

Maternal overfeeding during lactation, rather than overweight per se, impairs the metabolic response to fed/fasting changing conditions in the post-weaning offspring

Running title: Early metabolic inflexibility and AMPK α 1

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Abbreviations: O-PCaf, offspring of post-cafeteria dams; O-CAF, offspring of cafeteria diet-fed dams during lactation; O-C, offspring of control dams; PN, postnatal day; TOFI, thin-outside-fat-inside; WAT, white adipose tissue.

Abstract

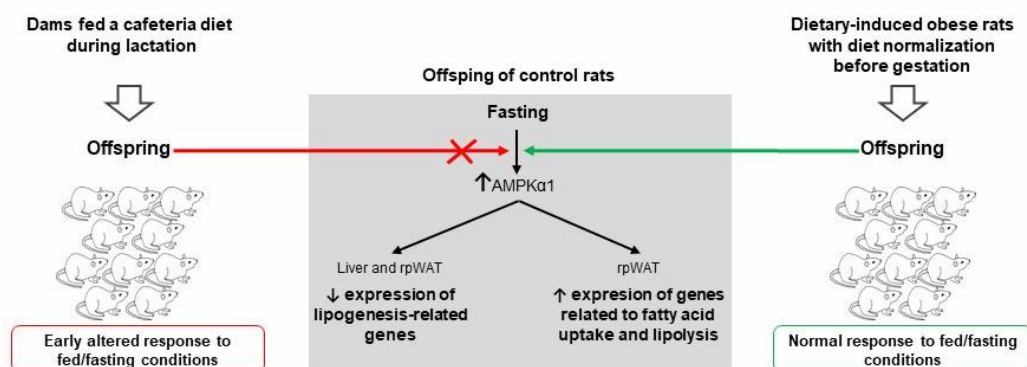
Scope. We studied the metabolic response to fed/fasting changing conditions at early age in rats with different predisposition to obesity-related alterations due to maternal conditions during the perinatal period.

Methods and Results. Offspring of dams made obese by cafeteria diet and moved to a normal fat diet one month before gestation (O-PCaf, with an apparently normal phenotype in adulthood), and offspring of cafeteria diet-fed dams during lactation (O-CAF, with a thin-outside-fat inside phenotype), together with the offspring of control dams (O-C), were studied at early age. Fasting was associated with downregulation of lipogenesis-related genes in liver and rpWAT, and upregulation of genes related to lipolysis and fatty acid uptake in rpWAT in O-C animals. The response to fed/fasting conditions was impaired in O-CAF, but not in O-PCaf animals. The fasting-induced increase in the expression of *Prkaa1* in liver and rpWAT, and the corresponding increase of hepatic AMPK α 1 protein levels of O-C animals were attenuated in O-CAF rats, while no alterations were found in O-PCaf animals versus controls.

Conclusion. Maternal intake of a cafeteria diet during lactation causes early alterations in the offspring, impairing their metabolic flexibility in response to fed/fasting changing conditions, which could contribute to hinder energy homeostasis maintenance.

Graphical Abstract.

Maternal intake of a cafeteria diet during lactation, rather than maternal obesity itself, impairs the response to fed/fasting changing conditions in the offspring



1. Introduction

A sufficient energy supply is essential for life. The capacity to adapt fuel oxidation to fuel availability is termed metabolic flexibility, which involves a healthy combination of underlying biochemical mechanisms. In turn, impaired ability to adapt tissue-specific substrate oxidation to fuel availability, known as metabolic inflexibility, reflects metabolic alterations, which has been suggested to occur early in the pathogenesis of insulin resistance, and is closely associated with type 2 diabetes and obesity [1-6]. This alteration may be caused by deficient or inappropriate nutrition during sensitive stages of development and, for example, previous results from our group have shown that a short period of exposure to a cafeteria diet in early infancy in rat offspring is enough to disturb the metabolic response to fed/fasting conditions in liver and white adipose tissue [7].

The liver plays a pivotal role in the storage and release of carbohydrates and in fatty acid synthesis, making it a key tissue in the handling of metabolic homeostasis during fed to fasting transition and *viceversa*. The adaptation to fasting involves the progressive shift from carbohydrate toward lipid oxidation, generally characterized by increased lipolysis, lipid oxidation, and ketone body synthesis, along with a decrease in glucose uptake and oxidation by peripheral tissues [8, 9]. The breakdown of triglycerides through the lipolytic pathway to provide fatty acids and glycerol as fuels to peripheral tissues is activated during fasting in the white adipose tissue (WAT) [9, 10]. In both tissues, liver and WAT, various energy/nutrient-sensing pathways are involved to ensure the maintenance of nutrient homeostasis. Among them, the principal monitor of cellular energy sensor is a widely-expressed serine/threonine protein kinase, the AMP-activated protein kinase (AMPK) [11]. Once activated, AMPK phosphorylates multiple targets leading to an inhibition of energy consuming biosynthetic pathways, and activation of catabolic pathways [11].

We have described that maternal consumption of a cafeteria diet throughout lactation in rats produces lasting effects in the metabolic health of their offspring, related to the so-called thin-outside-fat-inside (TOFI) phenotype [12]. Interestingly, cafeteria diet removal in obese rats before gestation may prevent the expected lasting effects in offspring which appear in the adulthood [13]. Thus, the offspring of cafeteria and post-cafeeteria dams are animal models that may provide a useful tool to

study early potential alterations in metabolic flexibility of the offspring in response to fed/fasting changing states. Therefore, the objective of this study was to analyse the capacity of adaptation to acute changes in feeding conditions (fed/fasting states) at an early age in two animal models with different predisposition to obesity-related metabolic alterations due to maternal conditions during the perinatal period. Subsequently, we studied the offspring of dams fed a cafeteria diet during lactation, and the offspring of rats made obese by cafeteria diet, after dietary normalization one month before gestation (post-cafeeteria dams). We focused on the expression of key genes in two insulin-sensitive tissues (liver and adipose tissue) to assess early signs of metabolic inflexibility, and on the relationship between fasting and AMPK.

2. Experimental Section

2.1. Ethics statement

All animal experimental procedures followed in this study were reviewed and approved by the Bioethical Committee of the University of the Balearic Islands, and guidelines for the use and care of laboratory animals of the University were followed.

2.2. Animals and experimental design

The study was performed in young rats from two previous independent studies related to maternal obesity models [12, 13]. The scheme of both experimental designs was depicted in Figure 1.

Model 1: Offspring of dams fed a cafeteria diet during lactation (O-CAF)

In this experimental approach, we studied at weaning (postnatal day (PN) 22) the response to fed/fasting conditions in the offspring of cafeteria diet fed dams during lactation (O-CAF). In addition, the offspring of control dams (O-C) were analysed. Briefly, virgin dams fed with a standard chow diet (control) (3300 kcal kg⁻¹; Panlab, Barcelona, Spain) were mated to control males. At PN1, litters were adjusted to 10 pups per dam and dams were assigned to either control or cafeteria group (n=8). Control dams continued with the control diet and dams of the cafeteria group were exposed to a cafeteria diet in addition to the standard chow. The cafeteria diet included: biscuits with a Majorcan sausage ('sobrasada') and with liver pate, salted peanuts, chocolate, candies, carrots, fresh bacon,

cheese, sugared milk (20% w/v) and a Majorcan pastry ('ensaimada') [12]. Dams fed with the cafeteria diet consumed a diet with lower protein and higher fat content versus controls [12]. Littermates from the same cohort of animals were used in a previous study aimed to characterise the lasting effects of the maternal intake of a cafeteria diet during lactation [12].

