

# Excess perigestational folic acid exposure induces metabolic dysfunction in post-natal life

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## Abstract

The aim of this study was to understand whether high folic acid (HFA) exposure during the perigestational period induces metabolic dysfunction in the offspring, later in life. To do this, female Sprague–Dawley rats (G0) were administered a dose of folic acid (FA) recommended for pregnancy (control, C, 2 mg FA/kg of diet,  $n=5$ ) or a high dose of FA (HFA, 40 mg FA/kg of diet,  $n=5$ ). Supplementation began at mating and lasted throughout pregnancy and lactation. Body weight and food and fluid intake were monitored in G0 and their offspring (G1) till G1 were 13 months of age. Metabolic blood profiles were assessed in G1 at 3 and 13 months of age (3M and 13M respectively). Both G0 and G1 HFA females had increased body weight gain when compared with controls, particularly 22 (G0) and 10 (G1) weeks after FA supplementation had been stopped. G1 female offspring of HFA mothers had increased glycemia at 3M, and both female and male G1 offspring of HFA mothers had decreased glucose tolerance at 13M, when compared with matched controls. At 13M, G1 female offspring of HFA mothers had increased insulin and decreased adiponectin levels, and G1 male offspring of HFA mothers had increased levels of leptin, when compared with matched controls. In addition, feeding of fructose to adult offspring revealed that perigestational exposure to HFA renders female progeny more susceptible to developing metabolic unbalance upon such a challenge. The results of this work indicate that perigestational HFA exposure affects long-term metabolic phenotype of the offspring, predisposing them to an insulin-resistant state.

## Key Words

- ▶ dietary supplementation
- ▶ fetal programming
- ▶ folic acid
- ▶ metabolic syndrome
- ▶ pregnancy

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## Introduction

Folate is a generic term that designates a family of naturally occurring B-group vitamins derived from folic acid (pteroylglutamate, FA). FA is the fully oxidized and synthetic form of the vitamin used in supplements and in food fortification.

Folates are critical for the synthesis of purines and thymidylate, and for the generation of S-adenosyl-methionine, a major methyl donor for DNA methylation processes, thus being essential compounds for cell division, for the epigenetic regulation of gene expression and, very probably, for fetal programming (Burdge & Lillycrop 2010).

Besides this emerging role of folate in fetal programming, its importance during fetal development is unequivocal, as not only is low maternal folate intake associated with low birth weight and neural tube defects (NTDs), but also supplementation with FA during the periconceptional period reduces the incidence of such outcomes (Luccock 2000).

The effectiveness of FA in prevention of NTDs, and the inadequate compliance with FA supplementation by women of childbearing age resulted in the mandatory fortification of all enriched grain products with FA, in the USA, in the late 1990s. After the implementation of this policy, food fortification with FA was demonstrated to produce a general increase in serum folate levels (with concomitant decrease in homocysteine levels) in a US representative population sample (Pfeiffer *et al.* 2005) and to associate negatively with the prevalence of NTDs (Honein *et al.* 2001).

By January 2009, mandatory fortification with FA had been introduced in 52 countries worldwide. At that time, several EU countries permitted voluntary fortification, though none had implemented mandatory fortification (ESCO 2009), given the uncertainty about potential health hazards. In addition, the WHO currently recommends supplementation with 0.4 mg/day of FA to women during pregnancy and the first 3 months *post partum*. As a result of all these public health policies, it is an emerging concern that FA intake may be exceeding the recommended upper levels, as has been recently described by several authors (Bailey *et al.* 2010, Hoyo *et al.* 2011, Gomez *et al.* 2013), with possible implications for serum FA levels (Brown *et al.* 2011).

To date, several studies report the programming effects of maternal FA supplementation on the offspring's health, mainly regarding autism (Castro *et al.* 2014), asthma, insulin resistance, and cancer (see Burdge & Lillycrop

(2012) for a review). Results of studies of fetal programming of cancer by high FA exposure are somewhat conflicting. Indeed high intrauterine exposure to FA has been shown to increase mammary cancer risk in rodent offspring (Ly *et al.* 2011) while exerting protective effect against colorectal cancer risk (Sie *et al.* 2011).

Metabolic syndrome (MetS) is a condition of high and increasing prevalence worldwide that comprises a constellation of risk factors for cardiovascular disease and type 2 diabetes, such as central obesity, glucose intolerance, insulin resistance, dyslipidemia, and hypertension. MetS may be determined during fetal development due to nutritional, hormonal, or metabolic inputs from the mother (Sinclair *et al.* 2007, Xita & Tsatsoulis 2010).

Based on this rationale and because there is a major gap in the current knowledge regarding the long-term metabolic effects of exposure to high doses of FA, the driving hypothesis of this work was as follows: does high FA supplementation during the perigestational period result in fetal programming of metabolic dysfunction during post-natal life?

To address this central question, we studied the effect of a perigestational supplementation of the diet of Sprague–Dawley (SD) female rats with high levels of FA on the long-term metabolic phenotype of their offspring.

## Materials and methods

### Materials

The study materials include D-fructose, D-glucose (Sigma–Aldrich); ketamine chlorhydrate (Imalgène 1000, Merial Portuguesa, Rio de Mouro, Portugal); medetomidine hydrochloride (Domitor, Orion Pharma, Orion Corporation, Espoo, Finland); treatment diets (control (C) or high folic acid (HFA), Research Diets, Inc., New Brunswick, NJ, USA); and standard diet (SAFE, Augy, France).

### Animals and diets

All animal procedures were approved by the Directorate of Health and Animal Protection Services of the General Veterinarian Direction of the Portuguese Government. A diagram of the study design is depicted in Fig. 1.

Ten 7-week-old (225 g initial body weight) female SD CD rats (generation G0) (Charles River Laboratories, Barcelona, Spain) housed individually were divided into a control (C, *n*=5) and a high FA supplemented

