Maternal methionine supplementation in mice affects long-term body weight and locomotor activity of adult female offspring

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Abstract
Methionine is a precursor of S-adenosylmethionine, the main donor of methyl radicals for methylation of DNA and other compounds. Previous studies have shown that reduced availability of methyl radicals during pregnancy/lactation decreased offspring perigonadal white adipose tissue (PWAT) and body weight. Therefore, we aimed to evaluate the effects of methionine supplementation during early development, a time of great ontogenic plasticity, by assessing the biometric, biochemical and behavioural parameters of the offspring of adult Swiss female mice supplemented with 1% methionine in water 1 month before pregnancy, during pregnancy or pregnancy/lactation. After birth, the offspring were distributed into three groups: control (CT), methionine supplementation during pregnancy (SP) and methionine supplementation during pregnancy and lactation (SPL), and were followed until postnatal day (PND) 300. No changes were observed in offspring birth weight in both sexes. At PND 5, 28 and 90, no differences in body weight were found in females; however, at PND 300, SP and SPL females showed an increase in body weight when compared with the control group. This increase in body weight was accompanied by a total and relative increase in PWAT, and a decrease in locomotor activity in these groups. No differences in the body and organ weights were found in male offspring. In conclusion, the increased availability of methyl radicals during pregnancy and lactation impacted long-term body composition and locomotor activity in female offspring.

Key words: Fetal programming: Diet: Methionine: Mice: Body weight

Since the discovery of a link between maternal and early-life malnutrition and an increased risk to the health of adult offspring, different nutritional manipulations (supplementation or restriction) have been tried to understand the role of individual components in this relationship(1,2). It is known that nutrition state influences epigenetic regulation(3) and that some nutrients can directly affect the methionine–homocysteine pathway, with S-adenosylmethionine (SAM) being an important product in this respect. SAM availability during early development may affect methylation patterns that lead to short- and long-term consequences, as it is the major donor of methyl radicals for the methylation of DNA, RNA, proteins and other compounds(4).

S-Adenosylhomocysteine (SAH) is the product of SAM demethylation and is further hydrolysed to adenosine and homocysteine (HCY) in a reversible reaction, wherein the thermodynamics favour SAH synthesis rather than HCY production (whose excess is related to oxidative stress). In the transsulphuration pathway, HCY is catabolised by a series of pyridoxine-dependent reactions that result in cysteine (CYS), which may be further used in GSH synthesis and other processes. Moreover, several vitamins such as riboflavin, folate, cobalamin and choline, as well as methionine, have an effect on the availability of SAM by affecting the remethylation of HCY to methionine(4).

The relationship between maternal folate and cobalamin deficiency and increased risk of neural tube defects is well established(5). In addition, in a previous study by our group looking at the consequences of imbalances in this pathway, we observed that riboflavin, folate, cobalamin and choline deficiency during early development resulted in a long-term reduction in the weight of perigonadal white adipose tissue (PWAT) in the male mice offspring(6). These findings seem to be related to a decrease in adipose tissue expansion during early development, such as that observed in undernourished mice during the lactation phase(7).

Abbreviations: CT, control group; CYS, cysteine; HCY, homocysteine; PND, postnatal day; PWAT, perigonadal white adipose tissue; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SP, methionine supplementation during pregnancy; SPL, methionine supplementation during pregnancy and lactation.

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As different aspects of early B-vitamin deficiency and their association with morphometric and biochemical changes have already been evaluated\(^6\),\(^8\), in this study, we focus on assessing the consequences of methionine supplementation during early development. The effect of methionine restriction has been studied in adult mice and has been shown to be associated with lower body and fat tissue weight and increased longevity\(^9\),\(^10\). Thus, to better understand the consequences behind manipulations affecting the availability of the methyl radical, the goal of this study was to evaluate the effects of methionine supplementation during pregnancy and pregnancy/lactation on biometric, biochemical and behavioural parameters of the offspring.

