# EFFECTS OF INTERMITENT FASTING AND CHRONIC SWIMMING EXERCISE ON BODY COMPOSITION AND LIPID METABOLISM

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EFFECTS OF INTERMITENT FASTING AND CHRONIC SWIMMING EXERCISE ON BODY COMPOSITION AND LIPID METABOLISM

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Intermittent fasting protocol (IFP), has been suggested as a strategy to change body metabolism and improve health. The effects of IFP seem to be similar to aerobic exercise, having a hormetic adaptation according to intensity and frequency. However, the effects of combining both interventions are still unknown. Therefore, the aim of the present study was to evaluate the effects of IFP with and without endurance exercise training on body composition, food behavior, and lipid metabolism. Twenty weeks old Wistar rats were kept under an inverted circadian cycle of 12 hours with water ad libitum and assigned to four different groups: control group (CON; ad libitum feeding and sedentary); exercise group (EX; ad libitum feeding and endurance training); intermittent fasting group (IF; intermittent fasting and sedentary); intermittent fasting and exercise group (IFEX; intermittent fasting and endurance training). After six weeks, the body weight of IF and IFEX animals decreased without changes in food consumption. Yet, the body composition between the two groups was different, with the IFEX animals containing higher total protein and lower total fat content than the IF animals. The IFEX group also showed increases in total HDL cholesterol and increased intramuscular lipid content. The amount of brown adipose tissue was higher in IF and IFEX groups; however, the IFEX group showed higher expression levels of UCP-1 in this tissue, indicating a greater thermogenesis. The IFP combined with endurance training is an efficient method for decreasing body mass and altering fat metabolism, without inflicting losses in protein content.

Keywords: Aerobic Exercise; Endurance Training; Fat Metabolism; Thermogenesis; Fasted Exercise;
INTRODUCTION

Intermittent fasting protocol (IFP), a changing in feeding periods characterized by fasting and feeding cycles, has been suggested as a strategy to change body metabolism and improve health (Horne et al. 2015; Tinsley and La Bounty 2015). Many potential beneficial phenotypes are associated with IFP, such as improvements in body weight and energy expenditure (Halberg et al. 2005), blood lipid profile (Benli Aksungar et al. 2005), heart function (Ahmet et al. 2005), eating behavior (Chausse et al. 2014), trophic factors and cognitive ability (Halagappa et al. 2007; Li et al. 2013). Mechanistically, IFP seems to reduce body weight and increase energy expenditure by activating brown adipose tissue (BAT) nonshivering thermogenesis as well as browning of white adipose tissue (WAT).

Thus, the brown-like adipocyte, which is found in small quantities in WAT and in large amounts in BAT, is a potential therapeutic target against obesity. Exercise and diet intervention can modify the lipid metabolism and stimulate the expression and activation of this adipocyte in BAT and WAT (De Matteis et al. 2013). The efficiency of adipocyte thermogenic activation is related to the amount of UCP-1 in the tissue. This protein uncouples ATP production and promotes thermogenesis. Additionally, Qiang et al. (2012) showed that Sirt1 dependent deacetylation of Pparγ can promote browning of WAT, increase BAT hypertrophy, and regulate thermogenesis in these tissues.

It is believed that IFP promotes a survival response to stress similar to that induced by exercise (Tapia 2006). Similar to IFP, exercise has been shown to induce a hormetic cellular stimulus to adaptation resulting in mitochondrial biogenesis by Sirt1/AMPK pathway activation (Longo and Mattson 2014).
Despite those similarities, there are also many different beneficial phenotypes associated with IFP and exercise such as Sirt1 activation, resulting in a greater lifespan and fat mobilization by modulation of PPAR (Hayashida et al. 2010), a protein modulated by AMPK (Longo and Mattson 2014), indicating that a combination of both stresses could result in stronger beneficial actions to metabolic health. Indeed, some studies have found increased mitochondrial activity and exacerbated utilization of intramyocellular stored lipids by the combination of IFP and exercise (Loon et al. 2003; Van Proeyen et al. 2011), suggesting an enhanced lipid oxidative metabolism. However, the effects of combining these two types of stress on BAT and WAT metabolic activity were not yet properly investigated. Therefore the aim of the present study was to evaluate the effects of IFP combined with endurance exercise training on body composition, food behavior and lipid metabolism in rats.
METHODS

Our experimental method was designed to investigate how combining IFP (six weeks) with exercise training (swimming) affects body composition and lipid metabolism of Wistar rats (Figure 1).

FIGURE 1 MUST BE HERE

Ethical Aspects

This research was performed according the Principles of Laboratory Animal Care formulated by the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985), and was approved by local Ethics Committee in Animal Research (N. 337/2015).