Model 2: Offspring of postcafeteria dams (O-PCaf)

In a second experimental approach, we studied at weaning (PN26) the response to fed/fasting conditions in the offspring of control (O-C), and post-cafeteria dams, which after a period of 100 days with cafeteria diet, they were submitted to dietary normalization for one month before mating [13]. We will refer to the offspring of post-cafeteria dams as O-PCaf. Littermates from the same cohort of animals were used in a previous study aimed to characterise the lasting effects of maternal diet normalization before mating [13].

In both experimental designs, at PN21, animals were weaned on the same diet as that of their dams during lactation till sacrifice and offspring were killed at PN22 (Model 1) and PN26 (Model 2) under *ad libitum* feeding conditions or after 12-h fasting, by decapitation during the first 2 h at the beginning of the light cycle. Liver and retroperitoneal white adipose tissue (rpWAT) were rapidly removed and frozen in liquid nitrogen and stored at -70°C until analysis.

2.3. RNA extraction

Total RNA was extracted from liver and rpWAT with Tripure Reagent (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions. Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (Nadrop Technologies Wilmington, DE, USA) and its integrity confirmed using agarose gel electrophoresis.

2.4. Real-time quantitative RT-PCR analysis

Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was used to measure mRNA expression levels of selected genes in the liver and rpWAT. Specifically, we analysed protein kinase AMP-activated catalytic subunit alpha 1 (*Prkaa1*), protein kinase AMP-activated catalytic subunit alpha 2 (*Prkaa2*), sterol regulatory element binding transcription factor 1 (*Srebf1*),

fatty acid synthase (*Fasn*), stearoyl-Coenzyme A desaturase 1 (*Scd1*), acetyl-CoA carboxylase alpha (*Acaca*), and glucokinase (*Gck*) in liver; and *Prkaa1*, *Prkaa2*, peroxisome proliferator-activated receptor gamma (*Pparg*), *Srebf1*, *Fasn*, lipoprotein lipase (*Lpl*) and patatin-like phospholipase domain containing 2 (*Pnpla2*) in rpWAT. 18S ribosomal RNA (18S) and GDP dissociation inhibitor 1 (*Gdi*) were used as reference genes.

0.25 µg of total RNA (in a final volume of 5 µl) was denatured at 65 °C for 10 min and then reverse transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 20°C for 15 min, 42 °C for 30 min, with a final step of 5 min at 95 °C in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystem, Madrid, Spain). Each PCR was performed from diluted cDNA template, forward and reverse primers (1 µM each), and Power SYBER Green PCR Master Mix (Applied Biosystems, CA, USA). Primers for the different genes analyzed are provided in Supplementary Information Table S1. All primers were obtained from Sigma. Real-time PCR was performed using the Applied Biosystems StepOnePlus™ Real-Time PCR Systems (Applied Biosystems) with the following profile: 10 min at 95 °C, followed by a total of 40 two-temperature cycles (15 s at 95 °C and 1 min at 60 °C). In order to verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle (Ct) was calculated by the instrument's software (StepOne Software v2.2.2, Applied Biosystem) and the relative expression of each mRNA was calculated as previously described [14].

2.5. Western blot

Western blot was performed to determine the protein levels of AMPKα1 and AMPKα2 in the liver and rpWAT of male animals. Tissue was homogenized at 4°C in 1:5 (w:v) in RIPA buffer containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher, Rockford, IL, USA). 25 µg (for liver) of RIPA protein extracts were separated in a 4-20% SDS-PAGE (Criterion™ TGX™, Bio-Rad Laboratories, Madrid, Spain), and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). After blocking, membranes were incubated with the corresponding primary antibodies (see Supplementary Information Table S2), and then with the infrared-dyed secondary anti-IgG antibodies (Li-COR Biosciences, Lincoln, NE, USA). For infrared detection, membranes were scanned in

Odyssey Infrared Imaging System (LI-COR Biosciences), and the bands were quantified using the software Odyssey v3.0 (LI-COR Biosciences). β -Actin was used as transfer and loading control.

2.6. Statistical Analysis

No blinding was carried out for data analysis. Each study was considered separately and results on gene expression or protein levels were expressed versus their corresponding controls. In addition, male and Female animals were considered separately. Data are expressed as the mean $\pm$ SEM (n=8-12). Data were checked for normality using Shapiro-Wilks normality test. For multi-group comparisons, Levene's test was performed to assess whether the variance is equal between groups; if the variance was heterogeneous, data were log-transformed before analysis. Two-way analysis of variance (ANOVA) was used to determine, separately for each sex (male and female), the effects of different factors: maternal group (cafeteria diet versus control or post-cafeteria versus control) and feeding conditions (*ad libitum* versus fasting). Single comparisons were assessed by U-Mann Whitney test. The test used for each comparison is indicated in the footnote of the Figures. The analyses were performed with SPSS for Windows (SPSS, Chicago, IL). Threshold of significance was defined at $p < 0.05$.

3. Results

3.1. Effects of fasting in body weight

We have previously described that the offspring of cafeteria-diet fed dams during lactation (O-CAF) displayed a TOFI phenotype and metabolic alterations in adulthood, since they showed greater fat accumulation but no higher body weight [12]. Interestingly, in a second animal model, we have described that elimination of the cafeteria diet in obese rats one month before gestation can prevent the detrimental effects in offspring associated with maternal obesity [13]. Here we analyzed the response to an overnight fasting in O-CAF and O-PCaf animals at early ages. O-CAF animals presented a lower body weight lost upon fasting than their controls, while the O-PCaf animals had the same weight lost upon overnight fasting as their controls (Supplementary Information Table S3).

3.2. Effects of fed/fasting conditions on the expression of known physiological target genes regulated by AMPK system in liver

Results showing the expression of selected target genes regulated by AMPK system and involved in energy metabolism in liver are summarized in Figure 2 for the Model 1 (O-C and O-CAF) and Figure 3 for the Model 2 (O-C and O-PCaf).

In model 1, both O-C and O-CAF groups showed decreased mRNA expression levels of lipogenesis-related genes (*Srebf1*, *Acaca*, *Fasn* and *Scd1*), and the glucose metabolism-related gene, *Gck*, under fasting conditions compared with *ad libitum* feeding conditions (Figure 2). However, under *ad libitum* feeding conditions, O-CAF rats showed lower expression levels of the abovementioned genes compared with their controls. Moreover, O-CAF male animals presented higher mRNA levels of *Srebf1* and *Gck* compared with controls under fasting conditions.

In model 2, O-PCaf rats exhibited a similar response to fasting conditions as O-C animals (Figure 3). Specifically, mRNA expression of lipogenesis-related genes (*Srebf1*, *Acaca*, *Fasn* and *Scd1*), and *Gck* decreased under fasting compared with *ad libitum* feeding conditions. Female O-PCaf animals presented lower mRNA levels of *Srebf1*, *Fasn* and *Scd1* versus their controls under *ad libitum* feeding conditions, whereas no differences between groups were found in males.

3.3. Effects of fed/fasting conditions on the expression of genes related to lipid metabolism in rpWAT.

Results showing the expression of selected genes related to lipid metabolism in retroperitoneal fat depot are summarized in Figure 4 for the Model 1 (O-C and O-CAF) and Figure 5 for the Model 2 (O-C and O-PCaf).

In model 1, O-C animals underwent a marked decrease in the expression levels of lipogenic genes (*Pparg*, *Srebf1*, and *Fasn*) under fasting conditions compared with *ad libitum* feeding conditions in rpWAT. However, only a mild decrease in the expression of these genes, or even no decrease (in the case of *Pparg* and *Srebf1* in females), was found in O-CAF animals. In fact, in females, the expression of the lipogenic genes (*Pparg*, *Srebf1*, and *Fasn*), under fasting conditions, was higher in O-CAF with respect to O-C rats. Furthermore, O-CAF animals, under *ad libitum* feeding conditions,

exhibited a lower expression of the lipogenic genes *Srebf1* and *Fasn* compared with controls. Regarding the expression of genes related to fatty acid uptake and lipolysis, 12-h fasting produced an increase in mRNA expression levels of *Cd36*, *Lpl* and *Pnpla2* in both male and female O-C animals. This mentioned response to fasting conditions was also observed in O-CAF females, but was impaired in O-CAF males. In addition, under feeding conditions, male and female O-CAF animals displayed greater expression levels of *Cd36* compared to O-C rats, whereas, under fasting conditions, male O-CAF rats exhibited lower expression of *Cd36*, *Lpl* and *Pnpla2* compared to O-C animals.

