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Time-restricted feeding combined with aerobic exercise training can prevent weight gain and improve metabolic disorders in mice fed a high-fat diet

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Key points

- Time-restricted feeding (TRF, in which energy intake is restricted to 8 h/day during the dark phase) alone or combined with aerobic exercise (AE) training can prevent weight gain and metabolic disorders in Swiss mice fed a high-fat diet.
- The benefits of TRF combined with AE are associated with improved hepatic metabolism and decreased hepatic lipid accumulation.
- TRF combined with AE training increased fatty acid oxidation and decreased expression of lipogenic and gluconeogenic genes in the liver of young male Swiss mice.
- TRF combined with AE training attenuated the detrimental effects of high-fat diet feeding on the insulin signalling pathway in the liver.

Abstract Time-restricted feeding (TRF) or physical exercise have been shown to be efficient in the prevention and treatment of metabolic disorders; however, the additive effects of TRF combined with aerobic exercise (AE) training on liver metabolism have not been widely explored. In this study TRF (8 h in the active phase) and TRF combined with AE (TRF+Exe) were compared in male Swiss mice fed a high-fat diet, with evaluation of the effects on insulin sensitivity and expression of hepatic genes involved in fatty acid oxidation, lipogenesis and gluconeogenesis. As in previous

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reports, we show that TRF alone (eating only between zeitgeber time 16 and 0) was sufficient to reduce weight and adiposity gain, increase fatty acid oxidation and decrease lipogenesis genes in the liver. In addition, we show that mice of the TRF+Exe group showed additional adaptations such as increased oxygen consumption ($\dot{V}_{O_2}$), carbon dioxide production ($\dot{V}_{CO_2}$) and production of ketone bodies (β-hydroxybutyrate). Also, TRF+Exe attenuated the negative effects of high-fat diet feeding on the insulin signalling pathway (insulin receptor, insulin receptor substrate, Akt), and led to increased fatty acid oxidation (*Ppara*, *Cpt1a*) and decreased gluconeogenic (*Fbp1*, *Pck1*, *Pgc1a*) and lipogenic (*Srebp1c*, *Cd36*) gene expression in the liver. These molecular results were accompanied by increased glucose metabolism, lower serum triglycerides and reduced hepatic lipid content in the TRF+Exe group. The data presented in this study show that TRF alone has benefits but TRF+Exe has additive benefits and can mitigate the harmful effects of consuming a high-fat diet on body adiposity, liver metabolism and glycaemic homeostasis in young male Swiss mice.

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Introduction

Obesity is a severe public health problem worldwide that has a significant economic and social impact with a consequent increase in morbidity and mortality (Gregg & Shaw, 2017). The main modifiable risk factors for the development and worsening of obesity are physical inactivity, increased caloric intake, low nutritional quality, behavioural factors and an obesogenic environment (Kushner, 2018; Li *et al.* 2018). Studies have shown that a high-fat diet negatively affects the metabolic health status, causing changes in the lipid and inflammatory profile, with consequent impairment in insulin sensitivity and glucose homeostasis (McDonald *et al.* 2011; Brunetta *et al.* 2020). Furthermore, previous investigations in the literature have shown that rodents exposed to a high-fat diet developed obesity and hepatic metabolism disturbances, such as increased glucose production and fat accumulation (Cintra *et al.* 2008; Oliveira *et al.* 2015; Pereira *et al.* 2020). In addition, increased body weight has been associated with reduced spontaneous physical activity, further compromising lipid and carbohydrate metabolism in obese rodents (Kohsaka *et al.* 2007; Hatori *et al.* 2012).

In contrast, it was shown that the adoption of time-restricted feeding (TRF) can protect obese rodents on a high-fat diet from an excessive increase in body adiposity and metabolic disorders (Hatori *et al.* 2012; Sherman *et al.* 2012; Chaix *et al.* 2014; Sundaram & Yan, 2016; Regmi & Heilbronn, 2020). The scientific evidence allows us to consider that TRF is associated with reductions in body weight, glycaemia and insulinaemia, as well as improvements in the lipid profile (reduction in the concentration of total cholesterol and triglycerides) and inflammatory signature (decrease in interleukin-1β, interleukin-6 and tumour necrosis factor-α), with consequent positive outcomes for insulin sensitivity (Sherman *et al.* 2012; Melkani & Panda, 2017; Aouichat *et al.* 2020; Regmi *et al.* 2021). An analysis performed on the liver showed TRF to attenuate pyruvate carboxylase and glucose 6-phosphatase expression and to increase glucokinase during the active phase in mice fed a high-fat diet (Hatori *et al.* 2012; Chaix *et al.* 2014). These previous findings have shown that the adoption of the TRF strategy for the prevention or mitigation of risk factors related to metabolic diseases is promising.

Aerobic exercise (AE) has also been used as a non-pharmacological intervention to prevent or treat disorders related to obesity (i.e. insulin resistance, hyperglycaemia and hepatic fat accumulation, among others) (De Cabo *et al.* 2014; Lustig *et al.* 2020). Results obtained from rodents showed that AE training improved insulin signalling in hepatic tissue (Heled *et al.* 2004; Marinho *et al.* 2012; Muñoz *et al.* 2018*b*). Previously, it was also demonstrated that AE training improved insulin sensitivity and attenuated liver fat accumulation in rats with obesity induced by a high-fat diet (Lee *et al.* 2006; Muñoz *et al.* 2017). Also, it has been shown that AE training can alter the expression of multiple genes in liver tissue, which can play an essential role in preventing diabetes in obese rats (Colombo *et al.* 2005). Interestingly, the effects of TRF combined with aerobic exercise on body adiposity and metabolic disorders in mice fed a high-fat diet have not yet been fully elucidated.

In the current study, we hypothesized that TRF combined with AE training could be a beneficial preventive intervention, with synergic effects, against obesity and metabolic disturbances associated with consuming a high-fat diet. Therefore, this study aimed to investigate the impact of TRF combined with AE training on the molecular markers related to insulin signalling, fatty acid oxidation, lipogenesis and gluconeogenesis in the liver of young male Swiss mice fed a high-fat diet.

Methods

Ethical approval

All animal protocols were approved by the Animal Ethics Committee (CEUA) of the Institute of Biological Sciences, UNICAMP – Campinas-SP (Protocol number 5185-1/2019) and were aligned with the National Council for Animal Experimentation Control (CONCEA). The authors of this study are aware of the ethical principles under which *The Journal of Physiology* operates and acknowledge that all procedures conducted follow the animal ethics checklist described in Grundy (2015).

Experimental animals

Male Swiss mice (*Mus musculus*), 4 weeks old (∼20 g), were obtained from the Multidisciplinary Centre for Biological Investigation on Laboratory Animal Science (CEMIB) – University of Campinas (UNICAMP). The animals were divided into four experimental groups for 10 weeks: lean sedentary mice fed a standard rodent commercial diet *ad libitum* (C-Ad) ($n = 7$); mice fed a high-fat diet *ad libitum* (HF-Ad) ($n = 7$); HF-Ad mice subjected to TRF ($n = 7$), and TRF mice subjected to a physical training protocol (TRF+Exe) ($n = 7$). Mice were housed in individual polyethylene cages at 21 ± 2°C, on a 12 h light–dark cycle with free access to water. The groups C-Ad and HF-Ad had free access to standard and high-fat diet, respectively, while TRF and TRF+Exe were subjected to the TRF protocol described below. The standard diet was a commercial pellet provided by Nuvilab (Quimtia, Colombo, PR, Brazil) and the nutritional composition provided by the manufacturer was 23% crude protein, 4% lipids and 5% fibre. The high-fat diet composition was 11.55% corn starch, 20% casein, 10% sucrose, 13.2% dextrinated starch, 4% soybean oil, 31.2% lard, 5% cellulose, 3.5% mineral mix, 1% vitamin mix, 0.3% L-cystine, 0.25% choline bitartrate (Cintra *et al.* 2012), according to the American Institute of Nutrition (AIN39-G) (Sundaram & Yan, 2016). The high-fat diet composition was described as a percentage of the mass (g/100 g). During the experimental protocol, the animals were weighed weekly, and the food intake was determined once a week, measuring the food weight in 24 h.

Time-restricted feeding protocol

Zeitgeber time (ZT) 0 was designated as lights-on time and ZT12 as lights-off time. The animals in the TRF and TRF+Exe groups were subjected to a TRF protocol. Access to diet occurred 4 h after the beginning of the dark cycle (ZT16) until the beginning of the light cycle (ZT0), totalling 8 h of access to diet (adapted from Hatori *et al.* 2012). Food access was regulated by transferring mice daily between cages with free access to food and water and cages with free access to water only. Thus, these animals had access to food for 8 h and performed fasting of 16 h daily (Fig. 1).