Samples

Wistar rats (twenty weeks-old) were kept under inverted circadian cycle of 12 hours (lights on at 7 p.m. and lights off at 7 a.m.) with water *ad libitum* and assigned into four different groups: control (CON; *ad libitum* feeding and sedentary and); exercise (EX; *ad libitum* feeding and endurance training); intermittent fasting (IF; intermittent fasting and sedentary); intermittent fasting + exercise (IFEX; intermittent fasting and endurance training).

Exercise

Endurance exercise training was performed 5 sessions per week, for six weeks. Every training session consisted of swimming exercise for 40 minutes in a 60cm
deep tank containing water at a constant temperature of 31ºC ± 2. Exercise training was performed at 10 p.m. in the dark cycle (Figure 1). In order to normalize density differences caused by different body compositions, training loads \( Tl \) were determined as 3% of total body mass \( Bm \) adjusted by the hydrostatic weight \( Hw \).

\[
Tl = 0.03 \times (Bm - Hw) \tag{1}
\]

To avoid hypothermia and brown adipose tissue activation, after training, the animals were dried with a towel and placed in a heated chamber until dry.

**Intermittent Fasting**

The IFP was performed continuously over six weeks, 18h/day, extending throughout the light period and half of the dark period (Figure 1). The *ad libitum* period began immediately after exercise, and lasted for 6 hours, until the start of the light cycle.

**Body Mass and Food Consumption**

Body weight was measured before and throughout the 6-week protocol period. Food intake was measured using semi-metabolic cages. Food Efficiency Ratio \( (FER) \) was calculated using the formula described below:

\[
FER = \frac{Fm - Im}{Tc} \tag{2}
\]

Where \( Fm \) is the final mass, \( Im \) is the initial mass and \( Tc \) is the total food consumption in the period.

**Euthanasia**
Animals were anesthetized with ketamine (90mg/kg) and xylazine (8mg/kg), and euthanized by exsanguination via cardiac puncture. The liver, muscle gastrocnemius and interscapular fat (brown adipose tissue) were then collected for analysis.

**Body Composition**

The bodies of the animals, minus the collected organs, were weighed and dried at 105°C for 48h, and then weighed again. The difference between the initial and final weight is equivalent to the total amount of body fluid of the animal. In sequence, the dry carcass, without the head, was totally crushed to form a homogeneous and particulate sample. Aliquots of this homogenous sample were used to perform, in triplicate, analysis of total protein using the micro-Kjehldal method (Ma and Zuazaga 1942), total fat quantified by distillation with petrol ether in a hot Soxleht balloon (Manirakiza et al. 2001) and total minerals by 600°C incineration in a muffle furnace.

**Intramuscular Lipid**

The red portion of the gastrocnemius muscle (500 mg), was homogenized in a test tube and lipid extracted with chloroform/methanol by the method of Bligh and Dyer (1959). After evaporation of the chloroform in a ventilated oven at 60°C, the glass tube was weighed to obtain the total weight of intramuscular lipid. The calculation was performed by the difference in the weighing beaker divided by the initial mass of tissue used.

**Hepatic Lipid Profile**
Liver (500 mg) was homogenized in an assay tube and lipid extracted as described above for the gastrocnemius muscle. After evaporation of organic solvents and weighing, lipids were resuspended in 1 ml of iso-propyl alcohol and utilized for the quantification of total HDL and LDL cholesterol using specific kits (Labtest, Minas Gerais, Brazil)

**Immunoblotting**

Immunoblotting was performed as previously described (Magdalon et al. 2016). Briefly, the interscapular brown adipose tissue was harvested, dissected, homogenized in a buffer containing 50 mM HEPES, 40 mM NaCl, 50 mM NaF, 2 mM EDTA, 10 mM Na₄P₂O₇, 10 mM C₃H₇Na₂O₆P, 2 mM Na₃VO₄, 1% Triton-X 100, and EDTA-free protease inhibitors, and centrifuged at 13,000 rpm for 10 min at 4°C. Protein quantification of the supernatant was performed by the colorimetric method of Bradford (Bio-Rad, Hercules, CA, USA). The supernatant was then mixed with Laemmli buffer (0.1% bromophenol blue; 1M sodium phosphate, pH 7.0, 50% glycerol and 10% SDS) and 30 ug of protein per sample were subjected to SDS-PAGE. Proteins were then transferred to a polyvinylidene difluoride membrane (0.2 µm in diameter), incubated with the following specific primary antibodies for proteins of the mitochondrial membrane respiratory chain function – OxPhos (Abcam Plc., #ab110413) and UCP-1 (Cell Signaling Technology Inc., #14670s), washed, incubated with secondary antibodies, and developed using a chemiluminescent kit (Bio-Rad, Hercules, CA, United States). Densitometric analyses were performed on the bands, using the Image Studio Digits V.4.0. software (Li-Cor, Lincoln, United States).
Statistical Analysis