Materials and methods

Animal treatment protocols

All animal experiments were conducted in accordance with the guidelines established by the Ethical and Practical Principles of the Use of Laboratory Animals\(^11\) and approved by the Institutional Animal Care and Use Committee of the Universidade Federal de São Paulo (no. 1169/08). Adult Swiss female mice were obtained from the Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia and were housed at the Department of Psychology facility in a ventilated system (365 × 207 × 140 mm, Tecniplast, Milan, Italy) with 3–5 animals/cage, maintained under standard laboratory conditions (12-h light–12-h dark cycle – lights on at 07.00 hours, controlled temperature (23 ± 2°C), humidity of 70–75 %, and access to food and water ad libitum). One month before pregnancy, twenty-seven female mice were randomly distributed into two groups: (a) standard diet (AIN-93M; \(n = 10\)) and (b) standard diet supplemented with 1 % L-methionine (M9625, Sigma-Aldrich\(^8\)) in water (AIN-93M + 1 % methionine; \(n = 17\)). This concentration was previously tested and shown to induce moderate hyperhomocysteinaemia\(^12\),\(^13\). The decision to have a higher number of animals in the experimental group was based on the observation that there is decreased fertility and litter size in females with hyperhomocysteinaemia\(^14\). From blood samples collected from the submandibular vein, homocysteine levels of both groups were dose after 20 d of treatment as an indirect proxy of increased methionine intake. A lancet (depth of lancet needle – 4 mm) was used to access the submandibular vein, which allows about 0·2 ml of blood to be promptly drawn without anaesthesia use\(^15\). One month after methionine supplementation was completed, male mice were placed in the females' home cages for mating (one male to three to five females). The time interval between blood collection and mating was established in order to allow the recovery of blood volume\(^16\) and minimise the stress involved in the collection procedure, making possible the normal conditions for mating. Although the male mice had access to the supplemented water during mating, this period was insufficient to cause any effect on the sperm involved in the fertilisation since the complete spermatogenic cycle was established to be about 8·6 d\(^17\). Gestational day zero was determined after confirmation of the presence of sperm in the vaginal smear.

Fifteen days later, the female mice were placed in individual cages and, after birth, all offspring were weighed (control: Females (\(n = 51\)), Males (\(n = 30\)); supplemented during pregnancy: Females (\(n = 63\)), Males (\(n = 55\)) and distributed into three groups: Control (CT) – the offspring of the dams that received a standard diet; the methionine supplementation during pregnancy group (SP) – the offspring of the dams receiving methionine supplementation during pregnancy and breastfed by CT dams; and the methionine supplementation during pregnancy and lactation group (SPL) – the offspring of the dams which continued to receive supplementation during lactation). The SP offspring were breastfed by CT dams. The adoption bias by the SP group was adjusted by performing adoptions within each group, CT and SPL. After distribution, the number of animals in each offspring group was CT = 53, SP = 61 and SPL = 44. Weaning occurred on postnatal day (PDN) 28, after which all the offspring groups were placed on a standard diet. Schematic of the overall study design is presented in Fig. 1.

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![Fig. 1. Overall study design. CT, control; SP, supplemented during pregnancy; SPL, supplemented during pregnancy and lactation; PND, postnatal day. Supplemented diet = standard diet + 1 % \(\mu\)-methionine in water.](http://doi.org/10.1077/600711421000000275)
**Locomotor activity box**

Locomotor activity was assessed using Opto-Varimex locomotor activity cages (20 × 30 × 40 cm), surrounded by horizontal photoelectrical sensors (Columbus Instruments). Recording was performed at PND 299 at regular intervals (every 5 min, for 30 min).