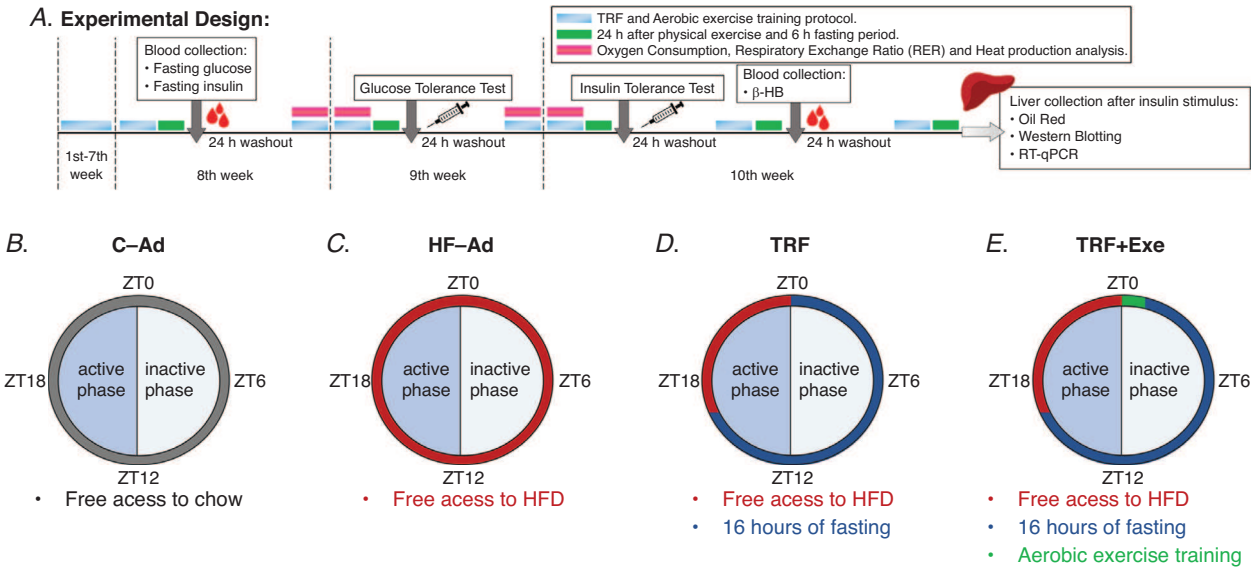

Figure 1. Experimental design
A, timeline of the whole experiment. The mice were subject to chow or a high-fat diet. The HFD groups had TRF and performed the aerobic exercise training protocol. In the 8th week, blood was collected to measure fasting glucose and insulin levels. In the 9th week, a glucose tolerance test was performed. An insulin tolerance test was completed in the 10th week, and another blood collection for β-hydroxybutyrate (β-HB) was performed. These analyses were performed 24 h after physical exercise, with 6 h of fasting. *B–E*, representations of C-Ad group (*B*), HF-Ad group (*C*), TRF group (*D*) and TRF+Exe group (*E*). [Colour figure can be viewed at wileyonlinelibrary.com]

Incremental load test

After adaptation to physical exercise on a treadmill (AVS Projects, São Carlos, São Paulo, Brazil) for 5 days at a 3 m min^{-1} speed, the animals in the TRF+Exe group performed an incremental load test. Animals ran on a non-inclined treadmill at an initial velocity of 6 m min^{-1}, with increments of 3 m min^{-1} every 3 min until exhaustion, defined when the mice touched the end of the treadmill five times in 60 s. The exhaustion velocity (EV) obtained in the test was used to determine the intensity of 60% of the EV of the 10-week chronic exercise. The incremental load test was repeated two more times, at the end of the fourth and seventh week of the protocol to correct training intensity due to improved performance. At the end of the 10-week protocol, the test was performed again. After the incremental load test, the animals rested for 24 h until the next training session. The training effects on mice over the course of the experiment were evaluated by a body weight-dependent method (body mass × EV). Also, mouse performances were evaluated by exhaustion velocity. Of note, no adverse outcomes were observed in any of the animals throughout the incremental load test (Fig. 2).

Aerobic exercise training protocol

The AE training lasted for 10 weeks, with an intensity of 60% of the EV obtained in the incremental load test. The animals were subjected to physical exercise for 7 days of the week after the feeding period. Therefore, aerobic exercise training was carried out from ZT0 to ZT1. The training volume gradually increased from 30 min in the first week to 45 min in the second week, to 60 min from the third to the tenth week. No adverse outcomes were observed in any of the animals throughout the AE.

Oxygen consumption, respiratory exchange ratio and heat production

Between the eighth and tenth weeks of the experiment, the mice were subjected to the Comprehensive Lab Animal Monitoring System (CLAMS-Oxymax) (Columbus Instruments, Columbus, OH, USA). Before the analysis, the animals were adapted to the device for 24 h in individual cages. On the day of the experimental evaluations, the TRF+Exe mice trained between ZT23 and ZT24, and the analyses started at ZT24. Throughout the experiment, the animals were kept in separate cages in a controlled temperature cycle. Oxygen consumption ($\dot{V}_{O_2}$), carbon dioxide consumption ($\dot{V}_{CO_2}$), respiratory exchange ratio (RER) and energy expenditure (EE) were analysed over 24 h. The EE value was calculated as $(3.815 + 1.232 \times \text{RER}) \times \dot{V}_{O_2}$ (Sasaki *et al.* 2014). The signal acquisition occurred at six points per hour, on average, during the entire 24 h. At the end of the analysis, the data were plotted in Microsoft Excel 2016 and grouped every hour.

Glucose tolerance test

After 6 h of fasting and 24 h after the last exercise session, the animals were submitted to a glucose tolerance test. After collection for basal glucose analysis, equivalent to time zero, animals received an intraperitoneal injection of 25% glucose solution (2 g kg^{-1}). Blood samples were collected via the tail vein at 30, 60, 90 and 120 min after the stimulation with glucose. Blood glucose was measured using a glucometer (Accu-Chek; Roche Diagnostics, Indianapolis, IN, USA). Subsequently, the area under the curve was calculated for each group. For these tests, blood samples were obtained following a tail snip (using surgical scissors), with bleeding suppressed between samples using a compression bandage (Johnson and Johnson, São Paulo, SP, Brazil). After the test, the animals continued to be monitored for up to 3 h.

Insulin tolerance test

After 24 h of the last physical exercise session, and 6 h of the fasting period, the insulin tolerance test was performed. After measuring fasting glycaemia, equivalent to time zero, 1.5 U kg^{-1} of insulin (Humulin R; Lilly, Indianapolis, IN, USA) was injected intraperitoneally. Then, blood samples were collected via the tail vein at 10, 15, 20, 25 and 30 min after the insulin stimulation to determine circulating glucose levels. Blood glucose was measured using a glucometer (Accu-Chek; Roche Diagnostics). The area under the curve was then

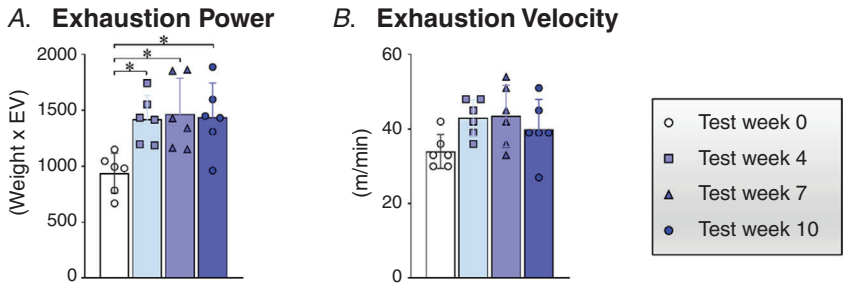

Figure 2. AE training increased animal running performance
A, exhaustion power of the fourth incremental load test performed during the aerobic physical training. *B*, exhaustion velocity of the fourth incremental load test performed during the aerobic physical training. The bars represent means and SD of test week 0, test week 4, test week 7 and test week 10 ($n = 6$). *Statistically significant, $P < 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com]

calculated for each experimental group. For these tests, blood samples were obtained following a tail snip (using surgical scissors), with bleeding suppressed between samples using a compression bandage (Johnson and Johnson). After the test, the animals remained monitored for up to 3 h.

Blood biochemistry analysis

Twenty-four hours after the aerobic exercise training, animals fasted for 6 h. Blood from the tail was utilized to measure the fasting glucose using a glucometer (Accu-Chek). In this collection, ∼100 μl of blood from the tail was centrifuged (1100 *g* for 15 min at 4°C). The serum was stored at −80°C to further measure insulin circulating levels by enzyme-linked immunosorbent assay (RayBiotech, Norcross, GA, USA; ELM-Insulin). The homeostatic model assessment for insulin resistance (HOMA-IR) was calculated using the formula: fasting plasma glucose (mmol l^{-1}) × fasting plasma insulin (μU ml^{-1})/22.5.