Results were tested for normality with the Kolmogorov-Smirnov test. Subsequently, Two-way Analysis of Variance – ANOVA followed by post-hoc Newman-Keuls analysis were performed to evaluate differences between groups. Significance level was considered $p < 0.05$. 
RESULTS

Figure 1 depicts the body weight, food intake, and energy efficiency of rats from the different groups. The IF rats displayed a trend for a decrease in body weight, whereas the IFEX rats had a significantly reduced body weight in the third and sixth weeks, when compared to CON and EX groups (Figure 2A). Despite the changes in body mass, no significant changes in food intake were observed among the groups, indicating perhaps an involvement of energy expenditure as a driver of the changes in body weight. Indeed, IF and IFEX rats displayed a reduced energy efficiency, an indicator of enhanced energy expenditure, in comparison to CON rats at week 6 of the protocol (Figure 2C).

FIGURE 2 MUST BE HERE

Regarding body composition (Table 1), IF did not induce changes in lipid, protein, minerals, or body water content. While EX and IFEX displayed reduced fat content and increased total protein, as compared with IF, and showed no changes in mineral or body water content when compared to CON rats.

TABLE 1 MUST BE HERE

Analyzing the hepatic lipid content (Table 2), showed no differences in total lipids or total LDL cholesterol. However, the IFEX group displayed increased HDL cholesterol when compared to the CON group.

TABLE 2 MUST BE HERE
There were muscular lipid content alterations only in the IFEX group (Figure 3), when we can note increases in the IFEX group compared to the IF and EX groups. This difference was influenced by fasting ($p = 0.0388$) and principally the interaction of fasting and exercise ($p = 0.0031$).

**FIGURE 3 MUST BE HERE**

It was observed in the analysis of the interscapulum BAT mass (Figure 4), that the IF and IFEX animals have increased BAT mass compared to CON and EX groups. The influence of diet in this difference was observed in the ANOVA test ($p = 0.0003$).

**FIGURE 4 MUST BE HERE**

Immunoblots of proteins related to thermogenesis in BAT (Figure 5), showed an increased expression of UCP-1 protein in EX and IFEX groups compared to IF and CON groups. This suggests that exercise has an influence on UCP-1 expression ($p < 0.0001$). The greatest significance of IFEX results indicates an increased influence of interaction of IFP and exercise in results. The influence of exercise ($p = 0.0028$) and IFP ($p = 0.0318$) are noted in NDUFB8 expression, a protein of respiratory chain complex I, which increases in the IFEX group, when compared with all groups. The proteins representing complexes III and V of the respiratory chain showed no statistical differences in expression.

**FIGURE 5 MUST BE HERE**
DISCUSSION

Body mass, Feed Efficiency and Food Consumption

The data analysis related to body mass (Figure 2A), showed a significant decrease in the IFEX group, suggesting that diet and exercise intervention was effective in lowering the body mass of rats. On the other hand, as shown in figure 2B, there are no differences in food consumption among all groups. These data together show that although IFEX rats have a decreased body mass, the food consumption was not changed. It should be pointed out that the amount of exercise and food consumption of the IFEX group are the same as the EX group, yet the final body masses are different.

The study of Chausse et al. (2014), has showed that intermittent fasting alters the metabolic pattern of rats, and modifies the food consumption and hypothalamic parameters of hunger control, which results in lower feeding efficiency and overeating. The authors discuss that these alterations may be caused by overexpression of neuropeptide Y, due to the diet. This peptide stimulates food intake, decreases thermogenesis, and increases plasma levels of insulin and corticosterone, thus making it a potential target to treat obesity (Zheng et al. 2013). In contrast, our results showed that the IF group, which consumed the same amount of food as the ad libitum groups, presented a lower feeding efficiency due to a decrease in body mass. These findings suggest that the increase in expression of neuropeptide Y alone can not account for the alteration in metabolism observed in the IF and IFEX groups.
Body Composition

The body composition of animals is altered only in protein and lipid compartments, suggesting that the interaction between fasting and exercise have an intrinsic relationship in these tissues (Table 1). It should be noted that exercise was efficient in controlling body composition, and decreasing body lipid independently of the feed profile. Lipid accumulation observed in the IF group seems to be reversed by exercise performed by the IFEX group. This change may be related to the regulation of fsp27 protein, which has already been shown to modulate fat storage in WAT, during cycles of 3 days of IFP (Karbowska and Kochan 2012). Furthermore, endurance exercise can negatively regulate fsp27 through AMPK activation (Zhang et al. 2014).