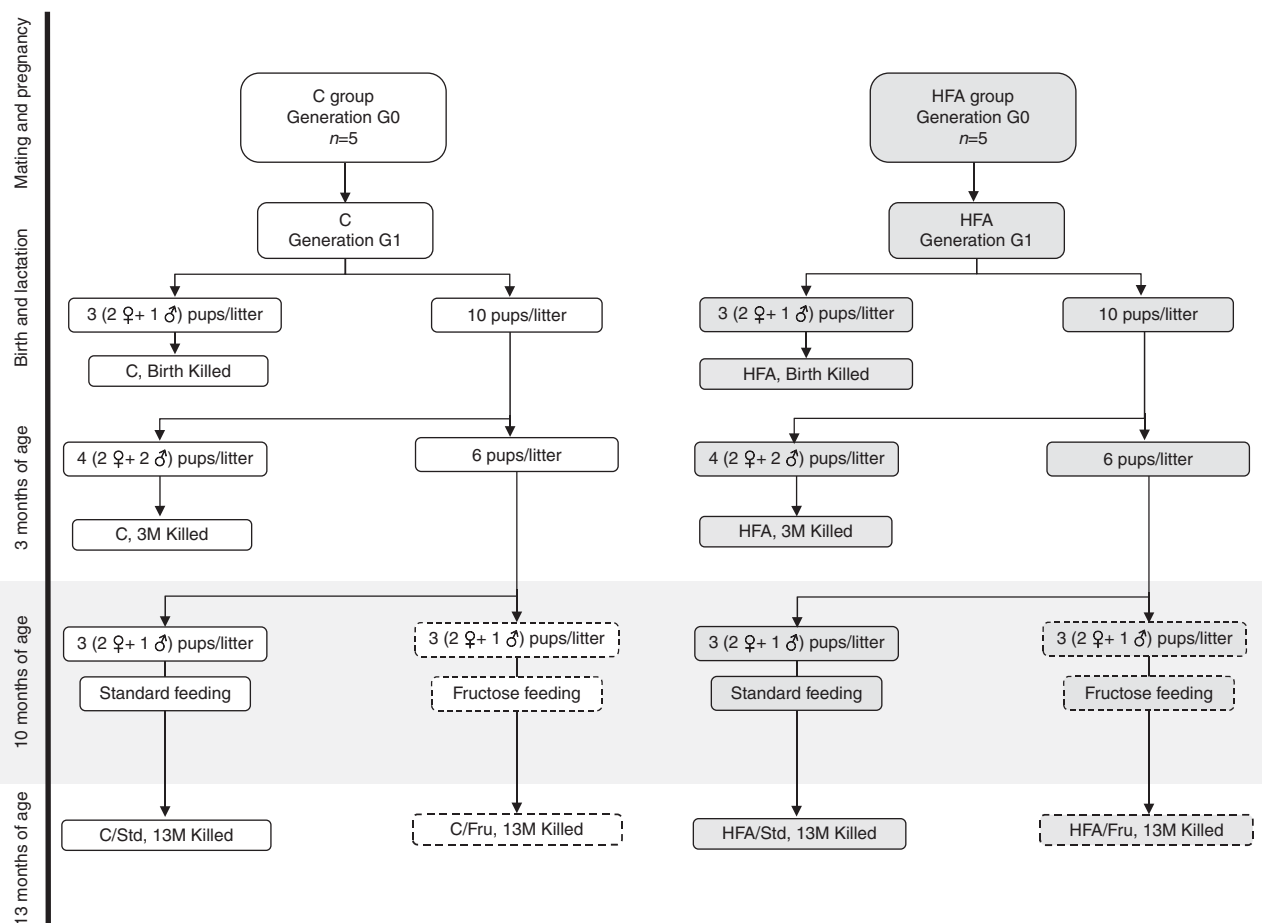
**Figure 1**

Diagram of the study design. In generation G0, control dams (C) were fed an adequate level of folic acid for pregnancy (2 mg FA/kg of diet) and HFA dams were fed a FA-enriched diet containing 40 mg FA/kg of diet. FA supplementation began during the mating period and lasted throughout pregnancy and lactation. Each generation G1 group (C and HFA) was divided, at 10 months of age, into a standard group fed the standard rat

diet (C/Std and HFA/Std) and a high-fructose group, fed a high dose of fructose (10% fructose in the drinking water) plus the standard rat diet (C/Fru and HFA/Fru). The animals were killed at birth, 3 months of age (3M), and 13 months of age (13M) for blood and tissue collection. The mean numbers of pups (total (females + males)) used per litter in each group are indicated.

(HFA,  $n=5$ ) group. C dams were fed a diet containing an adequate level of FA for pregnancy (2 mg FA/kg of diet) (Achon *et al.* 2000) and HFA dams were fed a FA-enriched diet containing 40 mg FA/kg of diet. Diets were isocaloric, isollipidic, isoproteic, and isoglycidic (see Supplementary Table 1, see section on supplementary data given at the end of this article for diet composition). FA supplementation began during the mating period and lasted throughout pregnancy and lactation (perigestational period). Each G0 female was mated with a 7-week-old SD male and mating was confirmed by vaginal plug detection, which elicited estimation of gestational age and date of delivery.

On weaning, all mothers (G0) and pups (G1, both males and females) were given the same standard diet containing 0.5 mg FA/kg of diet. This protocol ensured

that HFA and C diets were given only to G0, and so that G1 was exposed to HFA (or to C) diet only through placental feeding or lactation. Also the protocol approaches the situation of human pregnancy during which an extra-dose of FA is administered when compared with the non-pregnant state, when ingestion of FA is usually reduced.

Food and water were available and animals were allowed to eat and drink *ad libitum*. Animals were maintained in acclimatized rooms in 12 h light:12 h darkness cycles.

The body weight and food and water ingestion of G0 females were measured daily from the beginning of the study till weaning and every 5–7 days thereafter. For G1 animals, (both males and females) body weight, feeding and drinking were measured every 3 days from birth till the end of the study.

In order to assess whether perigestational HFA supplementation rendered G1 animals more susceptible to metabolic dysfunction upon a metabolic challenge, each G1 group (C and HFA, both males and females) was divided, at 10 months of age, into a standard group fed the standard rat diet (C/Std and HFA/Std) and a high-fructose group, fed a high dose of fructose (10% fructose in the drinking water) plus the standard rat diet (C/Fru and HFA/Fru).

For blood and tissue collection, the animals were killed by exsanguination under anesthesia with 50 mg/kg ketamine and 1 mg/kg medetomidine, as follows:

- i) G0 females were killed 13 months after delivery.
- ii) G1 animals (both males and females) were randomly selected from each treatment group and killed at: birth (B) (mean of three pups – two females and one male – per litter), 3 months of age (3M, young adulthood, mean of four pups – two males and two females – per litter) and 13 months of age (13M, late adulthood, mean of three pups – two females and one male – per litter).

In all cases, whole blood was collected by cardiac puncture and serum or plasma–EDTA was separated by centrifugation and kept at  $-80^{\circ}\text{C}$  until analyzed. The organs and tissues were dissected, weighed, and immediately snap frozen in liquid nitrogen, and then kept at  $-80^{\circ}\text{C}$  for future analyses.

#### Quantification of blood biochemical, metabolic, and inflammatory parameters

Serum glucose, uric acid, C-reactive protein (CRP), LDL, and HDL cholesterol were quantified using conventional methods with an Olympus AU5400 automated clinical chemistry analyzer (Beckman-Coulter, Izasa, Porto, Portugal). Homocysteine was measured by chemiluminescent immunoassay using an Architect i2000 SR automated analyzer (Abbott).

Serum folate was quantified by time-resolved fluorimmunoassay using an AutoDelfia automated analyzer (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland).

Plasma insulin, leptin, and adiponectin were quantified by ELISA using commercially available kits according to the manufacturer's instructions (insulin and leptin: Millipore, St Charles, MO, USA; adiponectin: Invitrogen Corporation). According to the instructions, if the difference between duplicate results of a sample was over  $>15\%$  (of the coefficient of variation), the sample was

repeated whenever it was possible. Sensitivities of the assays were 0.2 ng/ml (insulin), 0.04 ng/ml (leptin), and 50 pg/ml (adiponectin).