During the 10th week, we made a new blood collection for the analysis of β-hydroxybutyrate (β-HB). The animals in groups C-Ad, HF-Ad, TRF and TRF+Exe ($n = 4$) were fasted after the feeding window of the groups that underwent TRF (06.00 h). Blood was collected from the tail by dripping the blood into an Eppendorf tube (∼50 ul), performed at the beginning of the fast (fed state) and after 8 and 16 h of fasting. After each collection, the blood was centrifuged (1100 *g* for 15 min at 4°C). Subsequently, β-HB levels were measured using a colorimetric kit (Cayman Chemical Co., Ann Arbor, MI, USA).

The biochemical markers (total cholesterol, triglycerides, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase) were determined using commercial kits (Laborlab, São Paulo, SP, Brazil) using the serum collected in the liver extraction after decapitation (24 h after physical exercise, with 6 h of fasting).

Liver extraction, homogenization and determination of total protein content

After 24 h of the last physical exercise session and 6 h of the fasting period the animals received an intraperitoneal injection of ketamine chlorohydrate (90 mg kg^{-1}; Ketalar; Parke-Davis, Ann Arbor, MI, USA) and xylazine (10 mg kg^{-1}; Rompun; Bayer, Leverkusen, Germany) and then were euthanized by decapitation. Before tissue extraction, insulin was injected intraperitoneally (10 U kg^{-1}; Humulin R; Lilly, Indianapolis, IN, USA), and after 10 min the liver was collected and rapidly frozen in liquid nitrogen for storage at −80°C. Subsequently, the tissue was homogenized in extraction buffer (1% Triton X-100, 100 mM Tris (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride and 0.1 mg ml^{-1} aprotinin) at 4°C with a Bead Ruptor 12 Homogenizer OMNI (Omni International, Kennesaw, GA, USA) operated at maximum speed for 60 s. The lysates were centrifuged Eppendorf 5804R (Hamburg, Germany) at 12.851 *g* at 4°C for 15 min to remove insoluble material. The supernatant was used for the assay. Protein content was determined using the bicinchoninic acid method. The epididymal, retroperitoneal, mesenteric and inguinal white adipose tissues were harvested and weighed.

Western blotting analysis

After determining the total protein content, Laemmli buffer containing 100 mM dithiothreitol was added to the supernatant, and the samples were heated for 5–10 min (Laemmli, 1970). Then, samples with equal amounts of proteins (50 μg) were applied to a polyacrylamide gel for separation by SDS-PAGE and transferred to nitrocellulose membranes. Ponceau staining was applied to check membrane transfer. The blots were blocked with 5% dry milk at room temperature for 1 h and then incubated overnight at 4°C with the following primary antibodies: insulin receptor β (sc-711), insulin receptor substrate 1 (IRS1; sc-559), anti-phospho-Akt (Ser473) (sc-33 437), and anti-Akt (sc-8312) from Santa Cruz Biotechnology (Dallas, TX, USA); anti-glycogen synthase kinase 3 (GSK3) α/β (cat. no. 5676), anti-phospho-GSK3α/β (Ser9) (cat. no. 5558), anti-extracellular signal-regulated kinases 1 and 2 (ERK1/2; cat. no. 9102), anti-phospho-ERK1/2 (Thr202/Tyr204) (cat. no. 4370), anti-hormone-sensitive lipase (HSL) (cat. no. 4107), anti-phospho-HSL (ser565) (cat. no. 4137) from Cell Signaling Technology (Danvers, MA, USA); anti-phospho-insulin receptor (IR) (Tyr972) (07-838) from Millipore (Billerica, MA, USA); anti-phospho-IRS1 (Tyr612) (44816G) from Thermo Fisher Scientific (Waltham, MA, USA). The membranes were then incubated for 1 h with the specific secondary antibodies. The specific bands were visualized by enhanced chemiluminescence and acquired by the C-DiGit™ Blot Scanner (LI-COR, Lincoln, NE, USA). The bands were quantified by their areas using optical densitometry using the UN-SCAN-IT gel 6.1 software (Silk Scientific, Inc. Orem, UT, USA).

Histological analysis

During tissue extraction, part of the liver was frozen in pre-cooled isopentane for cryopreservation at −80°C.

Table 1. Primer sequences

Gene	Forward	Reverse
Ppara	5′-ACCACTACGGAGTTCACGCATG-3′	5′-GAATCTTGCAGCTCCGATCACAC-3′
Cpt1a	5′-AAAGATCAATCGGACCCTAGACA-3′	5′-CAGCGAGTAGCGCATAGTCA-3′
Acsl1	5′-ACACTTCCTTGAAGCGATGG-3′	5′-GGCTCGACTGTATCTTGTGG-3′
Acsl4	5′-GCACCTTCGACTCAGATCAC-3′	5′-CCAGGTTTGTCTGAAGTGGG-3′
Acsl5	5′-CGCCCCCATCTCCACTCCAG-3′	5′-GCTTCAAACACCCAACATCCCATTGC-3′
Fatp4	5′-GACTTCTCCAGCCGTTTCCACA-3′	5′-CAAAGGACAGGATGCGGCTATTG-3′
Fasn	5′-GAGGACACTCAAGTGGCTGA-3′	5′-GTGAGGTTGCTGTCGTCTGT-3′
Srebp1c	5′-GAGGACACTCAAGTGGCTGA-3′	5′-GGGAAGTCACTGTCTTGGTTGTT-3′
Cd36	5′-TGGAGCTGTTATTGGTGCAG-3′	5′-TGGGTTTTGCACATCAAAGA-3′
Fbp1	5′-TGCTGAAGTCGTCCTACGCTAC-3′	5′-TTCCGATGGACACAAGGCAGTC-3′
G6pc	5′-GCGCAGCAGGTGTATACTATG-3′	5′-CGTTCAAACACCGGAATCCA-3′
Pck	5′-AGAAGAAATACCTGGCCGCA-3′	5′-CTTAAGTTGCCTTGGGCATCA-3′
Pc	5′-AAGTTTGGTTGCGCGGAG-3′	5′-TCAGCATCATTAGTGTTGTCAGC-3′
Pgc1a	5′-GAATCAAGCCACTACAGACACCG-3′	5′-CATCCCTCTTGAGCCTTTCGTG-3′
Gapdh	5′-AACTTTGGCATTGTGGAAGG-3′	5′-ACACATTGGGGGTAGGAACA-3′

Liver slices of 10 μm were cut using a Leica cryostat (CM1850, Heidelberg, Germany) and placed on identified adhesion slides. Subsequently, slides were stained with Oil Red O (ORO) solution (Sigma-Aldrich, St Louis, MO, USA) for 25 min and stained with haematoxylin for 2 min. The slides were then washed and sealed with gelatin: glycerine solution (Mehlem *et al*. 2013). The images were obtained using the Leica Application Suite software. The stained area (red) was quantified using ImageJ (NIH, Bethesda, MD, USA) software.

RNA extraction and RT-qPCR

Liver tissue was homogenized in 400 μl Trizol (Thermo Fisher Scientific), and RNA content was extracted according to the manufacturer's instructions. A total of 2 μg of RNA was used for the cDNA synthesis using High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific). The cDNA samples were subjected to a quantitative real-time polymerase chain reaction (RT-qPCR) using 300 ng cDNA and 0.3 μM primers for *Ppara*, *Cpt1a*, *Acsl1*, *Acsl4*, *Acsl5*, *Fatp4*, *Srebp1c*, *Fasn*, *Cd36*, *Fbp1*, *G6pc*, *Pck*, *Pc* and *Pgc1a* with *Gapdh* as the endogenous control (Table 1) synthesized by Exxtend (Paulínia, SP, Brazil), and iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The data were evaluated using StepOne Software software (Thermo Fisher Scientific), calculating $\Delta\Delta C_t$.

Statistics

All results were presented as means ± SD, and statistical significance was determined when $P < 0.05$. Data that did not pass the normality test were analysed using the Kruskal–Wallis test complemented by the Dunn test. The Shapiro–Wilk test examined the normality. Data showing normality were analysed using Student's *t*-test when comparing two groups. One-way ANOVA was used to compare more than two groups and two-way ANOVA when groups were compared at different times, followed by Tukey's *post hoc* test. Graphing was done using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA).

Results

Benefits of TRF and TRF with AE interventions on weight gain, adiposity, and glucose homeostasis

During the experiment, body weight gain was statistically significantly the largest for the HF-Ad group (Fig. 3*A*). From the second week, the HF-Ad group's body weight was significantly higher than that of the TRF+Exe group. The HF-Ad group's body weight gain was also more remarkable after the third week than that of the TRF group, and, after the fifth week, compared to that of the C-Ad group. The TRF+Exe group had a significantly lower body weight than the C-Ad and HF-Ad groups after the second week. Interestingly, after the fifth week of the experiment, the TRF+Exe group showed significantly lower body weight than the TRF group (Fig. 3*A*). During the investigation, total body weight gain was substantially more significant in the HF-Ad group than in the C-Ad, TRF and TRF+Exe groups (Fig. 3*B*). Corroborating the total body weight findings, the mesenteric, epididymal and retroperitoneal weights were higher for the HF-Ad group than for the C-Ad and TRF+Exe groups (Fig. 3*C*). The total fat weight was higher for the HF-Ad group than for the C-Ad, TRF and TRF+Exe groups (Fig. 3*D*).