In model 2, control animals (O-C) showed a pattern similar to that described in Model 1 regarding expression levels of lipid metabolism-related genes under fed and fasted conditions. Specifically, male and female O-C animals from the Model 2 presented lower expression levels of lipogenic-related genes (*Srebf1* and *Fasn*), and greater expression of genes related to fatty acid uptake (*Cd36* and *Lpl*) and lipolysis (*Pnpla2*) under fasting conditions compared to *ad libitum* feeding conditions. Notably, O-PCaf animals showed a similar trend in response to fasting conditions. However, in male O-PCaf animals, the fasting-induced increase in *Cd36* expression was reduced in comparison to that of the control group, and, in the case of *Lpl*, and *Pnpla2*, changes were not significant by single comparisons (U-Mann Whitney test). Moreover, male O-PCaf animals displayed lower expression levels of genes related to lipogenesis (*Pparg*, *Srebf1* and *Fasn*) and lipolysis (*Pnpla2*) compared with their controls, more marked under fasting conditions.

3.4. Effects of fasting on mRNA expression and protein levels of AMPK catalytic subunits $\alpha 1$ and $\alpha 2$

The results presented above suggested the existence of an altered response to fed/fasting conditions in O-CAF animals, but not in O-PCaf animals, regarding the expression of known physiological targets genes regulated by AMPK system in liver and rpWAT. Therefore, we assessed whether mRNA expression levels of the two isoforms ($\alpha 1$ and $\alpha 2$) of the catalytic subunit α (*Prkaa1*, *Prkaa2*) in liver and rpWAT were also altered under fed/fasting conditions. We also analysed protein levels of AMPK $\alpha 1$ and AMPK $\alpha 2$ in liver of male offspring. Results are shown in Figure 6 (Model 1) and Figure 7 (Model 2).

In Model 1 (Figure 6), *Prkaa1* and *Prkaa2* mRNA levels in liver and rpWAT of O-CAF animals under *ad libitum* feeding conditions were similar to O-C animals. However, an altered response to fed/fasting conditions was observed in O-CAF animals regarding the expression of *Prkaa1* in both tissues with respect to their controls. Specifically, fasting led to an increase in *Prkaa1* mRNA expression levels in liver of O-C animals, whereas no effect (in females) or even a decrease (in males) was observed in O-CAF animals. Accordingly, the fasting-induced increase of AMPK α 1 levels observed in liver of control animals was also abolished in O-CAF. Moreover, fasting also increased *Prkaa1* mRNA expression in the rpWAT of O-C animals, while no effect (in males) or a less response (in females) was found in O-CAF. Regarding mRNA levels of the α 2 isoform (*Prkaa2*) no significant alterations were observed in O-CAF animals versus their controls, either in liver or rpWAT. Hepatic *Prkaa2* mRNA levels were higher in fasting conditions compared to feeding conditions in O-C and O-CAF animals. Conversely, decreased levels of *Prkaa2* mRNA were observed under fasting compared to feeding conditions in the rpWAT. Accordingly, no alterations were either found in AMPK α 2 at protein level in liver of O-CAF animals compared with controls.

In Model 2 (Figure 7), O-PCaf animals displayed lower expression levels of *Prkaa1* (especially males) and *Prkaa2* (only males) in rpWAT compared with O-C animals, but no differences were found in liver. Nevertheless, O-PCaf animals displayed a similar response to fed/fasting conditions as controls regarding the expression of both genes in liver and rpWAT. No differences between groups were either found regarding AMPK α 1 and AMPK α 2 protein levels in liver. Of note, in this model, the fasting-induced increase in *Prkaa1* mRNA expression in O-C animals was not reflected in the protein expression.

4. Discussion

Nutrition during the early stages of life can trigger adaptations that result in permanent changes in the physiology of the organism, and may program the susceptibility to develop chronic diseases in adulthood, such as obesity and associated metabolic alterations [15-18]. The present study aimed to examine the molecular response to fed/fasting conditions in the expression of key genes related to energy metabolism and regulated by AMPK in liver and rpWAT in the offspring of dams with

cafeteria diet during lactation, and in the offspring of obese dams submitted to a dietary normalization before gestation. The use of these two animal models may allow to discriminate the effects in offspring of maternal high-fat diet intake from maternal obesity *per se*, as well as to shed light into potential mechanisms involved in the different metabolic outcomes in both experimental models. It should be mentioned that these two experimental models were carried out independently. Thus, results for both models cannot be directly compared.

The AMPK is a key internal cell-energy sensor that controls nutrient flux. This is activated by a decrease in the energy state of a cell, reflected by an increase in the AMP/ATP ratio [11]. Thus, AMPK switches on catabolic pathways and switches off anabolic pathways [19]. For example, anabolic pathways such as lipogenesis and gluconeogenesis need ATP, so are invariably inhibited under fasting conditions, when AMPK is activated. The dysregulation of AMPK pathway may be a contributing factor to metabolic abnormalities associated with lipid accumulation on the onset of the metabolic syndrome [19, 20]. In fact, rodents with genetic-induced obesity show a diminished AMPK activity and insulin resistance in multiple tissues, and are more prone to present metabolic syndrome-associated diseases (reviewed in [20]). In clinical studies, a close link between dysregulation of AMPK activity and the development of WAT dysfunction associated with insulin resistance has been suggested [21, 22]. Accordingly, AMPK activity has been described to be lower in insulin resistant subjects than in their BMI-matched counterparts who were insulin sensitive [21]. Pro-inflammatory profile in visceral fat is also associated with decreased AMPK activity. However, whether the decrease in AMPK activity is a cause or a consequence of the inflammation is unclear. Considering that a low activity of AMPK has been revealed as a critical causal factor of inflammation, diabetes and obesity, it has been postulated that AMPK activation would have beneficial metabolic consequences for obese and diabetic patients, and has been considered as a potential target for their treatment [20, 23]. AMPK is activated by phosphorylation of the threonine residue 172 within the activation loop of the α -catalytic subunit, which contains the kinase domain. However, another possible mechanism for the decrease in AMPK activity under high-fat diets is the decrease in the transcription of the catalytic subunits of AMPK, as previously described [24, 25].