Serum interleukin 1 beta (IL1 $\beta$ ), interleukin 10 (IL10), and transforming growth factor-beta 1 (TGF $\beta$ 1) were measured by Luminex assay using the custom Milliplex Rat kits (Merck Millipore, Madrid, Spain), according to the manufacturer's protocols, by Luminex xMAP Multiplexing Technology platform.

#### Oral glucose tolerance assessment

Oral glucose tolerance tests (OGTT) were performed in animals fasted for 4 h using OneTouch Ultra Blood Glucose Meter (Lifescan Canada Ltd, Burnaby, British Columbia, Canada). Glucose was administered by gavage (2 g of glucose/kg body weight) and blood glucose was quantified at 0, 15, 30, 60, 90, and 120 min, by lateral tail vein puncture.

#### Bioelectrical impedance

Body fat was determined by bioelectrical impedance, using a Quantum/S bioelectrical impedance analyzer (RJL Systems, Akern SRL, Florence, Italy), according to methods published previously (Smith *et al.* 2009).

#### Blood pressure measurement

For 1 week before measurement of systolic blood pressure (SBP), animals were acclimated daily to the procedure room and handling. Indirect measurement of SBP in awake-restrained rats was carried out by the non-invasive tail-cuff method, using LE 5000 (Letica, Barcelona, Spain). To make the pulsations of the tail artery detectable, rats were kept at  $37^{\circ}\text{C}$  for 15 min before the measurements. After obtaining a stable pulse (300 HR), three to five consecutive measurements of SBP were taken and the average of them was considered for the analysis.

#### Calculations and statistics

As experimental exposure was performed upon the mothers (G0) and not the offspring (G1), the population size ( $n$ ) considered for statistical analyses refers to the number of litters rather than the number of pups. Given the size of the litters obtained, the greater amount of females than males obtained in both treatment groups, and the division of animal groups by sex, treatment and age, the mean number of pups representing each litter at birth and at 13M is three (two females and one male).

The mean number of pups representing each litter at 3M is four (two females and two males).

The Lee index, an equivalent of the BMI in humans, was calculated according to the equation: Lee index = body weight<sup>1/3</sup> (g)/nasal–anal length (cm) × 1000 (de Moura *et al.* 2009).

Data are expressed as means ± S.E.M. For comparisons between two groups, the unpaired *t*-test was used. For comparisons between more than two groups, one-way ANOVA followed by the Tukey's *post hoc* test was used. Data concerning different ages and different treatments (two variables) were analyzed using two-way ANOVA, followed by the Bonferroni *post hoc* test. Differences were considered significant for *P* < 0.05.

## Results

### Gestational and morphometric data

Gestational and morphometric data are given in Table 1. Although gestational data, such as mating efficiency, gestational age at birth, litter size, and the female-to-male ratio, do not differ between treatment groups, morphometric measures tend to be higher in the HFA group, achieving statistical significance for maternal Lee index and female birth weight.

We found that pancreas weight at birth of whole-offspring population was significantly higher in HFA group, when compared with controls ( $0.02378 \pm 0.00167$  versus  $0.03440 \pm 0.00064$  g in the C and HFA groups respectively; *n* = 3). This difference was not statistically significant when pancreas weight was stratified by sex. We found no differences in the weight of pup liver, heart, or brain at birth between treatment groups (data not shown).

In older animals (3 and 13 months of age), HFA supplementation was associated with an increased heart weight in female offspring (at 3 months of age:  $0.869 \pm 0.043$  g versus  $0.784 \pm 0.042$  g in the HFA and C groups respectively; and at 13 months of age:  $1.064 \pm 0.026$  g versus  $0.994 \pm 0.012$  g in the HFA and C groups respectively; *n* = 4–5), but not in male offspring (Supplementary Table 2, see section on supplementary data given at the end of this article). No differences between HFA and C groups at 3 or 13 months of age were found in the weight of pancreas, liver, brain, mesenteric, or gonadal adipose tissue (Supplementary Table 2).

### HFA mothers have increased weight gain and reveal hyperphagia and hyperdyspsia after the end of HFA exposure

Although maternal weight was not significantly different in the C and HFA groups at the time of delivery (Fig. 2 and Table 1), HFA mothers displayed increased weight gain from 22 weeks (154 days) after the end of the treatment period (weaning) until the end of the study (450 days).

In addition, HFA mothers ingested more food (Fig. 2B and C) and water (Fig. 2D and E) particularly after FA supplementation had been stopped, i.e. at weaning.

Interestingly, at the end of the study, HFA mothers had less gonadal (non-visceral) adipose tissue masses ( $18.40 \pm 0.54$  g versus  $20.82 \pm 0.83$  g in HFA versus C mothers respectively; *n* = 3–5) with a concomitant tendency (although not statistically significant) for greater mesenteric (visceral) adipose tissue masses when compared with control mothers ( $18.93 \pm 2.75$  g versus  $16.43 \pm 1.385$  g in HFA versus C mothers, respectively; *n* = 3–5; Supplementary Table 3, see section on

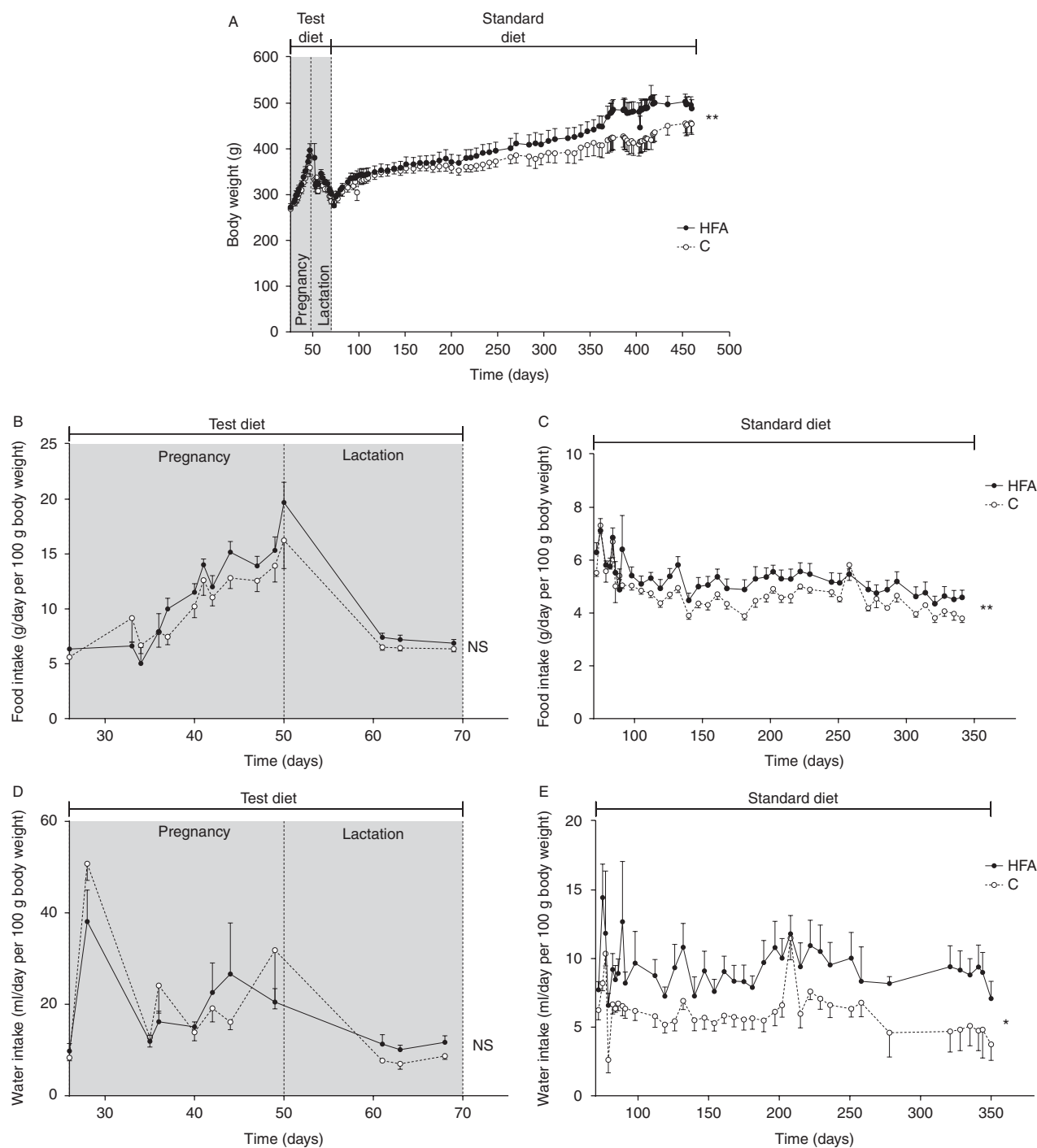
**Table 1** Gestational and morphometric data. Values represent means ± S.E.M. (*n*)