The 24-h food intake did not differ between the experimental groups (Fig. 3*E*). However, for the cumulative food intake during the 10 weeks, higher caloric intake was observed for the HF-Ad group than for the TRF+Exe group (Fig. 3*F*). Fasting blood glucose was significantly higher for the HF-Ad group than for the C-Ad group (Fig. 3*G*). Fasting insulinaemia was higher for the HF-Ad group than for the TRF+Exe group (Fig. 3*H*), and the HOMA-IR index was higher for the HF-Ad group than for the C-Ad, TRF and TRF+Exe groups (Fig. 3*I*). The HF-Ad group also showed greater glucose intolerance and reduced insulin sensitivity than the C-Ad group. The TRF and TRF+Exe groups were also preserved from these metabolic disorders induced by exposure to a high-fat diet (Fig. 3*J*–*M*). The TRF+Exe group had a smaller area under the glucose curve than the TRF group (Fig. 3*K*).

Effects of TRF and TRF with AE interventions on indirect calorimetry parameters

For the indirect calorimetry parameters, the TRF+Exe group had higher oxygen consumption ($\dot{V}_{O_2}$) than the C-Ad, HF-Ad and TRF groups in the light cycle, as well as higher oxygen consumption than the HF-Ad group in the dark cycle (Fig. 4*A* and *B*). The TRF+Exe group also showed higher carbon dioxide consumption ($\dot{V}_{CO_2}$) than the HF-Ad group in both the light and dark cycles (Fig. 4*C* and *D*). The respiratory exchange ratio (RER)

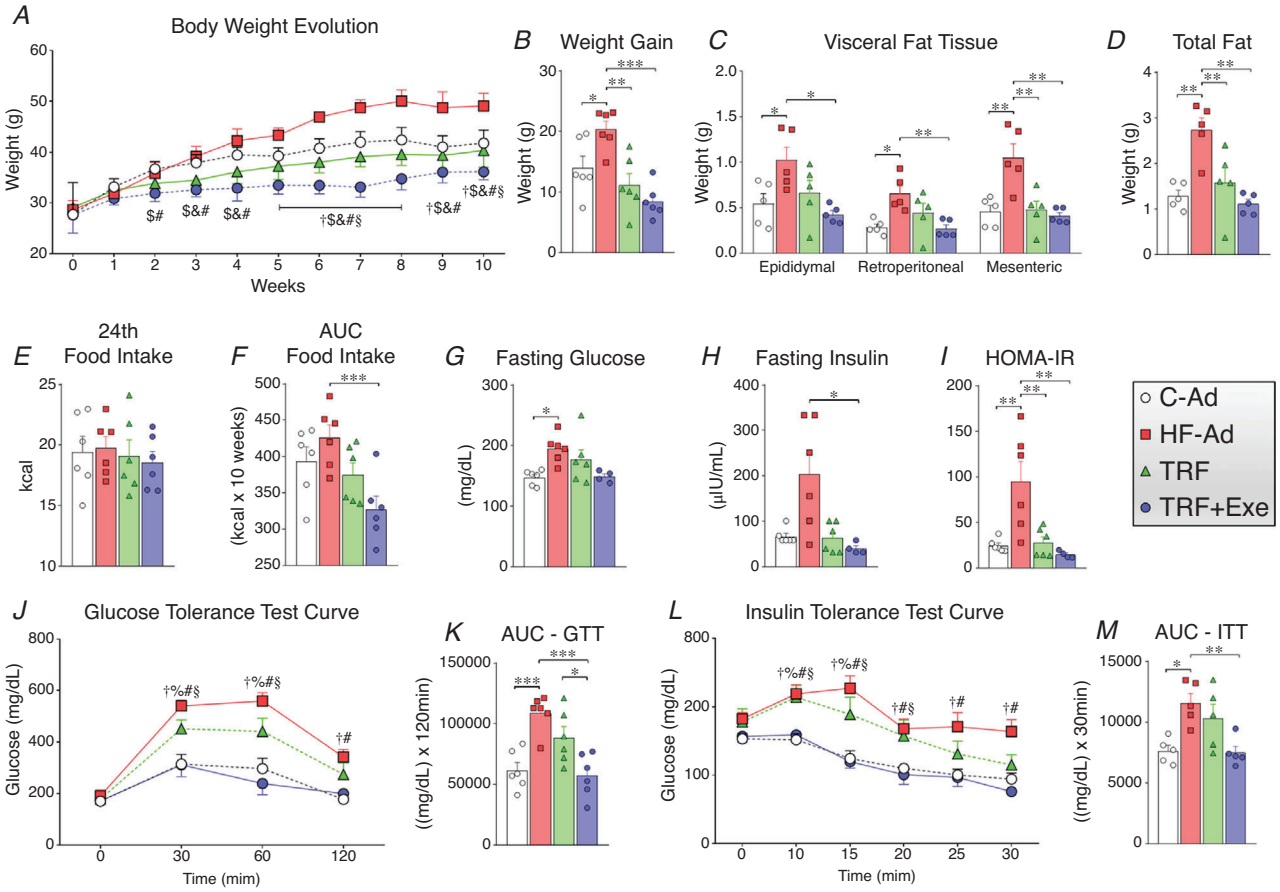

Figure 3. Time of feeding combined with aerobic exercise training regulates glucose homeostasis and influences body weight gain
A, body weight curve for 10 weeks ($n = 6$). *B*, weight gain (initial weight − final weight) ($n = 6$). *C*, weight of visceral adipose tissues ($n = 5$). *D*, total visceral fat ($n = 5$). *E*, food intake for 24 h ($n = 6$). *F*, the area under the curve of food intake ($n = 6$). *G*, fasting glucose ($n = 4$–6). *H*, fasting insulin ($n = 4$-6). *I*, HOMA-IR index ($n = 4$-6). *J*, glucose tolerance test curve ($n = 6$). *K*, the area under the curve (AUC) of the glucose tolerance test (GTT) ($n = 6$). *L*, insulin tolerance test curve ($n = 5$). *M*, area under the curve (AUC) of the insulin tolerance test (ITT) ($n = 5$). The bars represent the means and SD of C-Ad, HF-Ad, TRF and TRF+Exe mice. Statistical significance was as follows: *P <0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. For *A*, *J* and *L*, the statistical significance was: † for C-Ad *versus* HF-Ad group; % for C-Ad *versus* TRF; $ for C-Ad *versus* TRF+Exe; & for the HF-Ad *versus* TRF group; # for the HF-Ad *versus* TRF+Exe group; § for the TRF *versus* TRF+Exe group. [Colour figure can be viewed at wileyonlinelibrary.com]

