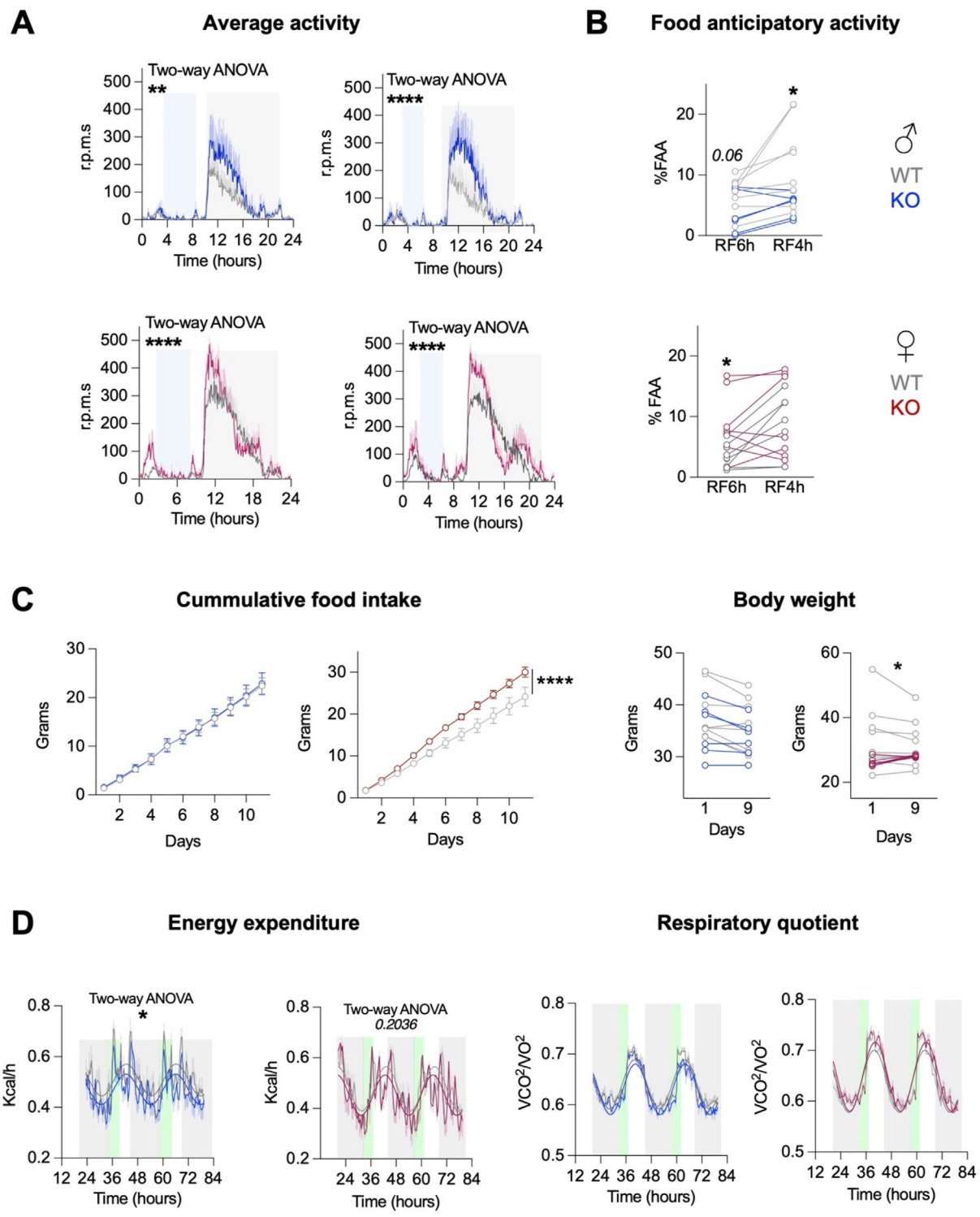


**Figure 8: Sex-specific behavioral and metabolic adaptations to TRF on a HFD in IL6KO mice. (A)** Average activity waveforms of control and IL6KO males (upper) and females (lower) during RF6h (ZT 4—10) and RF4h (ZT 4—8). Blue areas indicate feeding time. **(B)** FAA percentage in control and IL6KO males (upper) and females (lower). **(C)** Cumulative food intake and body weight in control and IL6KO males (left) and females (right) during TRF. **(D)** Energy expenditure and respiratory quotient hourly plots in control and IL6KO males and females during RF4h. Green areas indicate the time periods of FAA. Data are presented as mean ± SEM; $n = 6$—8; two-way ANOVA. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