**Sample collection**

Animals of both sexes were evaluated at PND 0, 5, 28, 90 and 300. The analyses at these time points allowed us to follow the effects of methionine supplementation during pregnancy and pregnancy/lactation in the short term (PND 0, 5 and 28) and in the long term (PND 90 and 300). All animals were euthanised by decapitation, and the total blood was collected in two types of tubes (Becton Dickinson) containing EDTA or heparin. The blood aliquot collected in tubes containing heparin was used for glucose, TAG and cholesterol measurement. Total blood in EDTA was stored on ice for up to 90 min and then centrifuged at 805 g for 10 min at 4°C. Plasma aliquots were stored at −80°C for HCY, CYS and GSH dosages. After the euthanasia, the whole brain was rapidly harvested and stored at −80°C for subsequent SAM, SAH and total GSH analyses/quantification. PWAT, pericardial white adipose tissue, liver, kidney, spleen, heart and brain weight (from animals at PND 28 and PND 300) were determined using an analytical balance (Bioprecisa Model-FA2104N), accurate to 0·001 g. The PWAT and pericardial white adipose tissue dissections followed the protocol described by Johnson & Hirsch(18) and Liu et al. (19), respectively. Both blood collection and killing were performed between 08.00 and 12.00 hours.

**Blood, plasma and tissue measurements**

Glucose, TAG and cholesterol were measured in heparinised blood using the commercially available Accutrend® Plus colorimetric system (Roche).

Plasma HCY, CYS and total GSH were analysed by HPLC based on the method developed by Pfeiffer et al. (20). Measurements were performed by fluorescence detection and isocratic elution in a column C18 Luna (5 mm, 150 mm, 64-6 mm), mobile phase (0·06 μ sodium acetate, 0·5 % acetic acid, pH 4·7 (adjusted with acetic acid), 2 % methanol) and a flow rate of 1·1 ml/min. The retention time was 3·6 min for CYS; 5·2 for HCY and 9·0 for GSH (21).

For tissue measurements, total brain or total dissected cortex was homogenised in PBS with the tissue homogenizer T10 basic IKA (Staufen, Germany). Before SAM and SAH injection in the HPLC column (LiChroCart C 18, 5 mm, 250 mm x 4 mm), the protein and debris were precipitated from the total homogenate tissue with HClO₄ and centrifuged. The mobile phase consisted of 50 ms sodium phosphate (pH 2·8), 10 ms heptane sulfonate and 10 % acetonitrile used at a flow rate of 1 ml/min. The retention time was 8·7 min for SAH and 13·6 min for SAM detected in a UV wavelength of 254 nm, a technique adapted from Blaise et al. (21,22). Total brain GSH measurements were performed using the same method described for measuring total plasma GSH.

**Statistical analyses**

Sample size was calculated by setting an effect size of 80 % at a level of significance of 5 % (G Power programme 3.1.9.7) and a power of 0·6 for females (n 15) and males (n 15) (Lenhard, W. & Lenhard, A. (2016). Calculation of effect sizes retrieved from: https://www.psychometrica.de/effect_size.html).

Data were analysed using Statistica software, version 10.0 (StatSoft). The concentration of SAH, SAM, GSH, HCY, CYS and SAM:SAH ratio was considered as dependent variables, and the independent variable was the group factor (Control (CT) – the offspring of the dams that received a standard diet; the methionine supplementation during pregnancy group (SP) – the offspring of the dams receiving methionine supplementation during pregnancy and breastfed by CT dams; and the methionine supplementation during pregnancy and lactation group (SPL), for females and males separately (sex was not considered as a variable), at every PND when measured).

Body weight and the absolute and relative weights of the organs were also considered as dependent variables, adopting the same independent variable described above. For the behavioural analyses, locomotor activity in the 30 min tested was considered as a dependent variable (for females and males separately) and the group as the independent variable. The sex of the animals was not considered as a variable, so the analyses were carried out separately for females and males.

All dependent variables were tested for normality (Shapiro–Wilk test), and when non-normal distribution was detected, the data were standardised by z-score before proceeding with the analyses. A general linear model was applied with a significance level of 5 % (P < 0·05). Duncan’s post hoc test was used.

**Results**

**Dams**

The increase of methionine availability and the consequent intracellular metabolism of this amino acid was inferred by HCY plasma concentration. Dams that were fed with a methionine-supplemented diet for 20 d showed an increase of 409 % in plasma HCY concentrations when compared with the control group (F(1,25) = 21·37; P = 9·9 × 10⁻⁴) (Fig. 2).