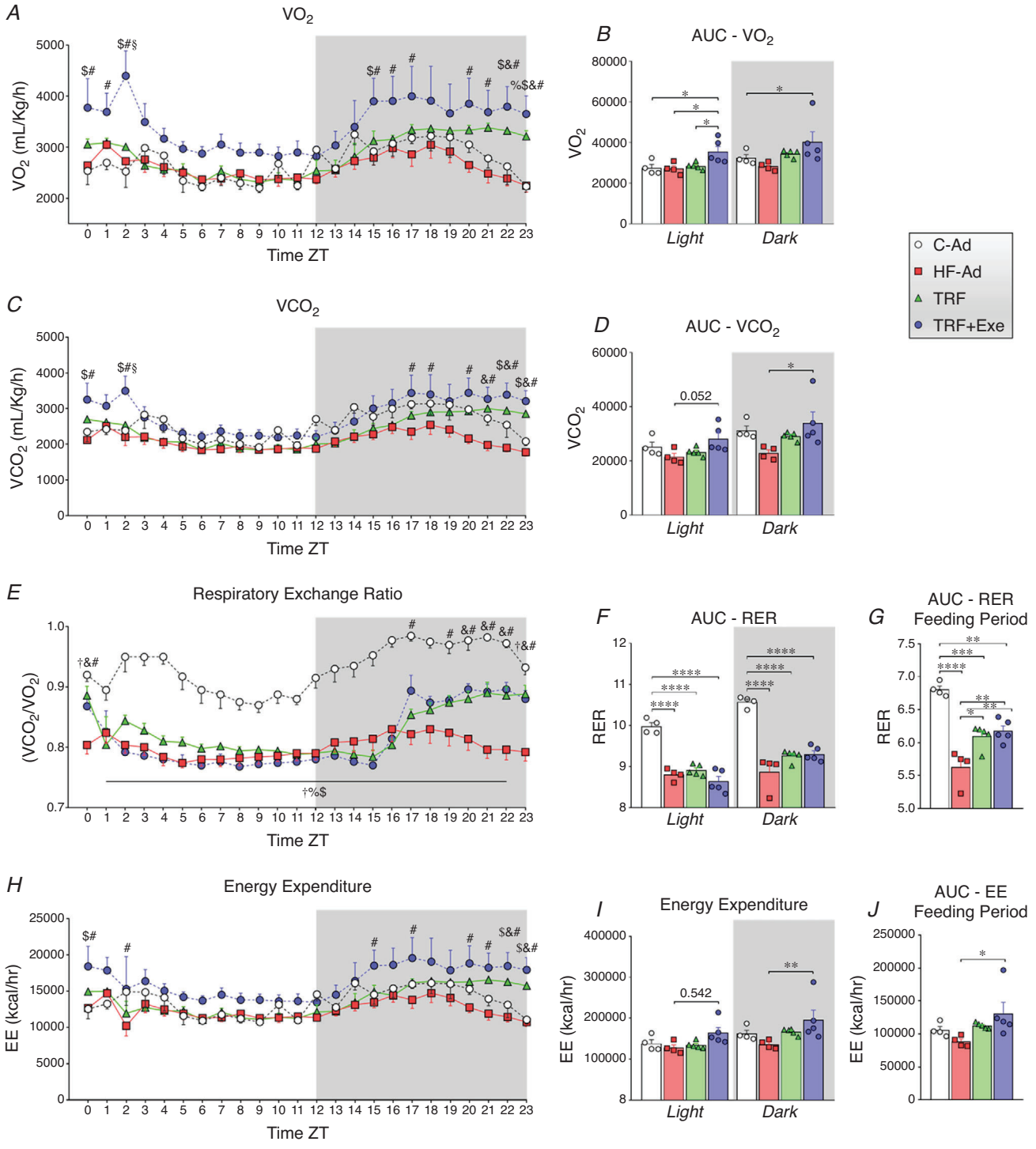

Figure 4. Effects of time-restricted feeding combined with aerobic exercise training on oxygen consumption, respiratory exchange ratio and heat production
A, $\dot{V}_{O_2}$ consumption for 24 h. *B*, $\dot{V}_{O_2}$ consumption during the light and dark cycles. *C*, $\dot{V}_{CO_2}$ consumption for 24 h. *D*, $\dot{V}_{CO_2}$ production during the light and dark cycles. *E*, respiratory exchange ratio for 24 h. *F*, respiratory exchange ratio (RER) during the light and dark cycles. *G*, RER during the feeding period. *H*, energy expenditure for 24 h. *I*, energy expenditure during the light and dark cycles. *J*, energy expenditure during the feeding period. Bars represent means and SD of C-Ad ($n = 4$), HF-Ad ($n = 4$), TRF ($n = 5$) and TRF+Exe ($n = 5$) mice. Statistical significance was as follows: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$. For *A*, *J* and *L*, the statistical significance was: † for C-Ad *versus* HF-Ad group; % for C-Ad *versus* TRF; $ for C-Ad *versus* TRF+Exe; & for the HF-Ad *versus* TRF group; # for the HF-Ad *versus* TRF+Exe group; § for the TRF *versus* TRF+Exe group. [Colour figure can be viewed at wileyonlinelibrary.com]

was lower for the HF-Ad, TRF and TRF+Exe groups than for the C-Ad group in the light and dark cycles. However, when we analysed the RER only in the period when the TRF and TRF+Exe groups were fed, it was found that the RER values for both groups were higher than those found for the HF-Ad group (Fig. 4*E*–*G*). The energy expenditure (EE) was higher for the TRF+Exe group than for the HF-Ad group in both light and dark cycles, and in the period when TRF and TRF+Exe groups were fed (Fig. 4*H*–*J*). No significant differences were observed in heat production and spontaneous activity (data not shown).

Liver and serum lipidaemia upon TRF and TRF with AE interventions

The liver plays a relevant role in balancing fat metabolism, regulating circulating levels of lipid, such as triglycerides (TG) and cholesterol. Also, fat accumulation in the liver is related to damage to the tissue itself and its function. Serum levels of TG and cholesterol were significantly higher for the HF-Ad group than for the C-Ad group. On the other hand, the TRF+Exe group had lower serum TG levels than the HF-Ad group (Fig. 5*A* and *B*). Regarding liver damage enzymes, increased alkaline phosphatase levels were found only in the HF-Ad group, not the C-Ad, TRF and TRF+Exe groups (Fig. 5*C*–*E*). For the analysis of lipid content in the liver tissue, the liver fragment histological images demonstrated a greater lipid staining area for the HF-Ad group than for the C-Ad group, indicating an increased liver fat accumulation. However, the TRF and TRF+Exe groups had a lower lipid staining area in the liver than the HF-Ad group. This lipid area was more significant for both groups than for the C-Ad group (Fig. 5*F* and *G*). In the analysis of β-hydroxybutyrate (β-HB), which together with acetone and acetoacetate is a

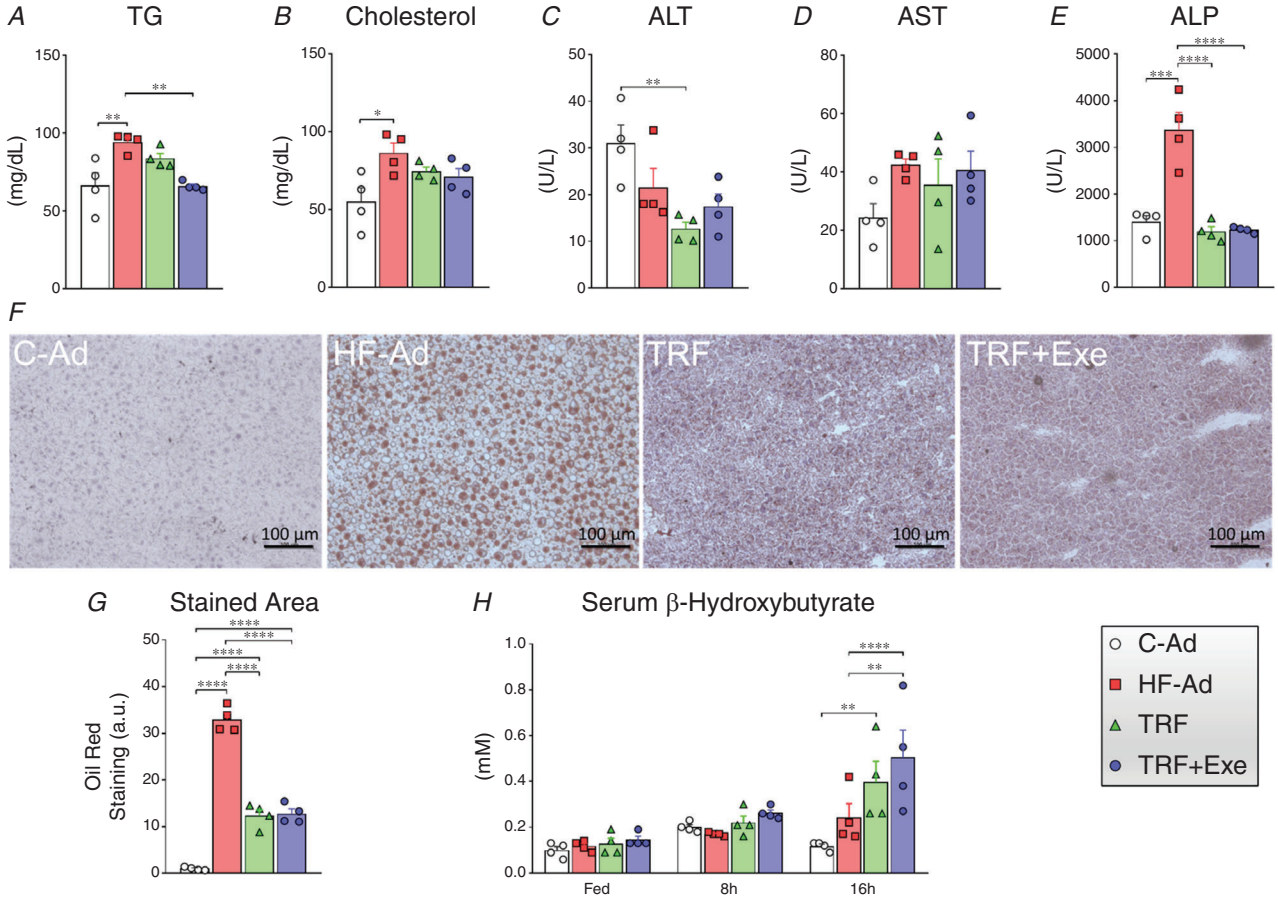

Figure 5. Time of feeding combined with aerobic exercise training alter lipids metabolism and attenuate lipid accumulation in the liver
A, triglycerides quantification in the serum. *B*, cholesterol quantification in the serum. *C*, alanine aminotransferase (ALT) in the serum. *D*, aspartate aminotransferase (AST) in the serum. *E*, alkaline phosphatase (ALP) in the serum. *F*, lipid marking by Oil Red Stain in liver sections. *G*, quantification of the area stained with Oil Red. *H*, quantification of β-hydroxybutyrate in serum with different fasting times. The bars represent means and SD of C-Ad, HF-Ad, TRF and TRF+Exe mice ($n = 4$). Statistical significance was as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. [Colour figure can be viewed at wileyonlinelibrary.com]

ketone body (Fig. 5*H*), the mice in the fed state compared to the 8 h fasting state did not present significant changes. After 16 h of fasting, β-HB levels were higher for the TRF group than for the C-Ad group. Also, the TRF+Exe group had higher β-HB levels than the C-Ad and HF-Ad groups.