 

## 3. DISCUSSION

Sex differences in circadian rhythms regulate energy homeostasis, feeding behavior, and sleep—wake cycles, playing a crucial role in metabolic regulation [26,31,32,38—41,59—61]. Here, we establish IL-6 as a key modulator of these sex-specific circadian adaptations, whose deficiency triggers either compensatory or maladaptive responses depending on physiological and nutritional conditions. These findings reveal critical mechanisms underlying resilience and susceptibility to circadian and metabolic disruptions.

Under STD conditions, IL6KO males exhibited reduced circadian rhythm amplitude during LD cycles and a lengthened free-running period, indicative of weakened light-driven entrainment and intrinsic circadian control. These disruptions were associated with reduced VIP levels, a neuropeptide essential for SCN synchronization and rhythm robustness [42]. Beyond its role in circadian regulation, VIP also plays a role in feeding behavior and metabolic cycles [62—66]. Thus, in the absence of IL-6, VIP deficiency may contribute to circadian rhythm instability, potentially disrupting the relationship between LA, FI and EE. This desynchronization, compounded by the generally restrictive influence of testosterone on circadian flexibility [60,67—69], may further impair the temporal integration of behavioral and metabolic rhythms. Previous studies on the effects of IL-6 on androgen levels have reported conflicting results. While elevated intratesticular androgens have been observed in IL6KO mice [43], IL-6 is also known to negatively regulate testicular function by disrupting the blood—testis barrier [70—72], impairing Sertoli and Leydig cell activity [71], and suppressing testosterone production in humans [73]. In our study, IL6KO males showed only a slight, non-significant reduction in circulating androgen levels suggesting possible tissue-specific regulation or systemic compensation. One possibility is that IL-6 actions on reproductive function follow a U-shaped dose—response curve, whereby both deficiency and excess impair steroidogenesis, potentially explaining the paradox of reduced circulating androgens despite IL-6's known inhibitory role. Nonetheless, our findings suggest that androgen modulation is unlikely to be the primary driver of the circadian and metabolic disruptions observed in IL6KO males. Rather, the dual loss of IL-6 and VIP signaling appears to play a more central role in rhythm desynchronization.

In contrast, IL6KO females maintained robust circadian rhythms under LD conditions, likely due to estrogen-driven SCN adaptability and overall greater circadian flexibility [61,74]. Females typically exhibit shorter circadian periods and higher-amplitude clock gene oscillations, which confer increased resistance to circadian disruption [26,38,40,41,59,61], suggesting a sex-dependent advantage in maintaining rhythm stability under physiological stress. However, under free-running conditions, this resilience was compromised, as indicated by a reduced periodogram amplitude, an increased circadian period, and decreased locomotor activity, reflecting vulnerability to desynchronization in the absence of external cues.

In addition, IL6KO females displayed altered feeding rhythms under LD cycles, including a lengthened circadian period, reduced total food intake, and a trend toward a lower mesor. These changes were accompanied by decreased EE and a trend toward reduced RQ, suggesting a compensatory, energy-conserving strategy that helps preserve temporal alignment between behavior and metabolism. The hormonal profile of IL6KO females, marked by elevated circulating progesterone under STD conditions, resembles the proestrus phase of the estrous cycle [75], which is typically associated with reduced locomotor activity, a longer circadian period, lower food intake, and reduced RQ, consistent with enhanced lipid oxidation [40,41,76,77]. Notably, IL-6 has been shown to suppress estrogen synthesis in the ovary by downregulating aromatase, the enzyme that converts androgens into estrogens [78]. However, in our study, estradiol levels were not significantly altered in IL6KO females, suggesting that IL-6 deficiency may affect other aspects of steroidogenic output, such as progesterone production. In summary, IL-6 deficiency triggers sex-specific adaptations: in females, elevated progesterone supports a partially adaptive, energy-conserving phenotype, while in males, the combined loss of IL-6 and VIP signaling leads to maladaptive circadian-metabolic desynchronization.