Present study shows that transcript levels of both AMPK α isoforms were not altered in liver and rpWAT in O-CAF animals under *ad libitum* feeding conditions, even though, these animals displayed at weaning greater fat accumulation compared to control offspring (previously published [12]). In addition, O-CAF animals at PN22 presented higher free fatty acids (NEFA) levels, under *ad libitum* conditions and higher leptinemia than controls [14]. O-CAF animals presented down-regulated expression of genes involved in lipogenic processes in liver and rpWAT, which could be explained by the higher fat content of milk due to maternal dietary conditions [12]. In fact, high-fat diet feeding has been shown to induce the expression of catabolism-related genes and reduce the expression of lipogenesis-related genes in the main tissues involved in the interorganal homeostatic response [26, 27]. Notably, the fasting-induced increase in the hepatic expression of *Prkaa1* (AMPK α 1), which was clear in control animals, was impaired in O-CAF animals, whereas a normal response to fasting conditions was observed in the expression of *Prkaa2* (AMPK α 2). These results were also found at protein level for AMPK α 1. However, the fasting-increase in *Prkaa2* mRNA was not observed at protein level. A plausible explanation for the difference between mRNA and protein levels could be the delay between transcriptional induction and protein levels increase, which is known to be protein specific, and could be of relevance during a dynamic adaptation process [28]

. In addition, a similar altered pattern in *Prkaa1* mRNA levels was observed in rpWAT. Thus, the altered response to fasting conditions regarding AMPK α 1 in O-CAF animals, could explain the alteration observed in the known physiological target genes regulated by the AMPK system. Specifically, in O-C animals, 12-h fasting produced a decrease in the expression of lipogenesis-related genes in liver and rpWAT and an increase in those controlling fatty acid uptake and lipolysis in rpWAT, whereas the response to fasting was generally impaired in O-CAF animals in both tissues. This is of note because an insensitivity to the transition from fed to fasting conditions regarding energy metabolism-related genes may contribute to increased adiposity in later life, and has been considered a hallmark of obesity [29]. In this regard, we have previously described that a short period of cafeteria diet exposure in early infancy is enough to disturb the metabolic response to fasting conditions and induces an exacerbated body fat accumulation, with no apparent effects on body

weight [7]. Interestingly, the expected detrimental effects in offspring are preventable by a dietary normalization of the dams before mating [13]. In fact, no significant differences were observed in the percentage of body fat at weaning and adult age in the offspring of post-cafeteria dams compared to controls [13]. Notably, in spite of certain differences in gene expression levels in comparison to their controls, O-PCaf animals showed increased expression of *Prkaa1* and *Prkaa2* in response to fasting conditions, similar to their controls. They also exhibited a similar trend to controls in the expression of energy metabolism-related genes in response to fasting conditions. Thus, the alteration in the expression of key metabolism-related genes in the offspring of dams submitted to an unhealthy diet during lactation, such as cafeteria diet, are probably mediated by a dysregulation of the AMPK system, and this alteration is prevented by maternal diet normalization before gestation. It could be speculated that this alteration could be a direct effect of a particular nutrient or a bioactive compound supplied through milk to the offspring, or the result of a combination of changes in maternal milk. In addition, considering that O-CAF display hyperleptinemia [12], another potential explanation could be a defective response to leptin action regarding the AMPK system, as described in obese state [30, 31]. Moreover, we have previously reported that O-CAF animals, but not O-PCaf animals, also display higher plasma levels of lipid-derived acyl carnitine species after weaning [32]. This was particularly evident under feeding conditions, and could be reflecting conditions of mitochondrial lipid overload due to the greater supply of lipids from breast milk [32]. The presence of increased levels of certain acyl carnitines has also been reported in obese individuals, and proposed as a marker of metabolic inflexibility in adulthood [33]. This would be in agreement with the altered response to fed/fasting conditions described here in O-CAF animals.

In addition, it is worth noting the existence of a tissue-dependent response to fasting conditions regarding the expression of *Prkaa2*. While fasting stimulates the expression of *Prkaa1* in both liver and rpWAT in control animals, mRNA expression levels of *Prkaa2* increased in liver and decreased in rpWAT under fasting conditions. In liver, both AMPK α 1 and AMPK α 2 have been described to contribute equally to total AMPK activity [34]. However, the two isoforms have been suggested to play different physiological roles within the cell, since they exhibit slightly different substrate

preferences [35]. Moreover, as described here, the $\alpha 1$ isoform seems to be more sensitive to maternal dietary conditions during lactation compared with the $\alpha 2$ isoform, and changes in the expression of *Prkaa1* may account for changes in the activity of AMPK and for the aforementioned alterations in the expression of AMPK-target genes found in O-CAF animals. Regarding WAT, it is known that the heterotrimeric complexes containing the $\alpha 1$ subunit accounts for most of the activity of AMPK in WAT from mice [36, 37] and humans [38], and our results are in accordance with the relevance of this subunit in the activity of the AMPK. Therefore, signs of metabolic inflexibility in the expression of *Prkaa1* and not *Prkaa2* found in O-CAF could serve as an early marker or may contribute to the obese phenotype observed in these animals at weaning and maintained in adult age.

All in all, present results show an altered response to fed/fasting conditions in the expression of genes regulated by the AMPK system in liver and rpWAT in the offspring of dams fed a cafeteria diet during lactation. This suggests the achievement of metabolic inflexibility already at early age in these animals. These alterations seem to be mediated by an impaired response of the AMPK $\alpha 1$ isoform to energy supply status. Interestingly, maternal dietary normalization one month before gestation in obese rats prevents the abovementioned detrimental effects in the offspring. The insensitivity to feeding conditions at early ages could constitute an early marker of later metabolic outcomes related to the increased adiposity observed later on in these animals.

Author's contribution

A. P., J. S and C. P. conceived and designed the experiments; C. A. P., H. C. and J. S. analyzed data; C. A. P. and H. C. performed research; C. A. P., J. S., C. P., and A. P. wrote the paper. All authors have revised the manuscript and approved the final version

Competing interest statement: The authors declare that they have no conflict of interest.

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Figure legends

Figure 1. Representative scheme of the animal models used in this study. The offspring were named according to maternal group. The first letter represents the term offspring and the second term represents the maternal group (C, control; CAF, cafeteria diet during lactation and PCaf postcafeteria dams).

Figure 2. Expression of selected genes related with energy metabolism (lipogenesis, fatty acid uptake, fatty acid synthesis, and glucose metabolism) in liver in postnatal day 22 of male (a) and female (b) offspring of dams fed a control (O-C) or a cafeteria diet during lactation (O-CAF) and killed under *ad libitum* feeding conditions or after 12-h fasting. mRNA levels were measured by RT-qPCR and expressed as a percentage of the value of O-C rats under *ad libitum* feeding conditions. Data are mean $\pm$ s.e.m. (n=8-12). Genes determined were: sterol regulatory element binding transcription factor 1 (*Srebf1*), acetyl-CoA carboxylase alpha (*Acaca*), fatty acid synthase (*Fasn*), stearoyl-Coenzyme A desaturase 1 (*Scd1*) and glucokinase (*Gck*). Statistics: F, effect of feeding conditions; D, effect of

maternal group (control/caféteria); FxD, interaction between feeding conditions and maternal group ($P < 0.05$, two-way ANOVA). #, Fasting *versus ad libitum* feeding conditions ($p < 0.05$, Mann-Whitney U-test). *, O-CAF *versus* O-C ($p < 0.05$, Mann-Whitney U-test).

Figure 3. Expression of selected genes related with energy metabolism (lipogenesis, fatty acid uptake, fatty acid synthesis, and glucose metabolism) in liver in postnatal day 26 of male (a) and female (b) offspring of control (O-C) or post-caféteria dams (O-PCaf) and killed under *ad libitum* feeding conditions or after 12-h fasting. mRNA levels were measured by RT-qPCT and expressed as a percentage of the value of O-C rats under *ad libitum* feeding conditions. Data are mean $\pm$ s.e.m. ($n=11-17$). Genes determined were: sterol regulatory element binding transcription factor 1 (*Srebf1*), acetyl-CoA carboxylase alpha (*Acaca*), fatty acid synthase (*Fasn*), stearoyl-Coenzyme A desaturase 1 (*Scd1*) and glucokinase (*Gck*). Statistics: F, effect of feeding conditions; D, effect of maternal group (control/post-caféteria); FxD, interaction between feeding conditions and maternal group ($P < 0.05$, two-way ANOVA). #, Fasting *versus ad libitum* feeding conditions ($p < 0.05$, Mann-Whitney U-test). *, O-PCaf *versus* O-C ($p < 0.05$, Mann-Whitney U-test).