**Offspring**

The increased availability of methionine during pregnancy did not significantly impact the birth weight of female offspring (F(1,112) = 2·66; P = 0·11), but male mice offspring showed a 6 % reduction in the birth weight (F(1,98) = 8·58; P = 4·4 × 10⁻³), (Fig. 3).

At PND 5 (F(2,12) = 0·87; P = 0·44), 28 (F(2,16) = 0·82; P = 0·60) and 90 (F(2,17) = 2·48; P = 0·09), no differences in body weight were found in the female offspring. The same results were observed for males at PND 5 (F(2,12) = 2·61; P = 0·11), 28 (F(2,16) = 0·94; P = 0·41) and 90 (F(2,17) = 0·70; P = 0·51).

However, at PND 300, SP and SPL females showed an increase in body weight when compared with control (F(2,17) = 7·46;
\[ P = 5.9 \times 10^{-3} \]. No significant differences in male offspring at PND 300 were observed \((F_{(2,16)} = 0.43; P = 0.66)\) (Fig. 4).

The results in respect of the total and relative weight of the organs at PND 28 are summarised in Table 1. No significant differences were observed between the groups for any of the organs evaluated, both in females and males.

In females at PND 300, no significant differences were observed in the absolute weight of the liver, spleen, kidney, pericardial white adipose tissue and heart. However, the relative weights of liver and spleen of the SPL offspring were lower than the SP and CT groups, and the relative weights of the kidneys and heart were lower in the SP and SPL groups when compared with the CT group \((P < 0.05)\). On the other hand, the weight of PWAT, both absolute and relative, was higher in the SP and SPL groups when compared with the CT group \((P < 0.05)\). No significant differences were observed between the groups for any of the organs evaluated in the males at PND 300 (Table 2).

Plasma dosages showed no significant changes in glucose, TAG, lactate and cholesterol in female \((F_{(2,17)} = 1.32; P = 0.28)\) or male \((F_{(2,16)} = 2.20; P = 0.062)\) offspring at PND 300 (Table 3).

At PND 0, we measured SAM and SAH (a product resulting from methionine demethylation), and GSH, a powerful cell protector.
antioxidant concentrated in the brain. No significant changes in the SAM, SAH and SAM:SAH ratio in both females ($F_{(1,14)} = 1.83; P = 0.20$) and males ($F_{(1,13)} = 0.65; P = 0.60$) were observed. In respect of GSH, no differences were observed in the female ($F_{(1,13)} = 0.94; P = 0.35$) or male mice ($F_{(1,13)} = 0.093; P = 0.76$), Table 4.

At PND 28, females from the SPL group showed an increase in plasma HCY concentrations when compared with the CT and SP groups ($F_{(2,16)} = 2.65; P = 0.037$). Male mice from this group also presented increased plasma HCY concentrations when compared with the CT and SP groups ($F_{(2,16)} = 7.27; P = 4.8 \times 10^{-3}$). No significant differences in CYS and GSH plasma concentrations were observed in females or males.

Evaluations of the brain cortex also showed no significant changes in SAM, SAH and SAM:SAH ratio in females ($F_{(2,15)} = 0.60; P = 0.73$) and males ($F_{(2,15)} = 0.87; P = 0.53$), or in GSH (females, $F_{(2,15)} = 2.75; P = 0.10$; males, $F_{(2,15)} = 0.19; P = 0.83$), as shown in Table 5.

For all groups, at PND 300, no changes were observed in plasma HCY, CYS and GSH in both females ($F_{(2,10)} = 1.26; P = 0.30$) and males ($F_{(2,10)} = 0.64; P = 0.70$). Likewise, there were no changes in brain cortex concentrations in SAM, SAH and SAM:SAH ratio in females ($F_{(2,10)} = 1.26; P = 0.31$) and males ($F_{(2,15)} = 0.74; P = 0.63$). Concerning GSH, no differences were observed in female ($F_{(2,15)} = 0.69; P = 0.51$) or male mice ($F_{(2,15)} = 0.75; P = 0.49$), Table 6.