|                                                        | C                   | HFA                 | P      |
|--------------------------------------------------------|---------------------|---------------------|--------|
| G0                                                     |                     |                     |        |
| Mating efficiency (days) <sup>a</sup>                  | 3.4 ± 1.0 (5)       | 2.0 ± 0.4 (5)       | 0.2476 |
| Gestational age at birth (days)                        | 21.0 ± 0.3 (5)      | 21.4 ± 0.2 (5)      | 0.3466 |
| Maternal body weight (g) <sup>b</sup>                  | 390.6 ± 9.0 (5)     | 413.8 ± 11.9 (5)    | 0.1572 |
| Maternal Lee index (g <sup>1/3</sup> /cm) <sup>c</sup> | 332.1 ± 4.5 (5)     | 346.0 ± 3.5 (5)     | 0.0393 |
| G1                                                     |                     |                     |        |
| Litter size ( <i>n</i> pups)                           | 15.8 ± 0.7 (5)      | 13.4 ± 1.6 (5)      | 0.2033 |
| Female-to-male ratio                                   | 1.8 ± 0.5 (4)       | 3.3 ± 1.5 (3)       | 0.2909 |
| Birth weight (g)                                       | 5.326 ± 0.451 (5)   | 6.491 ± 0.417 (4)   | 0.1066 |
| Female                                                 | 5.717 ± 0.113 (4)   | 6.659 ± 0.3515 (2)  | 0.0263 |
| Male                                                   | 5.802 ± 0.260 (4)   | 7.078 ± 0.628 (2)   | 0.0803 |
| Placental weight (g)                                   | 0.5553 ± 0.0897 (3) | 0.7438 ± 0.0596 (4) | 0.1268 |

<sup>a</sup>Corresponds to the number of days from mating to conception determined by vaginal plug detection.

<sup>b</sup>Body weight 3–4 days before delivery.

<sup>c</sup>Calculation based on maternal body weight 3 days before delivery.

**Figure 2**

G0 (maternal) body weight and feeding. Treatment diets (C or HFA) were given from beginning of mating till the end of lactation (grey background) when they were replaced with a standard rat diet in both groups. G0 body weight (A) and food (B and C) and water (D and E) intake, adjusted for body weight, were measured daily from the beginning of the study till weaning and every 5–7 days thereafter. The arithmetical means  $\pm$  s.e.m. (HFA,  $n=5$ ) and the arithmetical means  $\pm$  s.e.m. (C,  $n=5$ ) are shown.

Two-way ANOVA indicated a significant effect of HFA upon G0 body weight ( $P<0.0001$ ) and food ( $P<0.0001$ ) and water ( $P<0.0001$ ) intake after weaning. Age was also found to affect G0 body weight ( $P<0.0001$ ) and food ( $P<0.0001$ ) and water ( $P=0.0003$ ) intake. There was no interaction between the effects of HFA and age. \*\* $P<0.0001$ ; \* $P<0.05$ ; NS, not statistically significant.



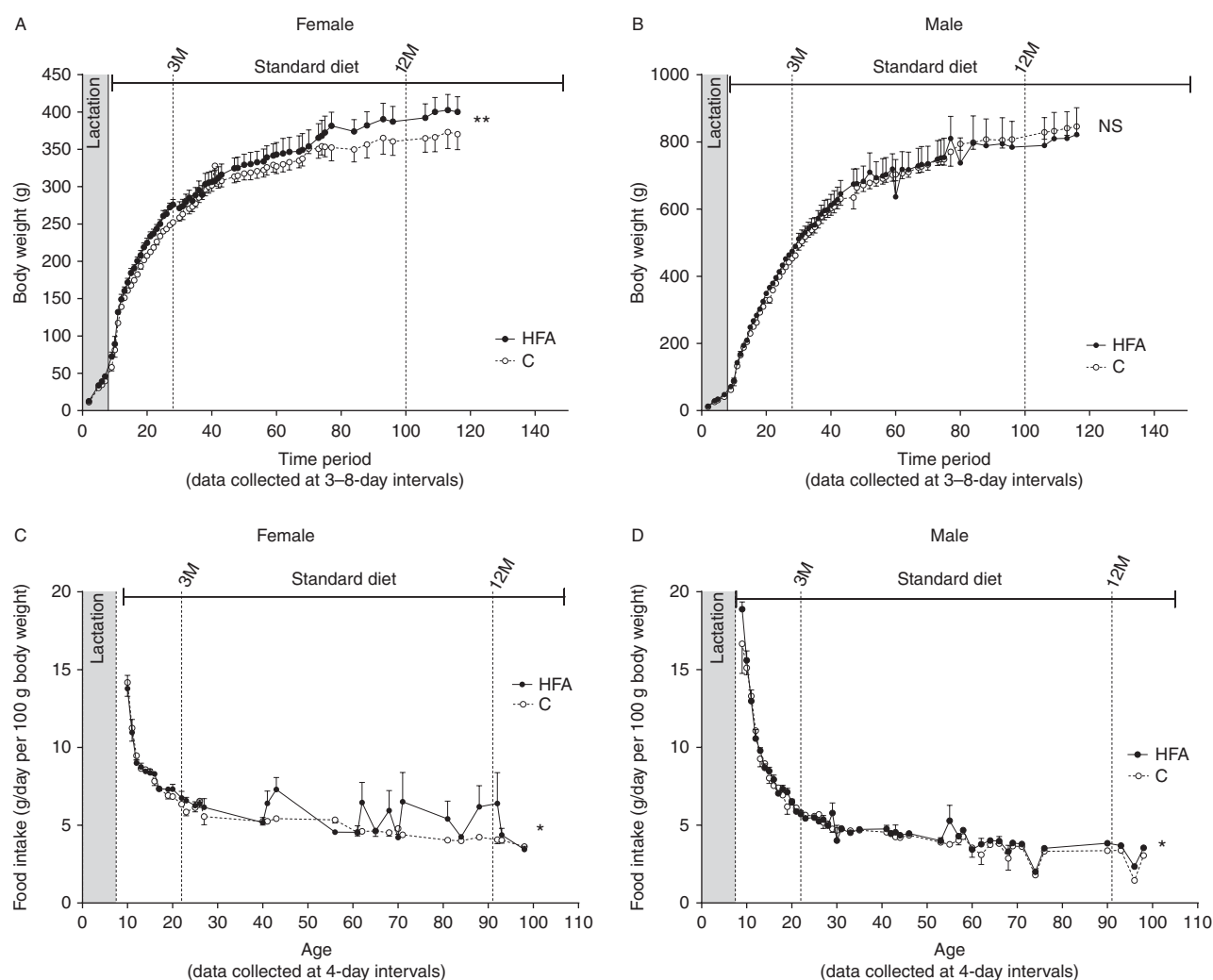
supplementary data given at the end of this article). Also, maternal fat mass at the end of the study was not significantly different between treatment groups (Supplementary Table 3).