Figure 4. Expression of selected genes related with lipid metabolism (lipogenesis, fatty acid uptake and lipolysis) in rpWAT on postnatal day 22 of male (a) and female (b) offspring of dams fed a control (O-C) or a caféteria diet during lactation (O-CAF) and killed under *ad libitum* feeding conditions or after 12-h fasting. mRNA levels were measured by RT-qPCR and expressed as a percentage of the value of O-C rats under *ad libitum* feeding conditions. Data are mean $\pm$ s.e.m. ($n=8-12$). Genes determined were: proliferator-activated receptor gamma (*Pparg*), stearoyl-Coenzyme A desaturase 1 (*Srebf1*), fatty acid synthase (*Fasn*), Cd36 molecule (*Cd36*), patatin-like phospholipase domain containing 2 (*Pnpla2*), and lipoprotein lipase (*Lpl*). Statistics: F, effect of feeding conditions; D, effect of maternal group (control/caféteria); FxD, interaction between feeding conditions and maternal group ($P < 0.05$, two-way ANOVA). #, Fasting *versus ad libitum* feeding conditions ($p < 0.05$, Mann-Whitney U-test). *, O-CAF *versus* O-C ($p < 0.05$, Mann-Whitney U-test).

Figure 5. Expression of selected genes related with lipid metabolism (lipogenesis, fatty acid uptake and lipolysis) in rpWAT on postnatal day 26 of male (a) and female (b) offspring of control (O-C) or post-cafeteria dams (O-PCaf) and killed under *ad libitum* feeding conditions or after 12-h fasting. mRNA levels were measured by RT-qPCR and expressed as a percentage of the value of O-C rats under *ad libitum* feeding conditions. Data are mean $\pm$ s.e.m. (n=11-17). Genes determined were: proliferator-activated receptor gamma (*Pparg*), stearyl-Coenzyme A desaturase 1 (*Srebf1*), fatty acid synthase (*Fasn*), Cd36 molecule (*Cd36*), patatin-like phospholipase domain containing 2 (*Pnpla2*), and lipoprotein lipase (*Lpl*). Statistics: F, effect of feeding conditions; D, effect of maternal group (control/post-cafeteria); FxD, interaction between feeding conditions and maternal group ($P < 0.05$, two-way ANOVA). #, Fasting *versus ad libitum* feeding conditions ($p < 0.05$, Mann-Whitney U-test). *, O-PCaf *versus* O-C ($p < 0.05$, Mann-Whitney U-test).

Figure 6. A) Expression of catalytic subunit $\alpha 1$ (*Prkaa1*) and $\alpha 2$ (*Prkaa2*) in liver and rpWAT in postnatal day 22 of male and female offspring of dams fed a control (O-C) or a cafeteria diet (O-CAF) during lactation and killed under *ad libitum* feeding conditions or after 12-h fasting. mRNA levels were measured by RT-qPCR and expressed as a percentage of the value of O-C rats under *ad libitum* feeding conditions. B) Protein levels of AMPK $\alpha 1$ and AMPK $\alpha 2$ in the liver of O-C and O-CAF male animals measured by western blot. Representative bands are shown. Protein levels were measured by western blot and expressed as a percentage of the value of O-C rats under *ad libitum* feeding conditions. Data are mean $\pm$ s.e.m. (n=8-12). Statistics: F, effect of feeding conditions; D, effect of maternal diet (control/cafeeteria) during lactation; FxD, interaction between feeding conditions and maternal group ($P < 0.05$, two-way ANOVA). #, Fasting *versus ad libitum* feeding conditions ($p < 0.05$, Mann-Whitney U-test). *, O-CAF *versus* O-C ($p < 0.05$, Mann-Whitney U-test).

Figure 7. a) Expression of catalytic subunit $\alpha 1$ (*Prkaa1*) and $\alpha 2$ (*Prkaa2*) in liver and rpWAT on postnatal day 26 of male (a) and female (b) offspring of control (O-C) or post-cafeteria dams (O-PCaf) and killed under *ad libitum* feeding conditions or after 12-h fasting. mRNA levels were measured by RT-qPCR and expressed as a percentage of the value of O-C rats under *ad libitum* feeding conditions. B) Protein levels of AMPK $\alpha 1$ and AMPK $\alpha 2$ in the liver of O-C and O-PCaf male

animals measured by western blot. Representative bands are shown. Protein levels were measured by western blot and expressed as a percentage of the value of O-C rats under *ad libitum* feeding conditions. Data are mean $\pm$ s.e.m. (n=11-17). Statistics: F, effect of feeding conditions; D, effect of maternal group (control/post-cafeteria); FxD, interaction between feeding conditions and maternal group ($P < 0.05$, two-way ANOVA). #, Fasting *versus* *ad libitum* feeding conditions ($p < 0.05$, Mann-Whitney U-test). *, O-PCaf *versus* O-C ($p < 0.05$, Mann-Whitney U-test).

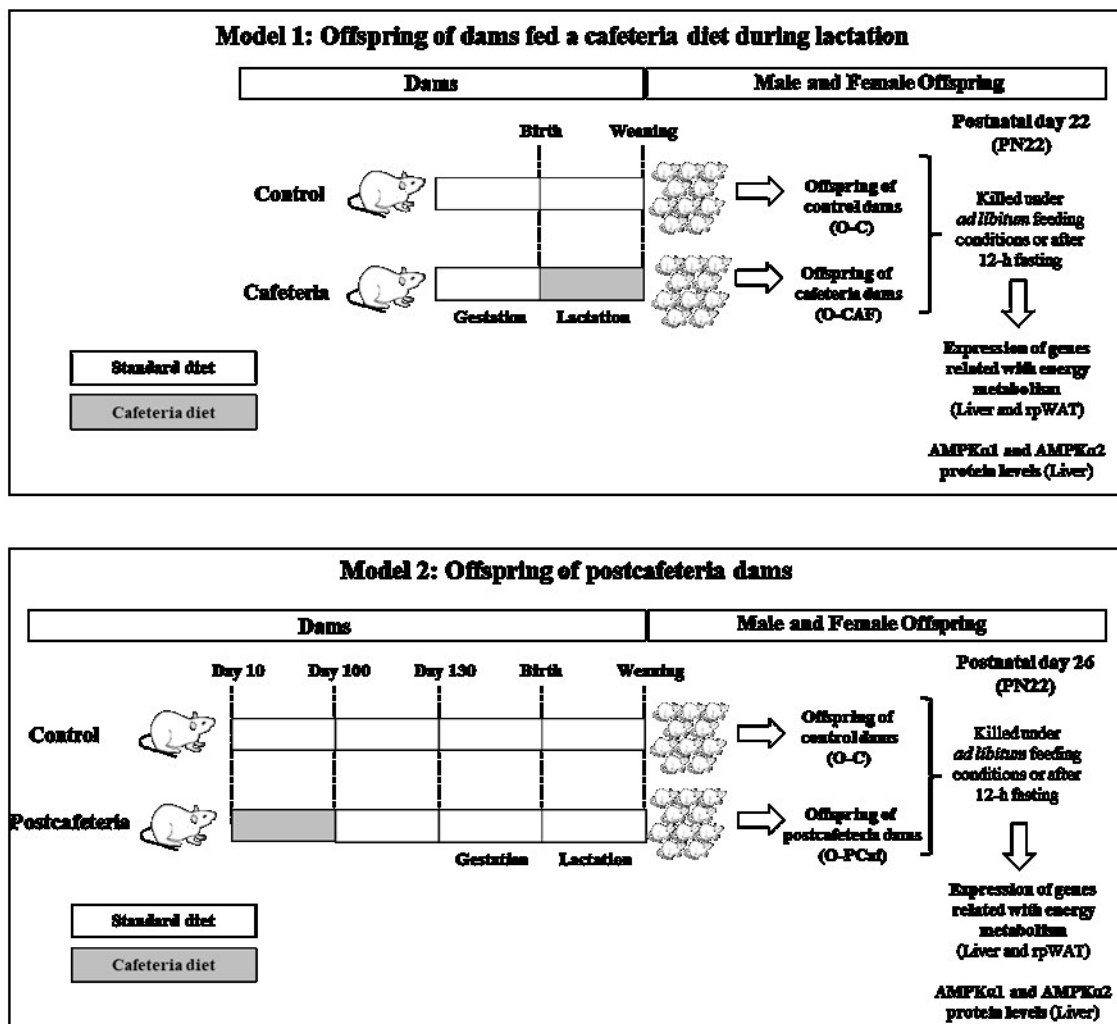
Figure 1.