With regards to total protein, the IFEX and EX groups showed increased levels of total protein, when compared to the IF group. Meaning that exercise prevents protein loss even during the IFP, and suggests that exercise can block protein degradation caused by fasting.

Together, these results show that despite IF and IFEX groups displaying the same pattern of alterations in body mass (Figure 1A), the mechanisms that lead to these alternations are different, and is likely the result of the cross-talk between exercise and intermittent fasting.

Hepatic and Muscular Lipid Content
The lipid content of the liver show, that even though there are no alterations in the total lipid amount among all groups, there is an increase in HDL cholesterol content observed in the IFEX group. These findings suggest that the transport of free fat acids is increased during IFP and exercise. A study by Varady et al. (2007) tested the effects of 24h IFP and showed an increase in free-fatty acids and a diminished size in fat cells of the inguinal and epididymal adipose tissue, which indicates a high consumption of these tissues in the absence of energy derived from the diet. Interestingly, we did not find these adipose cushions in the IFEX group in sufficient quantity for differentiation and dissection. This interpretation may be reinforced due to the fact that the amount of intramuscular lipid increased only in the IFEX group. The cellular adaptations to fasted exercise can include the translation of macronutrient preferences due to starvation, thus increasing consumption of lipid reserves and enhancing the lipid reserves in muscle. The intramuscular lipid reserves are an important substrate source during acute exercise and considered to be a beneficial adaptation to exercise in the fasted state (Loon et al. 2003). Van Proeyen et al. (2011) showed that six-weeks of fasted exercise stimulates lipid consumption in muscle, showed by increases in activity of both citrate synthase and β-Hydroxyacyl-CoA dehydrogenase. We believe that our results indicate an adaptation to fasted exercise, which led to an accumulation of intramuscular lipid that was used as a substrate during exercise. These positive results were also accompanied by an improvement in cellular oxidative stress in both tissues\(^1\), indicating that the strategy and the resulting increase in muscle lipid content does not appear to cause damage to the organism.

\(^{1}\) Supplementary material
**Brown Adipose Tissue**

The lessened food efficiency can be explained in part by the increased mass of BAT in IF and IFEX rats, and the increased expression of UCP-1 protein in the EX and IFEX groups. Both of these factors indicate a thermogenic adaptation of the tissue. The thermogenesis generated by BAT is considered a potential target in the treatment of obesity (Cypess and Kahn 2010). Together, the increased BAT mass by the IFP, and the increased UCP-1 content due to exercise can lead to augmented activation of thermogenesis, and contribute to greater energy expenditure in the IFEX rats. Several studies investigating the role of BAT in IFP indicate an influence in this tissue, but the results are still unclear. Desautels and Dulos (1988) have shown that IFP in a period of 1 day of fasting and 2 days of refeeding results in decreased of BAT mass and activity in mice. A study performed by López-Ibarra et al. (2015) show that after acute 24h of fasting, BAT expression of fatty acid transporters CPT1 and CPT2 and enzyme CoA dehydrogenase are increased compared to WAT, suggesting that under fasting conditions at physiological temperature the BAT increases the activation by gluconeogenesis. Our findings show that the greater expression of UCP-1 and BAT hypertrophy can be an adaptive response to chronic intermittent fasting and a potential target for increasing the metabolism expenditure.

**CONCLUSION**
The combination of intermittent fasting and moderate aerobic exercise is capable of modifying lipid metabolism, and can promote changes in body composition (i.e., inducing weight loss with maintenance of lean body mass). The metabolic alterations are probably the result of BAT thermogenesis adaptation and reduced food efficiency. The cycles of fasting and refeeding must be explored in order to understand how modulation of these cycles in combination with exercise and resting cycles could modify the mechanistic responses of lipid metabolism.

CONFLICT OF INTEREST
The authors report no conflict of interest associated with this manuscript.

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Table 1: Body percentage of lipids, proteins, minerals and water in rats submitted to 6 week intermittent fasting (IF), or exercise training (EX) or the combination of both (IFEX).