### HFA female offspring have increased weight gain and disturbed feeding behavior

Female offspring of HFA mothers not only revealed increased birth weight (Table 1), but also presented increased weight gain, evident from early adulthood and accentuating with aging (Fig. 3A). There were no

differences in weight gain between HFA and C groups for male offspring (Fig. 3B).

In line with these findings, HFA females developed increased food intake from 3 months after weaning onwards. Notably, this increase in food intake was not consistent over time, revealing disturbed feeding behavior in this female population (Fig. 3C). On the other hand, HFA supplementation increased food intake to a much smaller extent in male offspring (Fig. 3D), and there were no signs of disturbance in feeding behavior. There were no differences in water intake between HFA and C groups for female or male offspring (data not shown).



**Figure 3**

G1 (offspring) body weight and feeding. Treatment diets (C or HFA) were given to G0 from beginning of mating till the end of lactation (grey background) when they were replaced with a standard rat diet in both groups. Body weight of G1 females and males (A and B, respectively) and food intake of G1 females and males (C and D respectively) were measured every 3 days from birth till killing. The arithmetical means  $\pm$  s.e.m. (HFA,  $n=5$ )

and arithmetical means  $\pm$  s.e.m. (C,  $n=4$ ) are shown. Two-way ANOVA indicated a significant effect of HFA upon body weight of G1 females ( $P<0.0001$ ), and food intake of G1 females ( $P=0.0009$ ) and G1 males ( $P=0.0132$ ). Age was found to affect all the measured parameters ( $P<0.0001$ ). There was no interaction between the effects of HFA and age for any of the parameters. \*\* $P<0.0001$ ; \* $P<0.05$ ; NS, not statistically significant.

### No changes were found in blood pressure and fat mass of HFA when compared with C offspring

For G1 animals (whole-population, females and males) we found no differences in blood pressure at 3 or 13 months of age and in fat mass at 13 months of age between C or HFA groups (Supplementary Table 2).

### HFA offspring have impaired glycemic control

Blood general biochemistry with a particular focus on glycemic control markers was assessed in G1.

At 3 months of age we found no alterations in serum homocysteine, LDL, HDL, uric acid, or CRP in offspring (whole, female, and male) populations (data not shown). Serum folate levels tended to be higher in HFA offspring, albeit the difference was not significant ( $39.92 \pm 2.221$  nmol/l versus  $35.73 \pm 1.932$  nmol/l in HFA and C for the whole offspring population respectively;  $n=4-5$ ;  $40.77 \pm 1.911$  nmol/l versus  $35.45 \pm 1.930$  nmol/l in HFA and C female offspring respectively;  $n=4-5$ ; and  $39.74 \pm 3.401$  vs  $35.02 \pm 3.556$  in HFA and C male offspring respectively;  $n=3-5$ ).

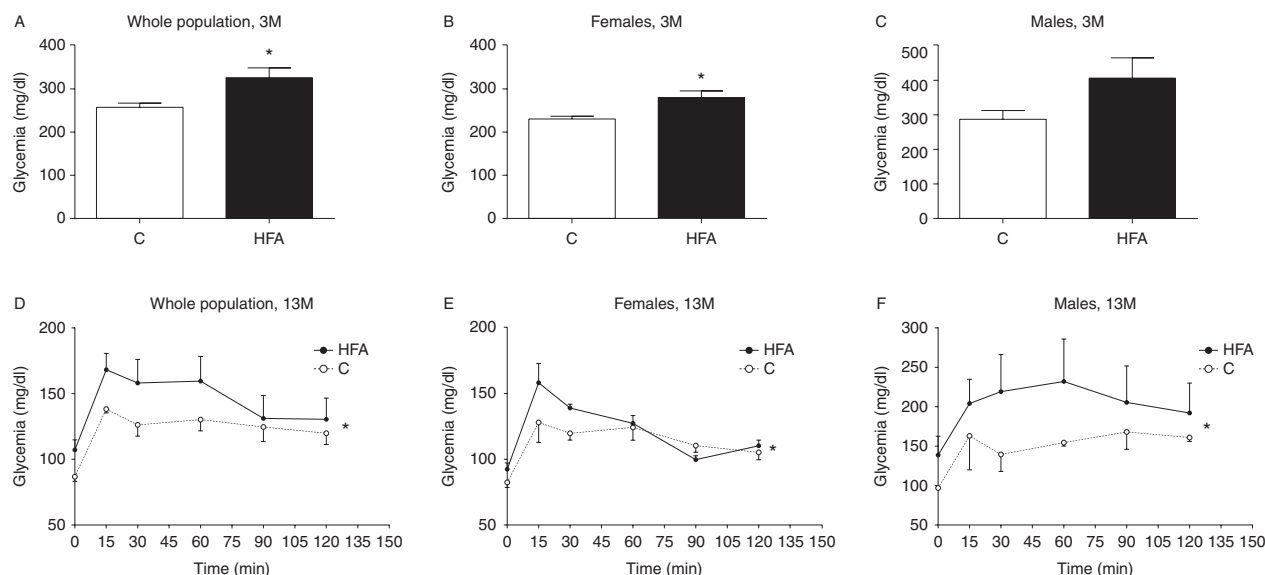
With respect to glycemic control, 3-month-old HFA offspring revealed increased glycemia when compared with matched controls (Fig. 4A). After stratification by sex, we observed that this difference is statistically significant

only in females (Fig. 4B, and C). Moreover, this impaired glycemic control is persistent with aging, as disclosed by decreased oral glucose tolerance in both sexes at 13 months of age (Fig. 4D, E, and F).