Building on prior evidence that HFD disrupts circadian and metabolic regulation in a sex-specific manner [24—26], our findings highlight a critical role for IL-6 in maintaining resilience under dietary stress. IL-6 deficiency in males leads to profound metabolic disruption, including reduced EE, LA, and RQ, accompanied by a delayed RQ acrophase, an increased EE period, a reduced mesor, and a lengthened circadian period of FI. These alterations indicate circadian-metabolic misalignment and impaired substrate utilization. Although IL6KO males maintain behavioral circadian stability under HFD, a likely compensatory response, this does not restore metabolic flexibility, leaving them highly susceptible to HFD-induced dysfunction. In contrast, IL6KO females preserve metabolic flexibility but exhibit increased circadian vulnerability. While HFD typically reduces VIP levels in females [79], IL6KO females maintain elevated VIP, likely as a compensatory mechanism to stabilize SCN rhythmicity. However, VIP elevation is insufficient to restore full circadian coherence, as evidenced by reduced periodogram amplitude. Instead, females appear to prioritize metabolic homeostasis, as indicated by their shorter RQ period, advanced acrophase, and reduced FI amplitude, adjustments consistent with enhanced substrate utilization and energy conservation.

Taken together, these findings suggest that IL-6 modulates the relationship between VIP signaling and circadian stability in a sex- and context-dependent manner. Under STD, reduced VIP in IL6KO males is accompanied by rhythm weakening, while females maintain both VIP levels and robust rhythmicity. Under HFD, VIP normalizes in males alongside preserved rhythms, suggesting compensation. In contrast, IL6KO females show elevated VIP but reduced rhythm amplitude, indicating a decoupling between VIP levels and SCN output. This divergence suggests that under metabolic stress, IL-6—deficient females reallocate physiological regulation toward maintaining metabolic balance, at the cost of circadian coherence.

This raises the question: why does HFD primarily disrupt metabolism in males but circadian rhythms in females in the absence of IL-6? One possibility is that estrogen-driven metabolic flexibility [80,81] allows females to maintain energy balance despite circadian instability, reducing their reliance on IL-6 under HFD conditions. Supporting this, astrocytic BMAL1 knockout models show that females maintain both circadian and metabolic resilience under STD conditions, whereas males exhibit impairments in both systems [26,38,39]. However, when challenged with HFD, female mutants develop a male-like phenotype, marked by weight gain, altered fat distribution, and impaired metabolic flexibility [26], suggesting that HFD overrides sex-specific metabolic advantages in the absence of IL-6. In males, behavioral circadian rhythms appear more stable than metabolic rhythms, potentially reflecting a system more reliant on VIP and testosterone-mediated circadian synchronization [60,67—69]. However, in IL6KO males, this rigidity, together with altered sex steroid profiles, including elevated progesterone without apparent compensatory effect, may compromise metabolic adaptability, increasing vulnerability to HFD-induced dysfunction.

 

Notably, a recent study identified a female-specific signaling axis involving estrogen receptor alpha (ERα), STAT3, and the co-activator Cited1 in arcuate POMC neurons, which mediates the anorexigenic effects of leptin and protects against diet-induced obesity in females [82,83]. Disruption of this ERα—STAT3—Cited1 complex leads to hyperphagia, reduced energy expenditure, and loss of female-specific metabolic protection, resulting in the emergence of a male-like phenotype under HFD. Given that IL-6 activates STAT3, its absence could impair this integrative pathway, weakening hypothalamic coordination of metabolic and circadian inputs. While IL6KO females do not replicate the obesogenic phenotype observed in Cited1-KO mice, they exhibit elevated SCN VIP levels and a hormonal profile with modest shifts toward increased androgens under HFD, suggesting an adaptive response that preserves energy balance despite circadian disruption. Together, these observations support a model in which IL-6 contributes to female-specific circadian and metabolic homeostasis by sustaining integrative signaling between immune, hormonal, and circadian systems, potentially via the STAT3—ERα—Cited1 axis.

While the role of IL-6 in diet-induced obesity remains debated, with studies reporting either no differences in body weight or reduced adiposity compared to controls [19], our findings align with those showing lower body weight in IL6KO mice on a HFD [28,29]. Given that HFD increases IL-6 levels and promotes chronic low-grade inflammation [27], its absence may provide partial protection against diet-induced obesity. However, IL-6 deficiency does not prevent all aspects of HFD-induced metabolic and circadian dysfunction. Specifically, IL6KO mutants exhibit glucose intolerance and systemic inflammation [19,28,29], weakened circadian rhythmicity in females, and altered metabolic cycles in males. These findings emphasize the complex interplay between immune signaling, metabolic adaptation, and circadian resilience. By identifying IL-6 as a key mediator of sex-specific trade-offs between metabolic and circadian homeostasis, we provide insights into immune-metabolic coordination and potential therapeutic strategies for mitigating diet-induced circadian misalignment.