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<thead>
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<th>CON</th>
<th>EX</th>
<th>IF</th>
<th>IFEX</th>
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<td>Lipids</td>
<td>7.36±0.49</td>
<td>5.37±1.08*</td>
<td>9.15±0.84</td>
<td>5.72±0.87*</td>
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<tr>
<td>Proteins</td>
<td>19.86±1.07</td>
<td>21.35±0.50*</td>
<td>18.41±0.67</td>
<td>22.88±0.90*#</td>
</tr>
<tr>
<td>Minerals</td>
<td>3.59±0.28</td>
<td>3.90±0.09</td>
<td>3.45±0.18</td>
<td>4.30±0.30</td>
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<tr>
<td>Water</td>
<td>67.56±1.54</td>
<td>67.87±0.84</td>
<td>66.91±0.23</td>
<td>64.88±0.98</td>
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* = p<0.05 to IF; # = p<0.05 to CON. Data was expressed by mean ± SEM.
Table 2: Hepatic total lipid and cholesterol, LDL and HDL (mg/g).

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<th>IF</th>
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<td><strong>Total Lipid</strong></td>
<td>38.53±0.77</td>
<td>36.0±2.81</td>
<td>43.6±3.67</td>
<td>43.75±1.71</td>
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<td><strong>Total Cholesterol</strong></td>
<td>1.925±0.23</td>
<td>2.63±0.41</td>
<td>2.509±0.43</td>
<td>2.714±0.60</td>
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<tr>
<td><strong>LDL Cholesterol</strong></td>
<td>1.596±0.19</td>
<td>2.17±0.40</td>
<td>1.987±0.43</td>
<td>1.943±0.66</td>
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<tr>
<td><strong>HDL Cholesterol</strong></td>
<td>0.329±0.06</td>
<td>0.456±0.02</td>
<td>0.521±0.05</td>
<td>0.770±0.16*</td>
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* = p<0.05 to CON. Data was expressed by mean ± SEM.
**Figure 1.** Dark-light cycle and weekly procedures of exercise and fasting during 6 weeks of training. Body Weight = (BW) and Food Efficiency Ratio = (FER)

**Figure 2.** Body weight (A), food intake (B) and energy efficiency (C) in rats under intermittent fasting (IF), or endurance swimming exercise training (EX), or combination of both (IFEX) for 6 weeks. * = p<0.05 and *** = p<0.001 to CON; # = p<0.05 and ### = p<0.001 to EX group; & = p<0.05 to IF group. Data was expressed by mean ± SD. N=6-7

**Figure 3.** Intramuscular lipid content of red portion of m. gastrocnemius.* = p < 0.05; ** = p < 0.001. Data was expressed by mean ± SEM. N=6-7

**Figure 4.** Relative mass of interscapulum fat brown adipose tissue.* = p < 0.05; ** = p < 0.001. Data was expressed by mean ± SEM. N=6-7

**Figure 5.** Brown Adipose Tissue Protein Expression of (A) UCP-1, and proteins of oxidative phosphorylation (B) NDUFB8 (Complex I), (C) UQCRC2 (Complex III), (D) ATP5A (Complex V) and MTCO1 (House Keeping). The data acquired of immunoblot assay was quantified by detection of wells color density + area of bands – Background density and normalized by endogenous protein. All the wells were filled with 30ug of protein. * = p < 0.05; ** = p < 0.001. Data was expressed by mean ± SEM in arbitrary units. N=6-7
Dark-light cycle and weekly procedures of exercise and fasting during 6 weeks of training. Body Weight = (BW) and Food Efficiency Ratio = (FER)

128x97mm (300 x 300 DPI)
Body weight (A), food intake (B) and energy efficiency (C) in rats under intermittent fasting (IF), or endurance swimming exercise training (EX), or combination of both (IFEX) for 6 weeks. * = p<0.05 and *** = p<0.001 to CON; # = p<0.05 and ### = p<0.001 to EX group; & = p<0.05 to IF group. Data was expressed by mean ± SD. N=6-7
Intramuscular lipid content of red portion of m. gastrocnemius. * = p < 0.05; ** = p < 0.001. Data was expressed by mean ± SEM. N=6-7.
Relative mass of interscapulum fat brown adipose tissue. * = p < 0.05; ** = p < 0.001. Data was expressed by mean ± SEM. N=6-7.

104x71mm (300 x 300 DPI)
Brown Adipose Tissue Protein Expression of (A) UCP-1, and proteins of oxidative phosphorylation (B) NDUFB8 (Complex I), (C) UQCRC2 (Complex III), (D) ATP5A (Complex V) and MTCO1 (House Keeping). The data acquired of immunoblot assay was quantified by detection of wells color density + area of bands – Background density and normalized by endogenous protein. All the wells were filled with 30ug of protein. * = p < 0.05; ** = p < 0.001. Data was expressed by mean ± SEM in arbitrary units. N=6-7