### HFA induces changes in hormone and adipokine blood levels in G1

Insulin and adipokines in plasma of 3M and 13M G1 animals were quantified. There were no differences in plasma insulin levels between C and HFA groups at 3M. However, at 13M, HFA supplementation increased insulin levels in female (but not in male) offspring, when compared with control (Fig. 5A, B, and C).

In addition, it can be observed that aging tends to reduce insulin plasma levels, which seems to be due to a reduction in pancreas weight with aging observed in the whole population (in the C group:  $1.985 \pm 0.148$  g versus  $1.811 \pm 0.088$  g at 3 and 13 months of age respectively;  $n=4$  and in the HFA group:  $2.298 \pm 0.157$  g versus  $1.769 \pm 0.118$  g at 3 and 13 months of age respectively;  $n=5$ ) and in female population (in C group:  $1.726 \pm 0.166$  g versus  $1.703 \pm 0.084$  g at 3 and 13 months of age, respectively;  $n=4$  and in HFA group:  $2.043 \pm 0.144$  g versus  $1.517 \pm 0.081$  g at 3 and 13 months of age, respectively;  $n=5$ ) in both treatment groups (Supplementary Table 2).

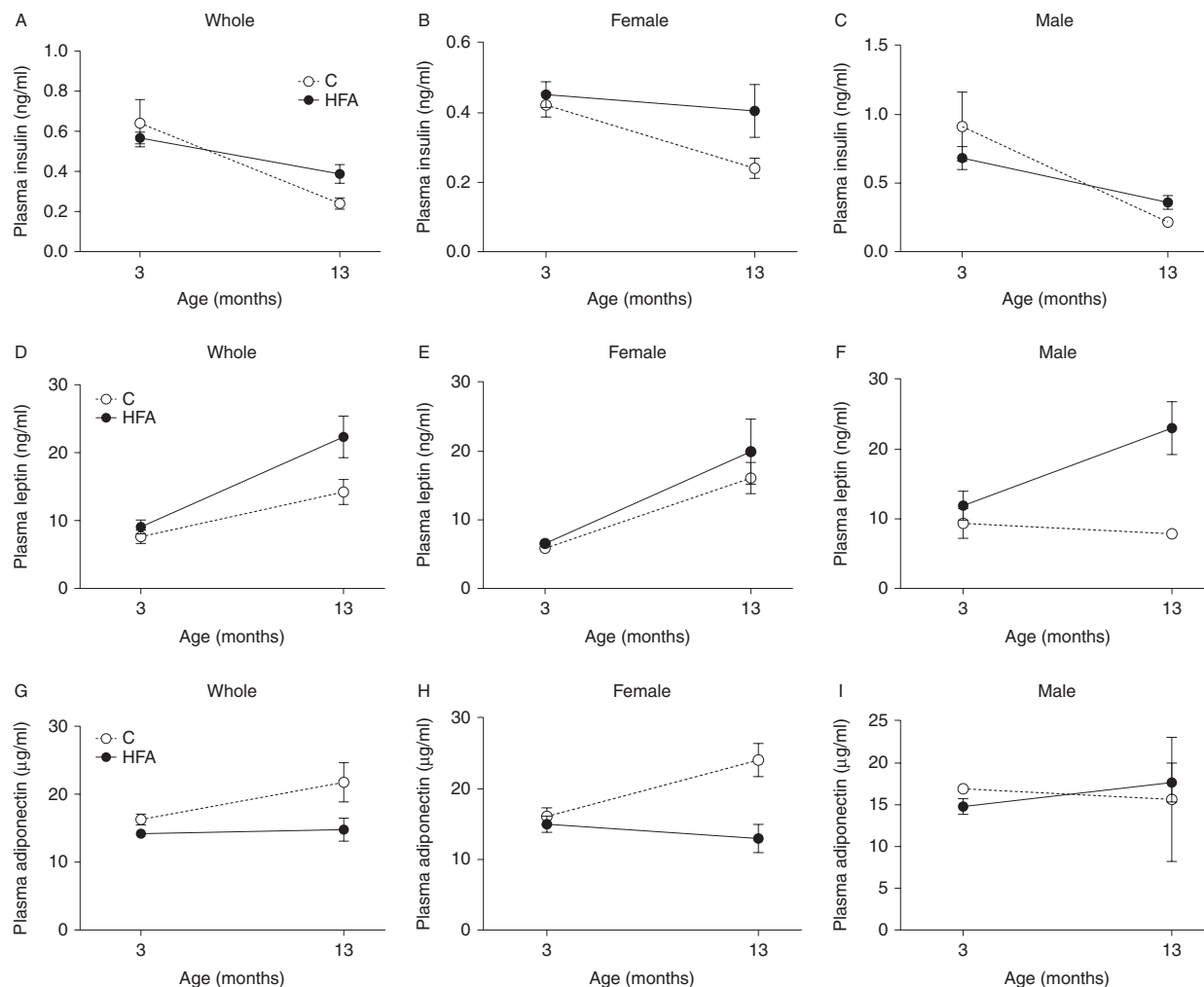


**Figure 4**

G1 (offspring) glycemic control. Plasma glucose was measured at 3 months of age in the whole (A), female (B), and male (C) G1 populations ( $n=4$  and  $5$  for C and HFA respectively). At 13 months of age, glucose tolerance was assessed in the whole (D), female (E), and male (F) G1 populations ( $n=2-4$  and  $3-5$  for C and HFA respectively). The arithmetical means  $\pm$  S.E.M. are shown.  $T$ -test

indicated a significant effect of HFA upon glycemia in the whole population and G1 females, at 3 months of age. Two-way ANOVA indicated a significant effect of HFA upon glycemia in oral glucose tolerance tests in the whole ( $P=0.0082$ ), female ( $P=0.0450$ ), and male ( $P=0.0364$ ) G1 populations. No interaction was found between the effects of HFA and time for OGT.  $*P<0.05$ .



**Figure 5**

Plasma levels of insulin (A, B, and C), leptin (D, E, and F), and adiponectin (G, H, and I) in G1 (offspring) whole (A, D, and G), female (B, E, and H), and male (C, F, and I) populations at 3 and 13 months of age. The arithmetical means  $\pm$  s.e.m. ( $n=4-5$  and  $1-5$  for 3 and 13 months of age, respectively) are shown. Two-way ANOVA indicated a significant effect of HFA upon plasma levels of insulin in female ( $P=0.0489$ ) (B) and a significant effect of age in whole ( $P=0.0011$ ) (A), female ( $P=0.0251$ ) (B), and male (C) ( $P=0.0401$ ) G1 populations. Two-way ANOVA also indicated a significant effect of HFA upon leptin plasma levels in whole ( $P=0.0200$ ) (D) and male ( $P=0.0253$ ) (F) G1 populations and a significant effect of age in whole ( $P=0.0002$ ) (D) and female ( $P=0.0004$ ) (E) G1 populations. No interaction was found between the effects of HFA and age upon plasma leptin levels. Regarding adiponectin, two-way ANOVA indicated a significant effect of HFA upon plasma levels of adiponectin in whole ( $P=0.0114$ ) (G) and female ( $P=0.0037$ ) (H) G1 populations. In G1 females an interaction between the effects of HFA and age upon adiponectin plasma levels ( $P=0.0124$ ) (G). No significant effect of age upon adiponectin plasma levels was observed.