Consequences of TRF and TRF with AE interventions on hepatic insulin signalling

Regarding the liver's insulin signalling pathway, the HF-Ad group showed decreased pIRS1^{Y612} and pAkt^{S473} compared to the C-Ad group, with no changes in pIR^{Y972}, pGSK3β^{S9}, pERK$^{T202/Y204}$ and pHSL^{S565} (Fig. 6*A* and *B*). On the other hand, the TRF+Exe group had increased pIR^{Y972}, pIRS1^{Y612}, pAkt^{S473}, pGSK3β^{S9}, pERK$^{T202/Y204}$ and pHSL^{S565} compared to the HF-Ad group (Fig. 6*C* and *D*). The TRF+Exe group had increased pIR^{Y972} and pGSK3β^{S9} compared to the TRF group (Fig. 6*C* and *D*) and the TRF group had increased pGSK3β^{S9} compared to the HF-Ad group (Fig. 6*D*). These results demonstrate that TRF+Exe attenuated the negative impacts of the high-fat diet liver's insulin-signalling pathway.

Hepatic regulation of lipid and glucose metabolism genes upon TRF and TRF with AE interventions

The mRNA for genes related to fatty acid oxidation, lipogenesis and gluconeogenesis in the liver were evaluated in the current study (Fig. 7). Considering

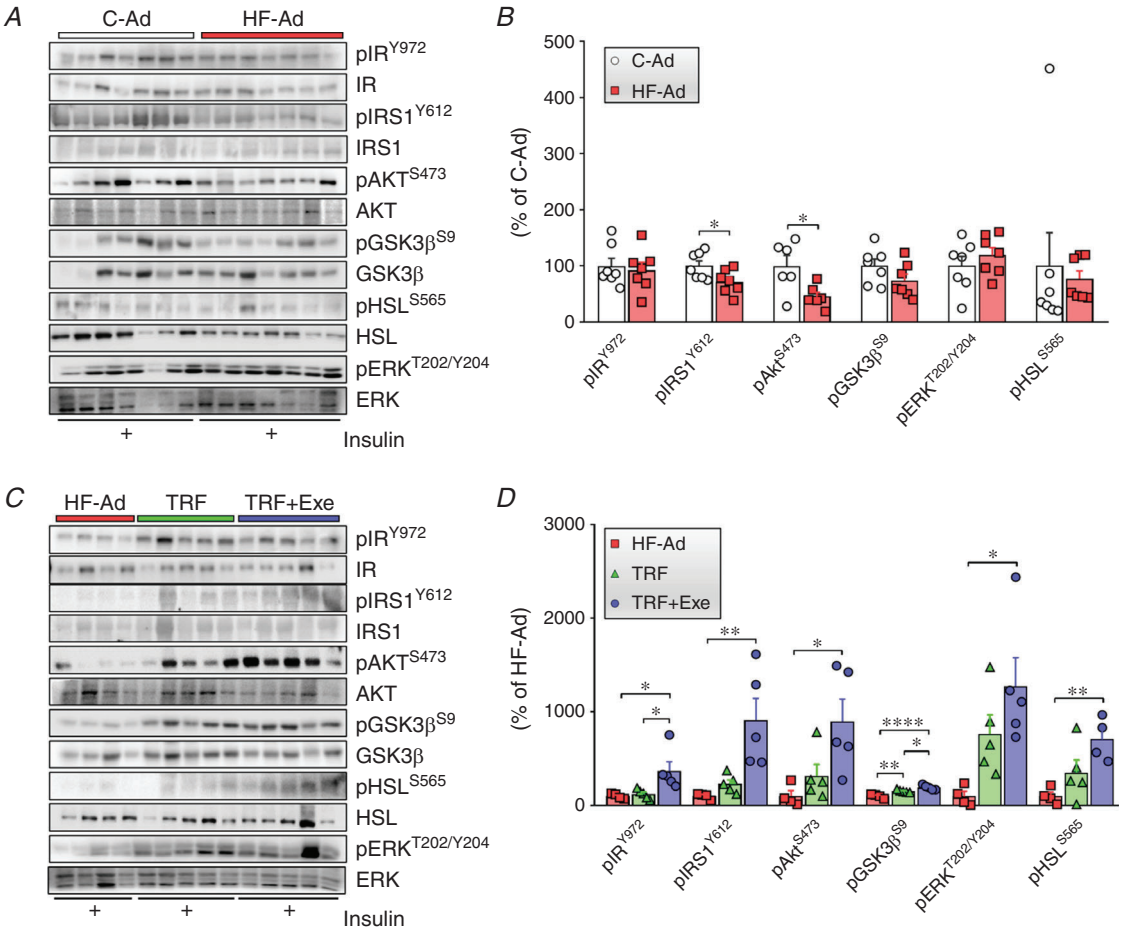

Figure 6. Time of feeding combined with aerobic exercise training improves insulin signalling in the liver
A, liver extracts with insulin stimulation (+ insulin) of the C-Ad and HF-Ad groups. *B*, proteins related to the insulin signalling pathway (pIR, pIRS1, pAKT, pGSK3B) and the fatty acid oxidation (pERK, pHSL) of the C-Ad ($n = 7$) and HF-Ad ($n = 7$) groups. *C*, liver extracts with insulin stimulation (+ insulin) of the HF-Ad, TRF and TRF+Exe groups. *D*, proteins related to the insulin signalling pathway (pIR, pIRS1, pAKT, pGSK3B) and fatty acid oxidation (pERK, pHSL) of the HF-Ad ($n = 4$), TRF ($n = 5$) and TRF+Exe ($n = 5$) groups. The bars represent means and SD of C-Ad, HF-Ad, TRF and TRF+Exe mice. Statistical significance was as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. [Colour figure can be viewed at wileyonlinelibrary.com]

the fatty acid oxidation genes, *Ppara* mRNA expression was higher for the TRF+Exe group than for the HF-Ad and TRF groups. The *Cpt1a* mRNA expression was significantly higher for the TRF and TRF+Exe groups than for the HF-Ad group (Fig. 7*A*). The *Acsl1*, *Acsl4* and *Acsl5* mRNA expression was higher for the HF-Ad group than for the C-Ad group (Fig. 7*A*). Regarding the lipogenesis genes, while the HF-Ad group had higher *Srebp1c* and *Cd36* mRNA expression than the C-Ad group, the TRF and TRF+Exe groups had lower *Srebp1c* and *Cd36* mRNA expression than the HF-Ad group (Fig. 7*B*). Finally, regarding the gluconeogenesis genes, the HF-Ad group had higher *Fbp1* and *Pc* mRNA expression than the C-Ad mice. On the other hand, the TRF group had lower *Fbp1* mRNA expression, and the TRF+Exe group had lower *Fbp1*, *Pck1*, and *Pgc1a* mRNA expression than the HF-Ad group (Fig. 7*C*). This demonstrates that interventions with TRF and TRF+Exe had a preventive effect on gluconeogenesis in relation to the group of HF-Ad mice. Figure 7*D* summarizes the main effects of the TRF and TRF+Exe interventions on molecular markers related to insulin signalling, fatty acid oxidation, lipogenesis and gluconeogenesis in the liver of young male Swiss mice fed a high-fat diet.

Discussion

The current investigation explored the effect of combining time-restricted feeding and aerobic exercise training (TRF+Exe) on the prevention of obesity-associated metabolic disorders in mice fed a high-fat diet. The TRF associated with AE training prevented body weight gain and glucose intolerance. Also, TRF+Exe preserved lipid homeostasis and insulin signalling in the liver. Besides demonstrating the protective effects of TRF+Exe against diet-induced obesity and metabolic diseases, our findings provided consistent proof that TRF+Exe offered additional metabolic benefits over TRF alone in preventing obesity, glucose intolerance and fatty liver disease.