IL-6 plays a critical role in regulating FAA by modulating the food-entrainable oscillator (FEO), which integrates circadian and metabolic signals in a sex- and diet-dependent manner. Our findings align with the circadian thermoenergetics hypothesis, which suggests that nocturnal animals shift activity from night to day during energetic stress to conserve energy [84]. Under standard conditions, IL6KO males exhibit exaggerated FAA at the expense of reduced nocturnal EE and nocturnal activity, consistent with an energy-saving adaptation. In contrast, IL6KO females acquire FAA more rapidly but normalize to WT levels by the end of the paradigm. While IL6KO females demonstrate metabolic flexibility through increased carbohydrate utilization during the daytime, they exhibit lower overall EE and a loss of the typical positive correlation between body weight and EE, suggesting a disruption in metabolic balance. These findings emphasize IL-6's crucial role in coupling circadian rhythms and metabolic cycles in a sex-dependent manner.

Although the exact location of the food-entrainable oscillator (FEO) remains unknown, it likely involves both central circuits and peripheral clocks that together integrate feeding cues and metabolic rhythms [6,85—87]. The persistence of sex differences in FAA regulation in gonadectomized mice [88] suggests that IL-6's role are not directly mediated by gonadal hormones. Instead, IL-6 may regulate these processes in a sex-specific manner via distinct central and peripheral mechanisms. Notably, in males, IL6R expression in muscle prioritizes circadian signals over feeding cues, indicating a stronger control by the central clock (SCN) over local entrainment. This is consistent with the lack of changes in IL-6 signaling in male muscle under TRF. In contrast, in females, IL-6 contributes to the integration of feeding cues into metabolic responses, promoting flexibility through substrate switching and compensatory activation of pSTAT3 in muscle. IL6KO females also adjust circadian proteins such as PER2 and CRY1 to preserve circadian coherence, an adaptation not seen in males. Together, these findings highlight IL-6 as a key modulator of metabolic and circadian coordination, with sex-specific roles in FAA regulation and TRF adaptation.

Under HFD conditions, IL6KO males fail to sustain FAA, with marked suppression that exceeds that of WT controls. They also lose the obesity resistance previously seen under ad libitum HFD, suggesting that TRF overrides this protective effect. In contrast, IL6KO females acquire FAA more rapidly, mirroring their response under STD, and adapt through metabolic flexibility, increased food intake, and a delayed RQ acrophase. These findings highlight a sex-specific role of IL-6 in circadian-metabolic plasticity: in males, IL-6 helps sustain FAA and metabolic regulation; in females, it coordinates substrate utilization and energy balance under feeding-time challenges. Understanding the mechanisms underlying feeding-time entrainment is essential for addressing circadian misalignment and its contribution to metabolic disorders, inflammation, and immune dysfunction [7—10].

Our study identifies IL-6 as a central integrator of circadian and metabolic regulation, acting in a sex- and context-dependent manner. This positions IL-6 as a promising therapeutic target for mitigating circadian misalignment, particularly in chronically stressed populations such as shift workers or individuals with metabolic syndrome. While chronic IL-6 overactivation promotes inflammation, targeted and time-specific modulation of IL-6 signaling, via IL-6 receptor antagonists or chronotherapy aligned with endogenous rhythms, may help restore circadian-metabolic homeostasis. Future studies should define the optimal timing, dosage, and tissue specificity of such interventions. Given the sex-specific responses observed in IL6KO mice, future therapeutic strategies may benefit from sex-based tailoring to maximize efficacy.