With regard to leptin, HFA supplementation significantly accounted for increased leptinemia in the whole population at 13 months of age (Fig. 5D). When stratifying by sex, the effect is significant only for male offspring (Fig. 5E and F).

It was also observed that the levels of leptin in the whole and female offspring populations increased with age (Fig. 5D and E).

Quantification of adiponectin also retrieved interesting results (Fig. 5G, H, and I). HFA caused a decrease in adiponectin plasma levels both in whole (Fig. 5G) and

in female (Fig. 5H) offspring populations, but not in male offspring (Fig. 5I), at 13 months of age.

Serum levels of IL1 $\beta$ , IL10, and TGF $\beta$ 1 were quantified in female offspring and did not show significant differences between treatment groups (data not shown).

Fructose intake stimulates weight gain in HFA females

### Fructose intake stimulates weight gain in HFA females

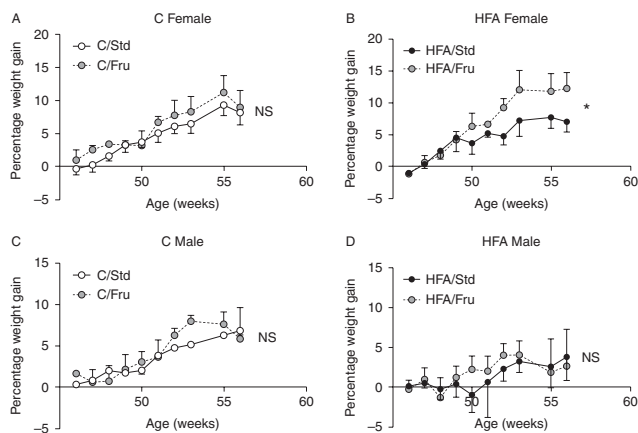
The effect of HFA perigestational supplementation with HFA upon the offspring's susceptibility to develop metabolic dysfunction in response to a metabolic challenge was

assessed by feeding fructose to 10-month-old offspring for 3 months, until the end of the study.

The fructose-fed SD rat (FFSD) is a well characterized animal model of the MetS (Oron-Herman *et al.* 2008) as these animals exhibit numerous features of the MetS, including insulin resistance, hyperinsulinemia, hypertriglyceridemia, and hypertension.

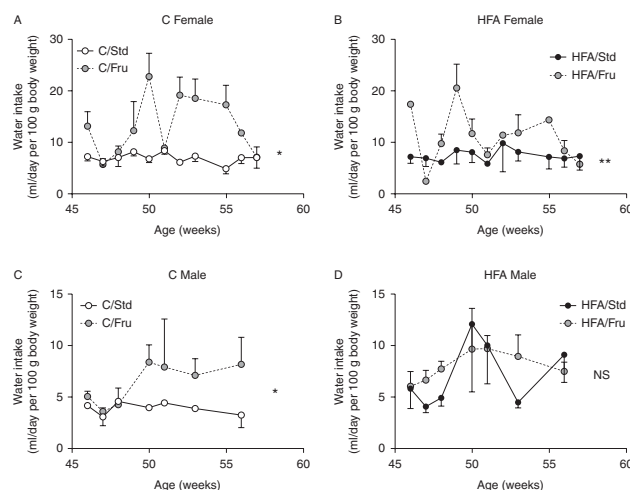
While fructose ingestion did not affect the profile of weight gain in C female offspring (no differences in percentage weight gain between C/Fru and C/Std, Fig. 6A), it resulted in an increase in weight gain in HFA-exposed female offspring (% weight gain is higher in HFA/Fru versus HFA/Std, Fig. 6B). C/Fru and HFA/Fru females ingested higher amounts of water, than C/Std (Fig. 7A) and HFA/Std (Fig. 7B) females, respectively, as expected, given the sweetness of fructose which confers higher palatability to the drinking water. This produced a compensatory decrease in food ingestion in both fructose-fed C and HFA females (Fig. 8A and B).

With regard to male offspring, feeding with fructose did not affect percentage weight gain in C or in HFA groups (Fig. 6C and D). Notwithstanding this, C/Fru males displayed increased water intake when compared with C/Std males (Fig. 7C), though no differences in water intake were observed between HFA/Fru and HFA/Std males (Fig. 7D).



**Figure 6**

G1 (offspring) body weight follow-up after feeding with fructose. Each G1 group (C and HFA) was divided, at 10 months (or 46 weeks) of age, into a standard group fed the standard rat diet (C/Std and HFA/Std,  $n=2-4$  and  $3-5$  respectively) and a high-fructose group, fed a high dose of fructose (10% fructose in the drinking water) plus the standard rat diet (C/Fru and HFA/Fru,  $n=3-4$  each). Body weight of G1 was registered once a week for C females (A) and males (C) and for HFA females (B) and males (D). The arithmetical means  $\pm$  S.E.M. are shown. Two-way ANOVA indicated a significant effect of fructose feeding upon percentage weight gain of HFA females ( $P=0.0193$ ) (B) and of age upon percentage weight gain ( $P<0.0001$ ) of C females (A), HFA females ( $P<0.0001$ ) (B), and C males ( $P=0.0033$ ) (C) in G1 populations. No interaction was found between the effects of Fru and age. NS, not statistically significant; \*,  $P<0.05$ .



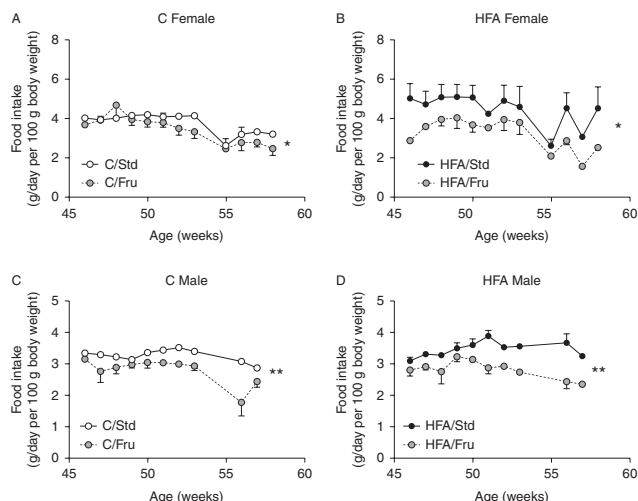
**Figure 7**

G1 (offspring) water intake follow-up after feeding with fructose. Each G1 group (C and HFA) was divided, at 10 months (or 46 weeks) of age, into a standard group fed the standard rat diet (C/Std and HFA/Std,  $n=2-4$  and  $3-5$ , respectively) and a high-fructose group fed a high dose of fructose (10% fructose in the drinking water) plus the standard rat diet (C/Fru and HFA/Fru,  $n=3-4$  each). Water intake by G1 was registered once a week for females (A and B) and males (C and D). The arithmetical means  $\pm$  S.E.M. are shown. Two-way ANOVA indicated a significant effect of feeding with fructose upon water intake by C females ( $P<0.0001$ , A), HFA females ( $P<0.0001$ , B), and C males ( $P=0.0499$ , C) of G1 populations. Two-way ANOVA also indicated a significant effect of age upon water intake by, C females ( $P=0.0055$ , A) and by HFA females ( $P=0.0466$ , B) of G1 populations, and interaction between the effects of high-fructose feeding and age in G1 C females ( $P=0.0022$ , A). \*\* $P<0.0001$ ; \* $P<0.05$ ; NS, not statistically significant.