After the fifth week, the body weight of the TRF+Exe group was lower than that of the TRF group. These findings corroborate investigations demonstrating the positive effects of TRF in body mass control (Hatori *et al.* 2012; Sherman *et al.* 2012) and reinforce TRF's role as a potential strategy for obesity treatment. When combined with AE training, these beneficial effects can be improved synergistically in mice fed a high-fat diet. Another study on Wistar rats performing the combination of TRF protocol with swimming exercise for 6 weeks also

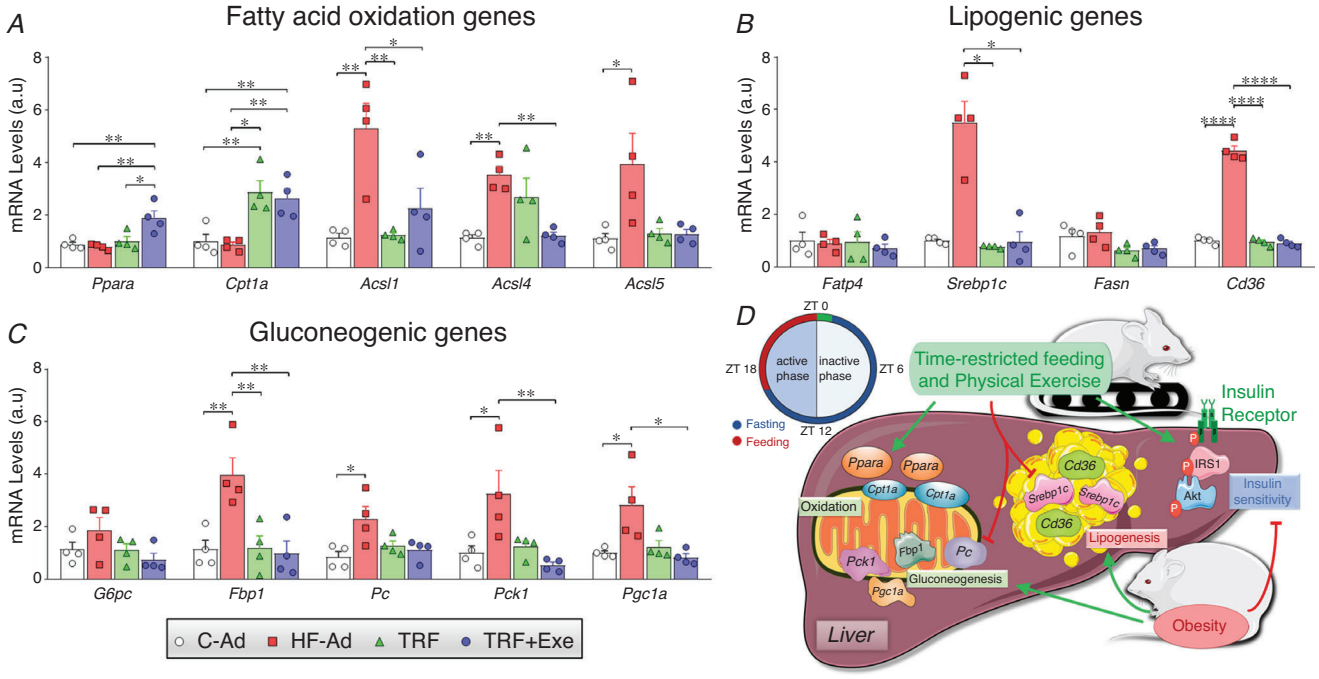

Figure 7. Time-restricted feeding combined with aerobic exercise training affect liver genes related to fatty acid oxidation, lipogenesis and gluconeogenesis
A, mRNA levels in genes related to fatty acid oxidation (*Ppara*, *Cpt1a*, *Acsl1*, *Acsl4*, *Acsl5*). *B*, mRNA levels in genes related to lipogenesis (*Fatp4*, *Srebp1c*, *Fasn*, *Cd36*). *C*, mRNA levels in genes related to gluconeogenesis (*G6pc*, *Fbp1*, *Pc*, *Pck1*, *Pgc1a*). The *Gapdh* housekeeping gene was used to normalize the mRNA levels. *D*, summary of the effects of TRF and aerobic exercise training on liver metabolism. The bars represent the means and SD of C-Ad, HF-Ad, TRF and TRF+Exe mice ($n = 4$). Statistical significance was as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. [Colour figure can be viewed at wileyonlinelibrary.com]

highlighted an additional effect in preventing increased body mass (de Moraes *et al*. 2017).

At least in part, the weight gain preventive effect in the TRF and TRF+Exe groups can be related to the lower cumulative caloric intake throughout the experiment. Restricting access to food is a strategy capable of reducing appetite and/or food intake in humans and rodents (Hatori *et al*. 2012; Lecheminant *et al*. 2013; Chausse *et al*. 2014; Wehrens *et al*. 2017; Gabel *et al*. 2018; Ravussin *et al*. 2019). It has been reported that intermittent fasting interferes with the metabolism and food intake of rats, with effects on hypothalamic hunger control mechanisms, leading to lower food efficiency and excessive intake (Chausse *et al*. 2014). On the other hand, other studies also suggest that the primary mechanism related to reducing weight gain with TRF is the increase in energy expenditure (Hatori *et al*. 2012; Bo *et al*. 2015; de Moraes *et al*. 2017). Rodents with free access to food for a restricted period of 8 h (i.e. at the beginning of their activity period) increase energy expenditure by 24 h, which was accompanied by increased thermogenesis in brown adipose tissue (Hatori *et al*. 2012). Our data show that the energy expenditure was higher for the TRF+Exe group, which might explain the reduction in body weight and fat depots for this group.

Furthermore, the aerobic exercise protocol's performance right after the end of the period of exposure to the high-fat diet may have contributed to the lower energy store in the form of fat. This association may be explained because the contracting muscle requires energy to maintain its activity. The analysis of indirect calorimetry showed that the TRF and TRF+Exe groups had a significantly lower RER ($\dot{V}_{CO_2}/\dot{V}_{O_2}$) during the period when food restriction occurred and higher RER during the period of exposure to food, which suggests a higher contribution of lipids in the food-deprived state and higher consumption of carbohydrates during the feeding state. These results were also observed in previous studies (Hatori *et al*. 2012; Woodie *et al*. 2018), indicating greater glycolysis and fat oxidation in response to the TRF. Besides, the TRF+Exe group had a higher $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ during the fed state and after an aerobic exercise training session. In a previous study, animals exposed to the TRF increased consumption of $\dot{V}_{O_2}$ during the fed state, which strengthens the idea that TRF increases oxidative metabolism (Chaix *et al*. 2019). Here, the results showed that exercise had intensified this increase in oxidative metabolism in mice submitted to TRF. Calorimetry test also showed a peak $\dot{V}_{O_2}$ after a period of running, reflecting a response to exercise known as excess post-exercise oxygen consumption (EPOC), which represents an additional energy expenditure to that generated during exercise, indicating an increase in oxidative metabolism (Gaesser & Brooks, 1984; Speakman & Selman, 2003; Pribyslavska *et al*. 2018). EPOC is a major metabolic modulator of body mass, and this response to AE training in our work may have contributed to the body mass control of the TRF+Exe group.

Exposure of the animals to a high-fat diet impaired carbohydrate metabolism, highlighted by the higher glucose and fasting insulin circulating levels and HOMA-IR index in the HF-Ad group, indicating a higher degree of whole-body insulin resistance. Similarly, other studies have also found adverse effects on glucose metabolism in animals fed a high-fat diet (Oliveira *et al*. 2015; Kuipers *et al*. 2019; Nakandakari *et al*. 2019; Brunetta *et al*. 2020). The adoption of TRF as a strategy to combat the high-fat diet-induced glucose metabolism disorders was relevant to the mice. Mice subjected to TRF improved glucose tolerance compared to the HF-Ad group. However, the effects were more robust in mice submitted to TRF associated with AE training. These animals were more tolerant to glucose than the HF-Ad and the TRF groups; TRF+Exe mice were also more sensitive to insulin than the HF-Ad group.

Regular exercise improves metabolism in various tissues. Therefore, the most pronounced improvement in glycaemic homeostasis found in the TRF+Exe group is probably due to increased glucose uptake capacity in other metabolic tissues (i.e. skeletal muscle, heart and fat) besides the liver (Stanford & Goodyear, 2016; Kraniou *et al*. 2004; Vettor *et al*. 2014; McGee & Hargreaves, 2020). For example, skeletal muscle contraction during physical exercise increases the energy demand, with consequent activation of the intramuscular metabolic pathways involved in the generation of ATP and general homeostatic regulation (Hawley *et al*. 2014; Febbraio, 2017). One of the adaptations involved with glycaemic improvement in response to exercise training is the increased expression of the type 4 glucose transporter (GLUT4), leading to enhanced insulin action and glucose uptake in skeletal muscle (Kraniou *et al*. 2004; Richter & Hargreaves, 2013). Therefore, regular physical exercise is an efficient stimulus to increase the expression of GLUT4 in skeletal muscle, contributing to better insulin action and decreased glycaemia, which were both observed in the TRF+Exe group.

Due to the possibility of TRF and AE having induced an increase in ketogenesis, we measured the serum levels of β-HB, the most abundant ketone body in mammals. Previous studies have shown that its function is not restricted to providing energy to other tissues. The role of β-HB is, directly and indirectly, to act as a signalling molecule affecting gene expression, lipid metabolism, neuronal function and metabolic rate (Newman & Verdin, 2017; Di Francesco et al. 2018; de Cabo & Mattson, 2019). Another study also indicated that β-HB could transmit energy from peripheral organs to the brain and be a peripheral signal for an increase in fasting food-anticipatory activity (Chaix, 2016). The depletion of hepatic glycogen levels during prolonged periods of fasting and exercise

promotes the migration of fatty acids from adipocytes to the liver, which are then metabolized through β-oxidation to produce β-HB, becoming the predominant energy source for other tissues, such as the brain and skeletal muscle (Newman & Verdin, 2017; Anton *et al*. 2018). Our study measured the levels of β-HB in the fed state and after 8 and 16 h of fasting. After 16 h of fasting, the TRF group had higher levels of β-HB than the C-Ad group, whereas the TRF+Exe group had higher β-HB levels than the C-Ad and HF-Ad groups. The β-HB data show that TRP+Exe stimulates ketone body production by increasing the levels of β-HB compared to the HF-Ad group. The effects of the increase in β-HB obtained through the combination of TRF with AE training should be explored in future studies to elucidate the impacts of this alteration on metabolism.