## 4. MATERIAL AND METHODS

### 4.1. Animals and treatments

IL6KO mice (B6.129S2-Il6tm1Kopf/J; RRID: IMSR_JAX:002650) were obtained from Jackson Laboratory and backcrossed eleven times onto the C57BL/6 background. Congenic wild-type controls (C57BL/6J) were also purchased from Jackson Laboratory. Both WT and IL6KO mice were bred in homozygous conditions [89], and the absence of full-length IL6 transcripts in IL6KO mice was previously confirmed [89]. Mice had *ad libitum* access to water and standard chow (Teklad-7913, Envigo) or a high-fat diet (HFD; 60% fat, 20% carbohydrate, 20% protein, 5.21 kcal/g; D12492; Research Diets, Inc). They were housed under a 12-h light—dark cycle (8 a.m.—8 p.m.) in a temperature- and humidity-controlled room at the University of Santiago de Compostela. Animal care complied with institutional guidelines, and all procedures were approved by the University Ethics Committee following European Union regulations (Project ID 15012/2021/011). Body weights were recorded weekly.

### 4.2. Indirect calorimetry and nuclear magnetic resonance

Energy expenditure, respiratory quotient, and spontaneous locomotor activity were measured using a calorimetric system (LabMaster; TSE Systems), as previously described [26,32,90,91]. After a 24-hour acclimation to the chambers, mice were monitored for 48—72 h. Whole-body composition was assessed via nuclear magnetic resonance [26,32] (EchoMRI; Houston, TX).

 

### 4.3. Circadian locomotor activity

IL6KO and control mice (5–6 months old) were housed individually in cages with running wheels (ENV-044; Med Associates Inc) [26,32,38]. To prevent interference between sexes, male and female mice were placed in separate ventilated cages within isolated racks, avoiding direct contact and olfactory interaction [26]. Mice were acclimated to running wheels for three days under a 12h:12h light–dark cycle before the experiment, which lasted 7–10 days under the same conditions. They were then placed in constant darkness (2–3 weeks) in black cages (Tecniplast), followed by another 5–7 days of light–dark cycles, as previously reported [26,32,38]. Running wheel activity was recorded in 5-min intervals using Wheel Manager software (SOF-860; Med Associates Inc) and analyzed with Actogram J [26,32,38]. Locomotor activity patterns were derived from 7 to 10 days of wheel-running data per mouse, considering potential variations in the estrous cycle. Activity profiles under light–dark (LD) and re-entrainment light–dark (rLD) conditions were plotted using zeitgeber time (ZT), while constant darkness (DD) data were adjusted for individual period variations and represented in circadian time (CT). Mean activity was averaged over 5-minute intervals for each mouse [26,32,38].

### 4.4. Food restriction experiments

5–6 months old C57BL/6 mice were divided into *ad libitum* or fasting groups. Fasted mice were placed in new cages without food 24 h before sacrifice to prevent access to remnants of chow. For TRF experiments, control and IL6KO mice were housed individually in cages with running wheels (ENV-044; Med Associates Inc) [26,32]. Locomotor activity was monitored for seven days in LD under *ad libitum* feeding. Body weight and food intake were recorded at ZT6, followed by a restricted feeding schedule for ten days. Mice had access to food from ZT4 to ZT10 for six days, and from ZT4 to ZT8 for four days. Daily food intake was measured by weighing pellets before and after food availability. Running wheel activity was recorded in 5-min intervals and analyzed with Actogram J [26,32].

### 4.5. Western blotting

Protein lysates from skeletal muscle, liver and hypothalamus were subjected to SDS-PAGE and electrotransferred onto a nitrocellulose paper (Protran, Schleicher and, Schuell, Dassel, Germany) and probed with antibodies against IL6 (Proteintech, 21865-1-AP) (Dilution 1:1000); IL6R (Invitrogen MA5-29721) (Dilution 1:1000); BMAL1 (Santa Cruz biotechnology, sc365645) (Dilution 1:1000); PER2 (Invitrogen, PA5-89045) (dilution 1:1000); CRY1 (Invitrogen, PA5-89349) (dilution 1:1000); STAT3 (Invitrogen, MA1-13042) (Dilution 1:1000); pSTAT3 (Cell Signaling, 9134S) (Dilution 1:1000) as we previously described. Membranes were then extensively washed and re-probed with antibodies against GAPDH (Proteintech, 60004-1-Ig) (Dilution 1:1000), as the loading controls. Immunoreactive bands were detected with a western light chemiluminescence detection system (ECL, GE Healthcare Bio-Sciences AB). Autoradiographic films (Fujifilm, Tokyo, Japan) were scanned, and the band signal was quantified by densitometry using ImageJ 1.33 software (NIH), as previously shown [92–98]. Values were expressed in relation to GAPDH. Representative images for all proteins are shown; in the case of the loading controls, a representative gel is displayed, although each protein was corrected by its internal control (GAPDH). In all the figures showing images of gels, all the bands for each picture are from the same gel, although they may be spliced for clarity.