Figure 2.**Model 1: Offspring of cafeteria fed dams during lactation**

Expression of selected genes related with energy metabolism in liver

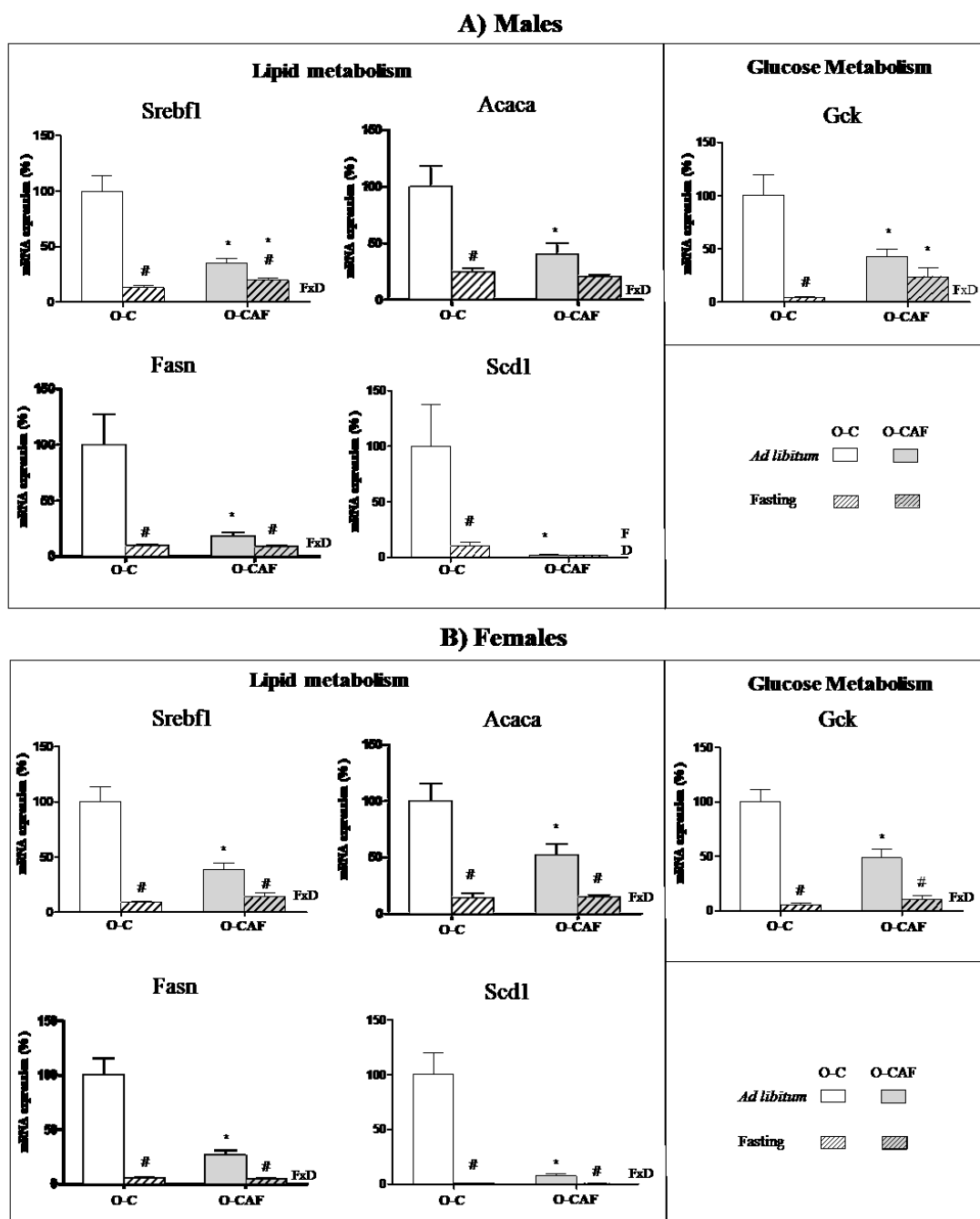


Figure 3.

Model 2: Offspring of Post-cafeteria dams
Expression of selected genes related with energy metabolism in liver