In respect of locomotor activity, the SP and SPL females presented lower locomotion when compared with the CT group ($F_{(2,20)} = 5.91; P = 9.7 \times 10^{-3}$). No differences were observed in the males ($F_{(2,10)} = 1.02; P = 0.38$) (Fig. 5).
Table 1. Organ weights (g) and relative organ weights (%) in female and male offspring at postnatal day (PND) 28 from dams supplemented with methionine during pregnancy and pregnancy/lactation (Mean values and standard deviations, n 4–8; General linear model)

<table>
<thead>
<tr>
<th></th>
<th>CT (n 4)</th>
<th>SP (n 8)</th>
<th>SPL (n 7)</th>
<th>F value</th>
<th>P value</th>
<th>CT (n 7)</th>
<th>SP (n 8)</th>
<th>SPL (n 7)</th>
<th>F value</th>
<th>P value</th>
</tr>
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<tbody>
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<td></td>
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</tr>
<tr>
<td>Liver (g)</td>
<td>1.4</td>
<td>0.1</td>
<td>1.4</td>
<td>0.3</td>
<td></td>
<td></td>
<td>1.3</td>
<td>0.2</td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>Liver (%)</td>
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<td></td>
<td>51</td>
<td>0.4</td>
<td></td>
<td>51</td>
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<tr>
<td>Spleen (g)</td>
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<td>0.1</td>
<td>0.1</td>
<td></td>
<td></td>
<td>0.1</td>
<td>0.3</td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>Spleen (%)</td>
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<td>0.1</td>
<td>0.6</td>
<td>0.4</td>
<td></td>
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<td>0.5</td>
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<td>0.5</td>
</tr>
<tr>
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<td>0.02</td>
<td>0.2</td>
<td>0.03</td>
<td></td>
<td></td>
<td>0.2</td>
<td>0.02</td>
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<tr>
<td>Kidney (%)</td>
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<td>0.6</td>
<td>0.04</td>
<td></td>
<td></td>
<td>0.6</td>
<td>0.03</td>
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<td>0.6</td>
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<tr>
<td>PWAT (g)</td>
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<td>0.3</td>
<td>0.1</td>
<td></td>
<td></td>
<td>0.2</td>
<td>0.1</td>
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<td>0.2</td>
</tr>
<tr>
<td>PWAT (%)</td>
<td>1.2</td>
<td>0.2</td>
<td>1.1</td>
<td>0.4</td>
<td></td>
<td></td>
<td>0.8</td>
<td>0.4</td>
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<td>0.9</td>
</tr>
<tr>
<td>PCWAT (g)</td>
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<td>0.1</td>
<td>0.02</td>
<td></td>
<td></td>
<td>0.1</td>
<td>0.03</td>
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<tr>
<td>PCWAT (%)</td>
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<td>0.1</td>
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</tr>
<tr>
<td>Heart (g)</td>
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<td>0.02</td>
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<td></td>
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<td>0.01</td>
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</tr>
<tr>
<td>Heart (%)</td>
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<td>0.5</td>
<td>0.1</td>
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<td></td>
<td>0.5</td>
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CT, control; SP, supplemented during pregnancy; SPL, supplemented during pregnancy and lactation; PND, postnatal day; PWAT, perigonadal white adipose tissue; PCWAT, pericardial white adipose tissue.

Table 2. Organ weights (g) and relative organ weights (%) in female and male offspring at postnatal day (PND) 300 from dams supplemented with methionine during pregnancy and pregnancy/lactation (Mean values and standard deviations, n 4–8; General linear model)