### 4.6. Immunofluorescence

Mice were anesthetized with ketamine/xylazine (150 mg/kg, 10 mg/kg) and perfused with ice-cold PBS followed by 4% PFA. Brains were post-fixed overnight in 4% PFA and subsequently sectioned into 30 μm thick slices using a cryostat (Leica). For immunostaining, sections were permeabilized with 0.3% Triton X-100 in PBS, blocked with 4% goat serum, and incubated overnight at 4 °C with the following primary antibodies: anti-VIP (PA5-78224, Invitrogen, dilution 1:500) and anti-IL6R (Santa Cruz Biotechnology, sc373708, dilution 1:200). After washing, sections were incubated for 2 h with secondary antibodies: Alpaca anti-Human IgG/Rabbit IgG VHH, Nano-Secondaries™ Recombinant, Alexa Fluor™ 647 (SRBAF647-1-100) or Alpaca anti-Mouse IgG1 VHH, Nano-Secondaries™ Recombinant, Alexa Fluor™ 647 (SMS1AF647-1-100). Slices were mounted with Prolong Gold and imaged using a confocal microscope (Leica TCS SP5) with 10x or 20× objectives. Quantification was performed using ImageJ by outlining the hypothalamus in DAPI-stained images and measuring immunostaining intensity. When analyzing multiple sections, the mean intensity from consecutive sections was used [26,32,38,39,93].

### 4.7. Measurement of serum steroid concentrations

Serum concentrations of progesterone, 17α-hydroxyprogesterone, androstenedione, testosterone, estradiol, and dihydrotestosterone were measured using a high-sensitivity liquid chromatography-tandem mass spectrometry assay [99]. Using mouse serum, the lower limit of quantitation (LLOQ) with the assay are 5 pg/mL, 20 pg/mL, 5 pg/mL, 5 pg/mL, 0.5 pg/mL, and 13 pg/mL respectively. Results under the LLOQ were calculated to be half of each lower limit of quantitation value to avoid overestimation of low values in the analysis.

### 4.8. Statistical analysis

Data are presented as mean ± SEM and analyzed using Prism 9 (GraphPad, San Jose, CA, USA). Statistical significance was assessed using two-tailed paired Student's t-tests for paired comparisons, one-way ANOVA with Tukey–Kramer post hoc for multiple group comparisons, and two-way repeated measures ANOVA with Bonferroni post hoc for repeated measures. Normality and equal variances were checked. Significance levels were set at $* = p < 0.05$, $** = p < 0.01$, $*** = p < 0.001$, $**** = p < 0.0001$. Rhythmic expression significance was determined by Cosinor analysis [26,32,38,39,92,93]. Samples deviating more than 2 s.d. from the group mean were excluded. In food-restriction experiments, pre-feeding activity duration was calculated as hours from the pre-feeding phase to mealtime. FAA ratio was the fold change in activity during the 4 h before feeding compared to the rest of the day, while nocturnal activity ratio was calculated by comparing nighttime activity with the rest of the day [26,32].

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## CRediT AUTHORSHIP CONTRIBUTION STATEMENT

**Antía González-Vila:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Ali Mohammad Ibrahim-Alasoufi:** Writing – review & editing, Methodology, Investigation, Formal analysis. **María Luengo-Mateos:** Writing – review & editing, Methodology, Investigation. **Víctor Pardo-García:** Writing – review & editing,

 

Methodology, Investigation. **Alejandro Diaz-López:** Writing — review & editing, Methodology, Investigation. **Belén Fernández-Rodríguez:** Writing — review & editing, Methodology, Investigation. **Matti Poutanen:** Conceptualization, Investigation, Resources, Writing — review & editing. **Claes Ohlsson:** Conceptualization, Investigation, Resources, Writing — review & editing. **Manuel Tena-Sempere:** Conceptualization, Investigation, Resources, Writing — review & editing. **Carlos Diéguez-González:** Writing — review & editing, Resources, Investigation, Conceptualization. **María del Carmen García-García:** Writing — review & editing, Resources, Investigation, Conceptualization. **Olga Barca-Mayo:** Writing — review & editing, Writing — original draft, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

## DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## DATA AVAILABILITY

Data will be made available on request.

## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2025.102171.

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