TRF and physical exercise can also have significant effects on the liver, a crucial organ for metabolism, and therefore are linked to the body's physiological changes and metabolic health. Hepatic fat accumulation occurs due to enhanced fat mass throughout the body, increasing the risk of heart and vascular diseases, as well as leading to non-alcoholic liver steatosis (Hatori *et al*. 2012; Chung *et al*. 2016; Melkani & Panda, 2017). In our histological analysis, we verified an accumulation of lipids in the liver of the HF-Ad group. In contrast, the TRF and TRF+Exe groups had their liver protected from this fat accumulation and presented a different phenotype from the HF-Ad group despite consuming the same diet. Other investigations also observed that exposure of mice to TRF and feeding a high-fat diet prevented liver fat accumulation compared to the group with *ad libitum* feeding (Hatori *et al*. 2012; Chung *et al*. 2016; Chaix *et al*. 2019). The differences in hepatic physiology between the TRF or TRF+Exe group and the HF-Ad group may be related to the increased oxidative metabolism in response to the TRF that promotes the use of fatty acids as an energy substrate during prolonged fasting. Thus, there was improved liver metabolism, increased oxidation and lipid lipolysis, and this prevented lipid accumulation in these animals. Serum dyslipidaemia is another consequence of high-fat diet-induced obesity (Namekawa *et al*. 2017; Udomkasemsab & Prangthip, 2019). Our study verified that the HF-Ad group showed hypertriglyceridaemia and hypercholesterolaemia, which was not observed in the TRF and TRF+Exe groups. These results found in the serum analysis and liver histology indicate that TRF concomitant with the AE training protocol contributed to lipid homeostasis and reduced fat accumulation. Previous studies have also found that TRF improves the lipid profile of rodents fed a high-fat diet (Hatori *et al*. 2012; Chausse *et al*. 2014; Chaix *et al*. 2019).

Furthermore, molecular analyses showed that mice exposed to a high-fat diet had decreased phosphorylation of IRS1 and Akt in the liver compared to control mice. This detrimental effect of exposure to a high-fat diet on the insulin signalling pathway in hepatic tissue has also been previously observed in rodents (Tremblay *et al*. 2001; Minokoshi *et al*. 2002; Lalli *et al*. 2008). The TRF group showed only an improvement in GSK3β phosphorylation compared to the HF-Ad group. On the order hand, the TRF+Exe group increased the hepatic phosphorylation of IR, IRS1, Akt, GSK3β, ERK and HSL, in relation to mice that received the high-fat diet. Other studies have shown that exercise can improve insulin signalling and positively influence liver metabolism (Heled *et al*. 2004; Trefts *et al*. 2015; Zhang *et al*. 2019). Based on our data, the combination of TRF with AE training seems to be an effective strategy to attenuate the negative effects of a high-fat diet on intracellular insulin action in the liver of mice. Mice exposed to a high-fat diet also increased the mRNA levels of lipogenic (*Srebp1c*, *Cd36*) and gluconeogenic (*Fbp1*, *Pc*) genes. However, the TRF group had decreased lipogenic gene expression (*Srebp1c*, *Cd36*). Interestingly, the mice submitted to the TRF and AE training protocols showed reductions in mRNA expression of lipogenic and gluconeogenic genes but increased mRNA expression of fatty acid oxidation genes (*Ppara*, *Cpt1a*).

TRF has been shown to reduce the expression of enzymes such as pyruvate carboxylase and glucose 6-phosphatase in the liver (Hatori *et al*. 2012; Chaix *et al*. 2014). These liver tissue adaptations were accompanied by reductions in glucose production in the liver and improvements in glycaemic homeostasis. Also, TRF is associated with a decrease in the genes for fatty acid synthase, stearoyl-CoA desaturase and fatty acid elongase and, consequently, with less storage of lipids (Hatori *et al*. 2012; Chaix *et al*. 2014). Physical exercise has been another strategy capable of attenuating hepatic glucose production in rodents fed a high-fat diet. This improvement promoted by physical exercise is related to regulating genes and critical proteins of gluconeogenesis such as phospoenolpyruvate carboxykinase and glucose 6-phosphatase (Pauli *et al*. 2014; Pereira *et al*. 2019, 2020; Gaspar *et al*. 2020). In our study, the combination of TRF with AE training prevented the increased expression of gluconeogenic genes in diet-induced obesity.

Furthermore, exercise has been shown to regulate lipid oxidation genes and fat accumulation in the liver (de Moura *et al*. 2013; Muñoz *et al*. 2018*a*,*b*; Gaspar *et al*. 2019). The decrease of free fatty acid (FFA) circulating levels in fasting and postprandial states, the increase of FFA uptake by skeletal muscle and the reduction of FFA uptake by the liver (Brouwers *et al*. 2016) are examples of the impact of physical exercise on the lipid profile that prevent non-alcoholic fatty liver disease (Shojaie *et al*. 2017). Here, TRF's combination with AE training seems advantageous for the circulating lipid profile of liver lipids in mice.

It is necessary to comment that in our study, the mice had access to food at night and, therefore, in their active phase. According to previous investigations, TRF in these conditions seems to promote more satisfactory results, mainly concerning the attenuation of weight gain and improvement of glycaemic homeostasis (Hatori *et al*. 2012; Lecheminant *et al*. 2013; Chaix *et al*. 2019). On the other hand, it was shown that when the TRF was applied in the daytime cycle and, therefore, in the animal's inactive phase, the results were more discreet or did not occur (Bray *et al*. 2010; Hatori *et al*. 2012; Rothschild *et al*. 2014; Freire *et al*. 2020). Thus, it becomes relevant to investigate whether the strategy adopted in our study of combining TRF with AE training would also be efficient if applied to the animal's daytime cycle. It is also necessary to highlight that AE training was performed at the end of the active cycle and after the feeding period of the mice. A previous experiment showed that eating in the morning or at noon followed by exercise in the evening could prevent weight gain more effectively than exercise in the morning followed by eating at noon or in the evening (Sasaki *et al*. 2014). Here, we verified the combination of TRF performed in the animal's night/active cycle combined with AE training right after the end of the TRF was effective in mitigating the metabolic impacts of exposure to a high-fat diet. Further investigations should evaluate whether different exercise types (resistance or high-intensity interval training) would minimize the adverse outcomes of eating a high-fat diet on liver metabolism and glycaemic homeostasis.

This study has several limitations. The result of the cumulative caloric intake in the TRF+Exe mice is expressive. Even with higher caloric expenditure, the animal presented a lower intake in the total of 10 weeks of the experiment. Thus, analysing the food intake on more days per week would be essential to confirm these findings. We also did not perform analyses at the hypothalamic level to assess whether adaptations to hunger control mechanisms occurred. Another limitation is the small number of mice used in our study (i.e. 4–7 animals/per group), which may have impacted the statistical significance of some relevant parameters such as food intake, insulin and fasting glucose. Consequently, most comparisons related to TRF's differential effects *versus* TRF+Exe seem to have little statistical power. Moreover, the inclusion of a group of mice exposed to a high-fat diet and submitted to physical exercise, without adding the TRF, should also be considered in future investigations and may help evaluate the additive effects of TRF's combined intervention with AE training.

In conclusion, these results demonstrated that a combination of TRF and AE training might be useful for minimizing the harmful effects of high-fat diet-induced obesity on glucose intolerance and fatty liver disease in young male Swiss mice.

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Additional information

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors of this study have no competing interests to declare.

Author contributions

R.F.L.V., V.R.M., R.L.J., F.O., S.C.B.R.N. and R.C.G. were responsible for the experiments, the data collection and tissue extraction. R.F.L.V., V.R.M. and J.R.P. were responsible for manuscript writing. R.F.L.V. and V.R.M. were responsible for the physiological, biochemical, WB, qPCR and histology analysis. A.S.R.S., D.E.C. and L.P.M. were responsible for the support technique and the manuscript review. J.R.P., L.P.M. and E.R.R. provided the laboratory support and discussed the manuscript. J.R.P., I.Z. and R.A.M. were responsible for the support technique and reviewed the manuscript. M.A.T., S.C.S. and V.R.M. assisted in histology and CLAM experiments and final review of the study. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Keywords

aerobic exercise training, insulin signalling, liver metabolism, obesity, time-restricted feeding (TRF)

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Statistical Summary Document