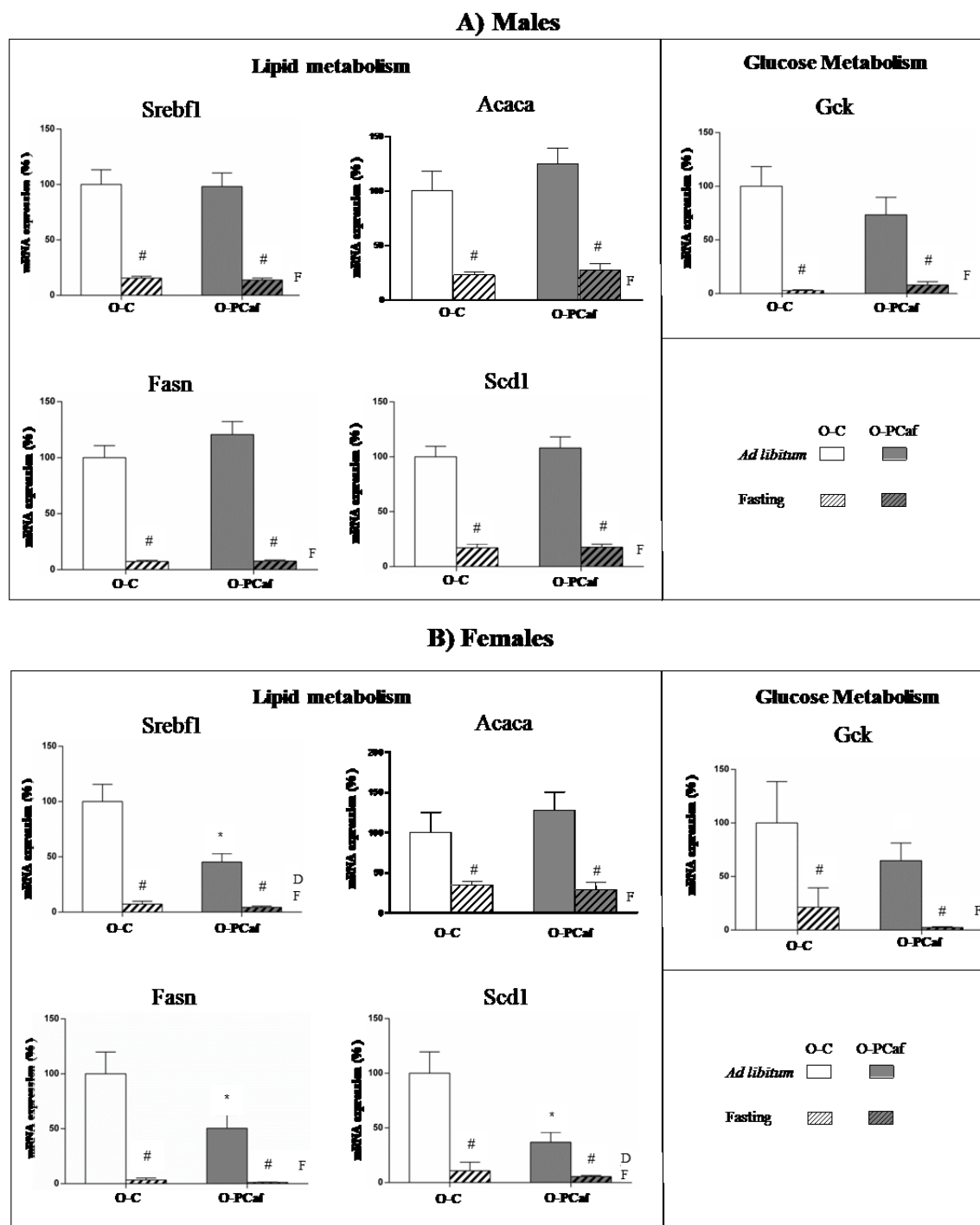


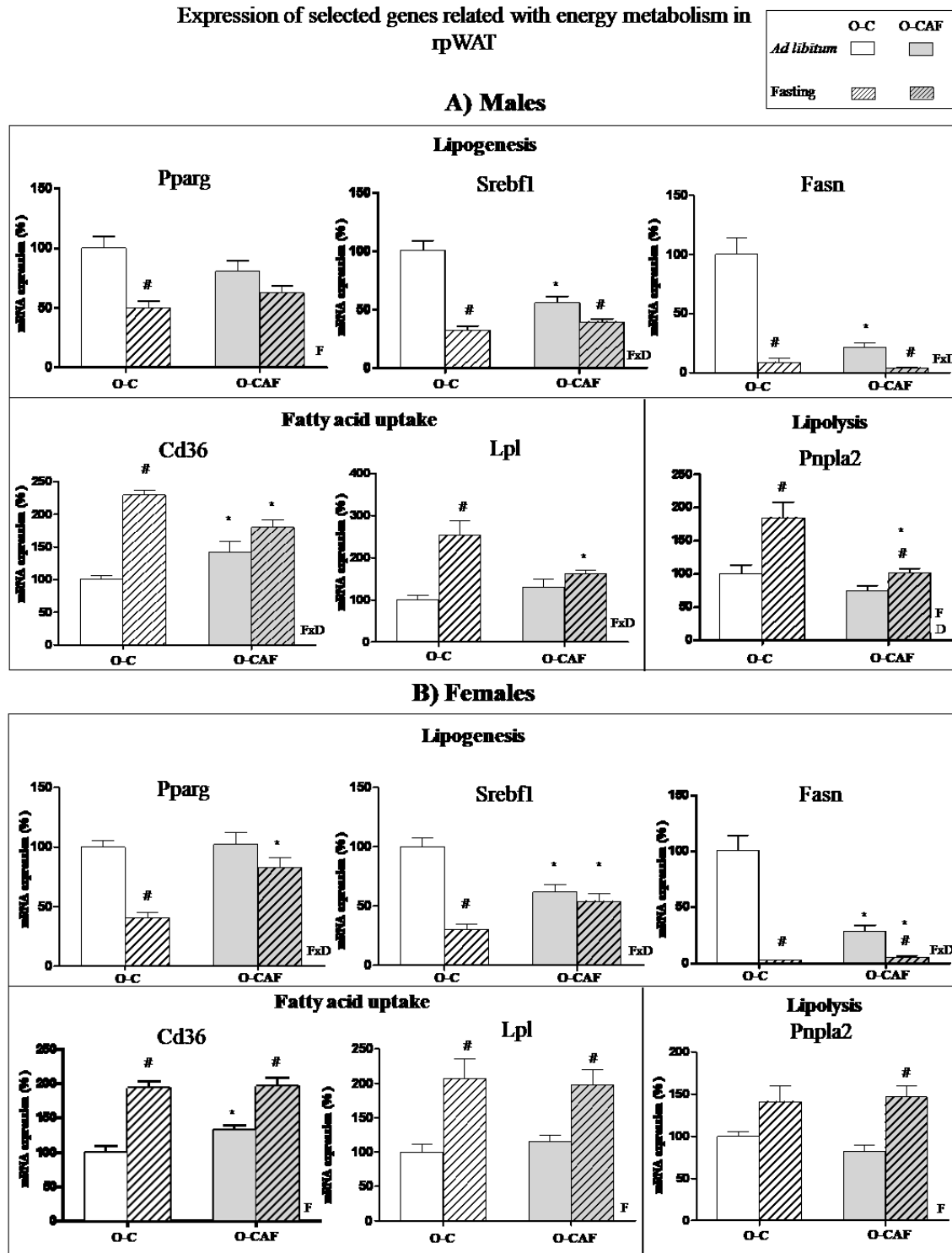
Figure 4.**Model 1: Offspring of cafeteria fed dams during lactation**Expression of selected genes related with energy metabolism in
rpWAT

Figure 5.

Model 2: Offspring of Post-cafeteria dams
Expression of selected genes related with energy metabolism in
ipWAT