<table>
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<th>SPL (n 6–7)</th>
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<th>P values</th>
<th>CT (n 8)</th>
<th>SP (n 5)</th>
<th>SPL (n 6)</th>
<th>F values</th>
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</tr>
<tr>
<td>Liver (g)</td>
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<td>0.2</td>
<td></td>
<td>1.8</td>
<td>0.4</td>
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<td>F_2(1.19) = 4.66</td>
<td>P = 4.5 · 10^(-3)</td>
</tr>
<tr>
<td>Liver (%)</td>
<td>39</td>
<td>0.7</td>
<td>3.3</td>
<td>0.4</td>
<td></td>
<td>3.0</td>
<td>0.3^*†</td>
<td></td>
<td>F_2(1.19) = 3.51</td>
<td>P = 0.018</td>
</tr>
<tr>
<td>Spleen (g)</td>
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<td>0.1</td>
<td>0.0</td>
<td></td>
<td>0.1</td>
<td>0.0</td>
<td></td>
<td>F_2(1.19) = 5.46</td>
<td>P = 1.8 · 10^(-3)</td>
</tr>
<tr>
<td>Spleen (%)</td>
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<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td></td>
<td>0.2</td>
<td>0.0^*†</td>
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<td>F_2(1.19) = 0.41</td>
<td>P = 0.72</td>
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<tr>
<td>Kidney (g)</td>
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<td>0.1^*†</td>
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<td>0.1</td>
<td></td>
<td>F_2(1.19) = 3.24</td>
<td>P = 0.024</td>
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<tr>
<td>Kidney (%)</td>
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<td>1.1</td>
<td>5.8</td>
<td>2.7^*</td>
<td></td>
<td>5.8</td>
<td>2.2^*</td>
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<td>F_2(1.19) = 3.16</td>
<td>P = 0.021</td>
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<td>3.6^*†</td>
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<td>F_2(1.19) = 0.19</td>
<td>P = 0.12</td>
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<tr>
<td>PCWAT (%)</td>
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<td>0.3</td>
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<td>F_2(1.19) = 0.19</td>
<td>P = 0.12</td>
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<tr>
<td>Heart (g)</td>
<td>0.2</td>
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<td>0.0</td>
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<td>F_2(1.19) = 0.19</td>
<td>P = 0.12</td>
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<tr>
<td>Heart (%)</td>
<td>0.4</td>
<td>0.1</td>
<td>0.3</td>
<td>0.1^*†</td>
<td></td>
<td>0.3</td>
<td>0.1</td>
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<td>F_2(1.19) = 0.19</td>
<td>P = 0.12</td>
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</table>

CT, control; SP, supplemented during pregnancy; SPL, supplemented during pregnancy and lactation; PND, postnatal day; PWAT, perigonadal white adipose tissue; PCWAT, pericardial white adipose tissue.

* Duncan's post hoc (P < 0.05), different from the CT group;
† Duncan's post hoc (P < 0.05), different from the SP group.
Small variations in the number of samples used in the different experiments carried out on PND 28 or PND 300 were related to experimental difficulties linked to data acquisition.

### Discussion

Over the past few years, our group has studied the effects of the manipulation of methyl radicals on the early development of offspring. First, we assessed the effects of hypomethylation, produced by a decrease in SAM availability in riboflavin, folate, cobalamin and choline-deficient diets during pregnancy and pregnancy/lactation\(^{14,21}\). In the current study, we evaluated the effects of hypermethylation produced by increasing the availability of methionine, a SAM precursor, during the same period.

The results of the previous studies related to an environment with a reduced availability of methyl suggested different responses between female and male mice, as there was only a reduction in body and PWAT weight in male offspring of mice submitted to vitamin B deficiency during pregnancy/lactation\(^{14}\). In the current study, the long-term effects of an environment with increased availability of methyl resulted in increased body and PWAT weight only in female offspring of mice supplemented with methionine during pregnancy and pregnancy/lactation, reinforcing the finding that sexes respond differently to variations in the availability of methyl radicals.

In fact, at PND 0, the female offspring of dams submitted to vitamin B deficiency during pregnancy show increased SAH levels, while male offspring show decreased SAM\(^{21}\). In the present study, no statistically significant changes were observed in SAM and SAH concentrations after birth, although we found a suggestive effect in that 34% increase observed in SAH concentrations in female offspring of supplemented dams, vs. approximately 18% in male offspring. We hypothesised that the increased SAH concentrations found in females in these two experiments may be a result of the greater need for methyl radicals to randomly inactivate one of the two X chromosomes present in each somatic cell during development\(^{23}\). This raises the question of whether this female biological characteristic would be a factor protecting them in an undernourished environment, a more plausible situation in real life than an over-supply of methionine.