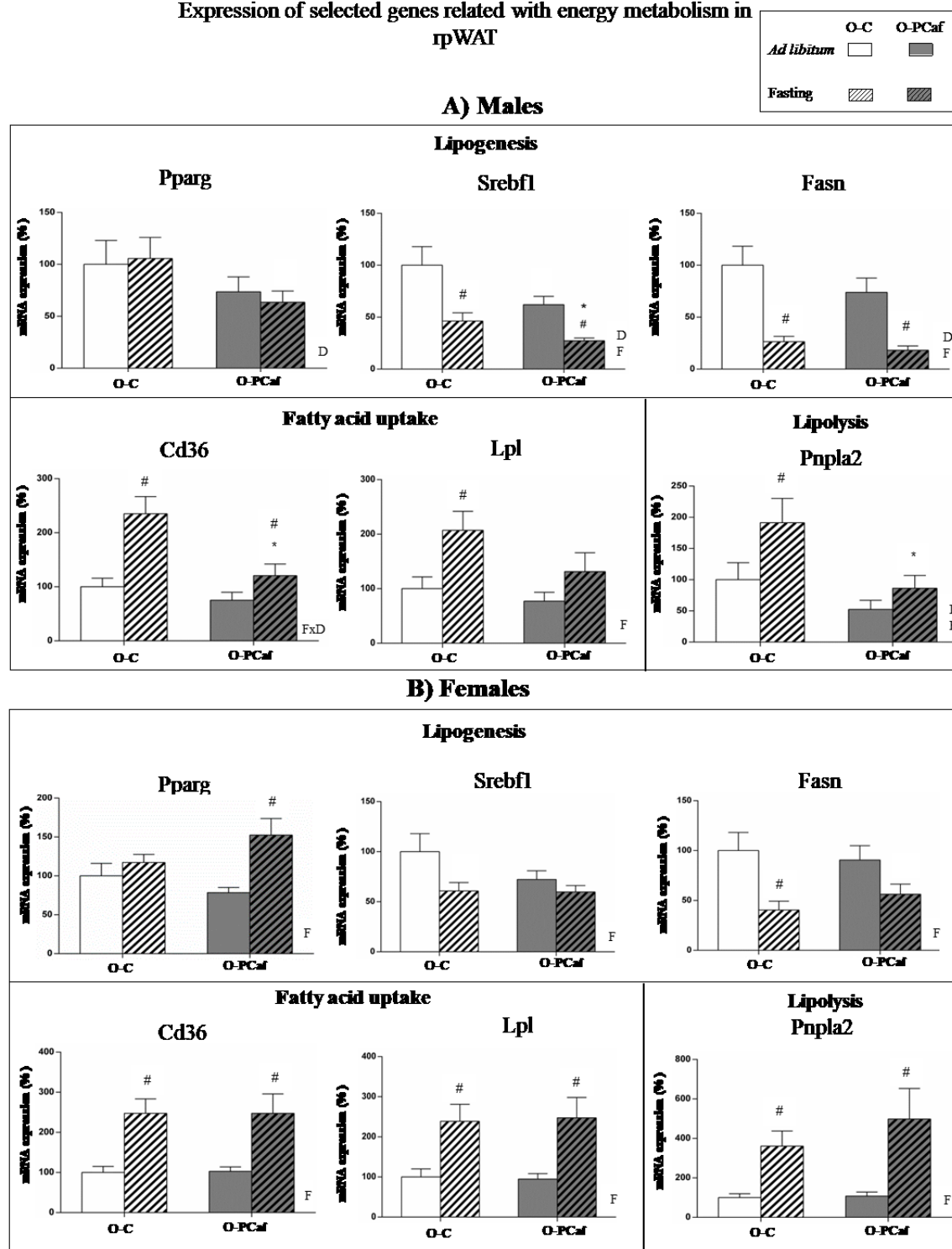


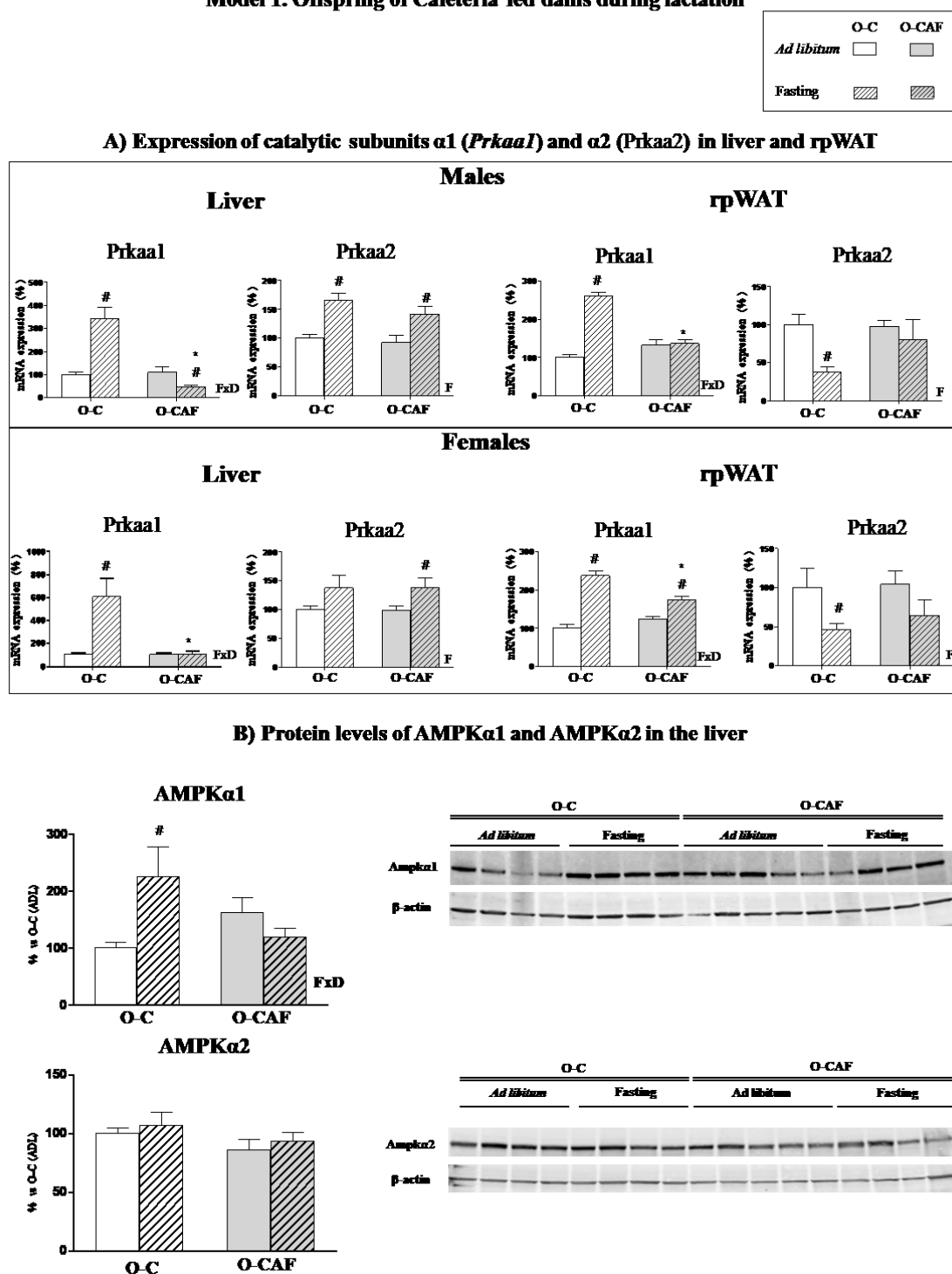
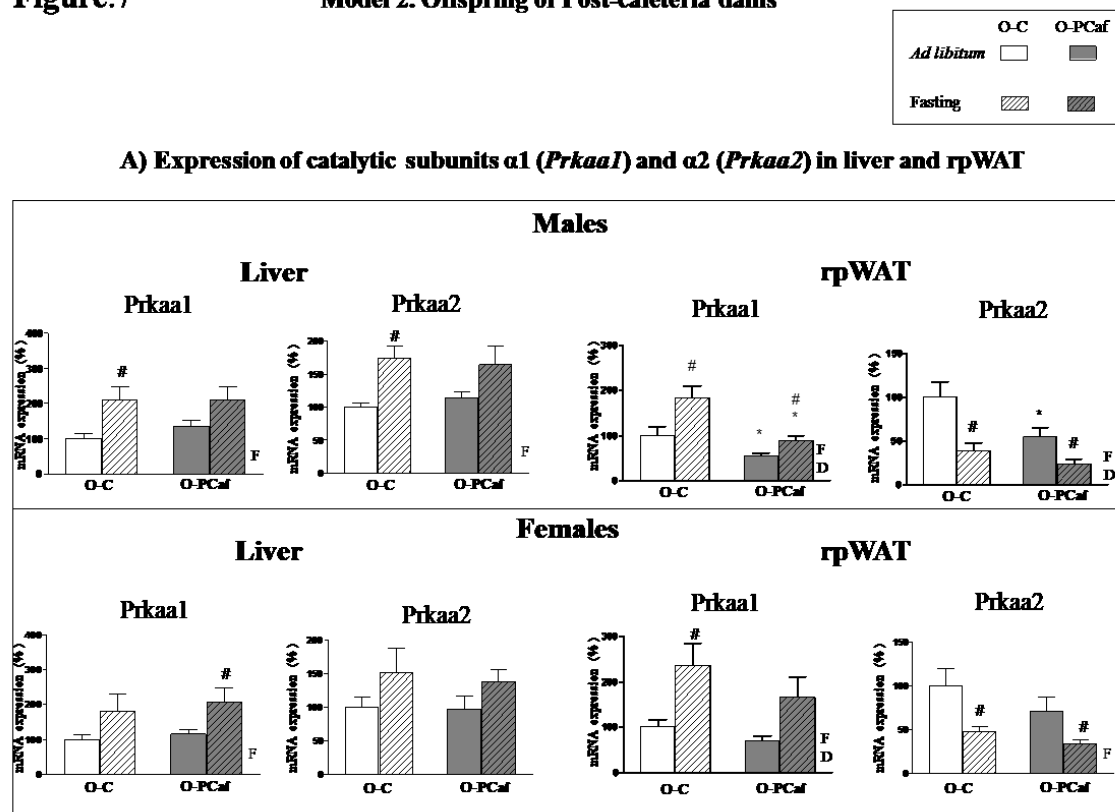
Figure 6.**Model 1. Offspring of Cafeteria fed dams during lactation**

Figure.7

Model 2. Offspring of Post-cafeteria dams

B) Protein levels of AMPK $\alpha 1$ and AMPK $\alpha 2$ in the liver