Interestingly, early vitamin B deficiency caused a reduction in birth weight in both female and male offspring; however, only males remained underweight throughout their development as a long-term result of the deficiency during pregnancy/lactation\(^{14}\). In the current study, both females and males presented a slight decrease in birth weight, but it was statistically significant only for males. However, evaluations at PND 5, 28, 90 and 300 showed no changes in male body weight. In females, body weight gain occurred after PND 90 and was found at PND 300 in both the SP and SPL groups. In a study of rats, maternal hyperhomocysteinemia induced by a high methionine diet was shown to slightly decrease offspring birth weight, but it was not significantly lower than the control values; on the day before euthanasia (PND 75), no differences in body weight were observed between the groups\(^{24}\). In another study, methionine supplementation (0.1%, 0.2%, 0.3% or 0.4% w/w)
associated with a protein-deficient diet (9% casein) during early development affected long-term body weight. The early protein deficiency decreased the long-term body weight per se, and the data suggested a trend towards reduced adult body weight related to increased methionine in the diet, which was reversed in the offspring of dams receiving the highest supplement of 0.4% w/w methionine. However, the authors draw attention to the fact that some dams in this group gave birth to litters of less than ten pups, while others had litters with about thirteen pups, and the ‘small litter appears to have improved fetal growth, possibly because they are better able to deal with excess methionine, or because the smaller litter ameliorates the effect of reduced protein.\(^{(25)}\) In the current study, the litter size was adjusted to eight animals per dam to exclude this effect.

Concerning organ weights, no changes were observed at PND 28 in females or males. At PND 300, no changes were observed in males, but females showed some alterations. The increases in total and relative PWAT in SP and SPL females were in line with increased body weight; however, the decrease in the relative weight of other organs appears to have resulted only from the increase in body weight, as no changes were observed in total organ weight. Despite the increase in body and PWAT weight, no changes in glucose, TAG, lactate and cholesterol were observed. Regarding the analyses of HCY, CYS, GSH,
SAM and SAH at PND 28, we observed only an increase in HCY concentrations in both females and males. The increase in this metabolite, an intermediate from methionine metabolism, was not accompanied by an increase in the concentrations of CYS and GSH, which are produced in the HCY transsulphuration pathway. A possible explanation for this is because the enzymes involved in the transsulphuration present Kms with at least an order of magnitude greater than the Km of the two HCY methyltransferases\(^{26}\). No changes in these metabolites were observed after weaning and exposition to a standard diet.

After weaning, all animals were fed with a control diet. So, the increased body weight in SP and SPL females may be related to the increased amount of methionine ingested during early development. It should be noted that these same animals also presented a decrease in locomotor activity. A study showed that acutely reduced locomotor activity was the major contributor to Western diet-induced obesity in mice. In this case, the decreased locomotor activity and consequent reduction in energy expenditure explained more than 60 % of the weight gain in these animals\(^{27}\).

In the current study, locomotor activity was evaluated only at PND 299; there is no data on locomotor activity throughout the experiment. However, we know that until PND 90, no changes in body weight were observed, which leads us to infer that the reduction in locomotor activity occurred after PND 90. On the other hand, we cannot say that there is a causal relationship between these two parameters as, in another study, a reduction in locomotor activity was also observed in male pups of vitamin B-deficient mothers, but they showed lower PWAT content and body weight\(^{30}\).

The current study and our previous study\(^{60}\) both showed the important role of methyl radicals in early life, for example, in the control of adipose tissue development and body weight. Therefore, the effects of dietary methyl donors must be carefully monitored and observed, not only because of the already established association with an increased risk of neural tube defects\(^{28}\) but also in relation to the important morphometric changes observed and the different effects that the availability of methyl radicals can have on males and females.

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V. C. S and V. D.’A. designed the study and wrote the protocol. V. C. S. and L. F. managed the experimental procedures and analyses. V. C. S. and J. R. S. V. undertook the statistical analysis, and V. C. S., J. R. S. V., L. F., A. C. O. and V. D.’A. wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

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