With respect to food intake, supplying fructose in the drinking water produced a decrease in food intake in both C and HFA males (Fig. 8C and D). Importantly, feeding with fructose was able to increase fat mass, at 13M, in HFA females, but it did not affect the amount of fat mass in the whole population or male offspring (Fig. 9). Fructose feeding did not affect blood pressure or organ weight of pancreas, liver, heart, gonadal, or mesenteric adipose tissue, measured at the end of the study (13 months of age) (data not shown).

## Discussion

In this study, we tested the hypothesis that exposure to high levels of FA during the early stages of development programs the offspring to develop metabolic dysfunction later in life. For this, we exposed SD female rats to a high dose of FA during the perigestational period, and we closely monitored the metabolic phenotype of the offspring from birth to 13 months of age.



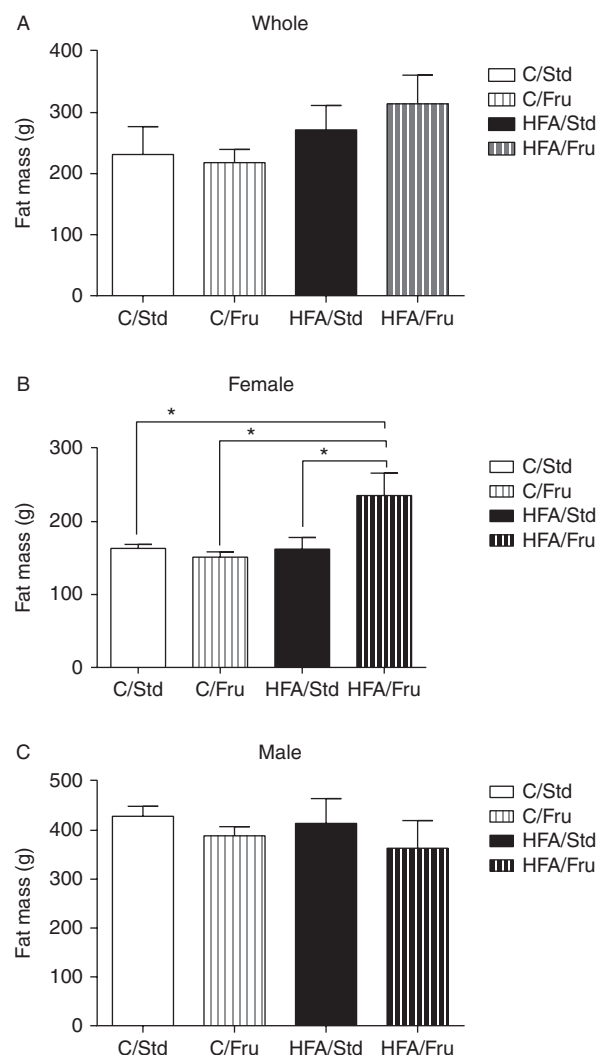
**Figure 8**

Food intake of G1 (offspring) follow-up after feeding with fructose. Each G1 group (C and HFA) was divided, at 10 months (or 46 weeks) of age, into a standard group fed the standard rat diet (C/Std and HFA/Std,  $n=2-4$  and  $3-5$ , respectively) and a high-fructose group, fed a high dose of fructose (10% fructose in the drinking water) plus the standard rat diet (C/Fru and HFA/Fru,  $n=3-4$  each). Food intake by G1 animals was registered once a week for females (A and B) and males (C and D). The arithmetical means  $\pm$  S.E.M. are shown. Two-way ANOVA indicated a significant effect of feeding with fructose upon food intake by C females ( $P=0.0139$ , A), HFA females ( $P=0.0003$ , B), C males ( $P<0.0001$ , C), and HFA males ( $P<0.0001$ , D) of G1 populations. Two-way ANOVA also indicated a significant effect of age upon food intake by C females ( $P<0.0001$ , A) and by C males ( $P=0.0081$ , C) of G1 populations. No interactions were found between the effects of high-fructose feeding and age.  $**P<0.0001$ ;  $*P<0.05$ .

The main findings of this study are summarized in Table 2. We can conclude that exposure to HFA during the perigestational period causes deterioration of the offspring's metabolic profile, particularly in females, and that the resulting overt metabolic dysfunction, revealed by increased weight gain, disturbed feeding behavior, glucose intolerance, increased insulin and decreased adiponectin plasma levels (in females), and increased leptin plasma levels (in males) develops in mid-to-late adulthood. Indeed, these alterations were observed many months after the treatment diets (C or HFA) had been replaced by the standard rat chow in both treatment groups, upon weaning, strikingly corroborating a long-term programming effect unrelated to the organoleptic features of the treatment diets. Importantly, at 3 months of age, folate levels in the serum of offspring were not significantly different between treatment groups.

Perigestational exposure to HFA was herein found to program particularly female offspring to ingest increased amounts of standard rat chow in an erratic, disturbed feeding behavior, which is likely to underlie the observed

increase in weight gain. This finding strongly corroborates the idea of fetal programming of feeding behavior. Several studies have revealed evidence that maternal nutrient restriction affects offspring feeding behavior, measured either by macronutrient preference or by total energy intake (Vickers *et al.* 2000, Bellinger *et al.* 2004, Bellinger & Langley-Evans 2005, Porto *et al.* 2009, Engeham *et al.* 2010). Curiously, many of those studies have also described a female-specific programming effect, either in fatty food preference (Bellinger & Langley-Evans 2005), in failure to



**Figure 9**

Fat mass of G1 (offspring) whole (A), female (B), and male (C) populations at 13 months of age. Each G1 group (C and HFA) was divided, at 10 months (or 46 weeks) of age, into a standard group fed the standard rat diet (C/Std and HFA/Std,  $n=2-4$  and  $3-5$ , respectively) and a high fructose group, fed a high dose of fructose (10% fructose in the drinking water) plus the standard rat diet (C/Fru and HFA/Fru,  $n=3-4$  and  $3-5$  respectively). Fat mass of G1 animals was quantified by bioelectric impedance before the animals were killed at 13 months of age. The arithmetical means  $\pm$  S.E.M. are shown.  $*P<0.05$ .

**Table 2** Summary of the main findings of the study

| HFA vs C         | G1 female |         | G1 male |         |
|------------------|-----------|---------|---------|---------|
|                  | 3M        | 13M     | 3M      | 13M     |
| Weight gain      | ↑         | ↑↑      | ↔       | ↔       |
| Food intake      | ↑         | ↑↑      | ↔       | ↑       |
| Heart weight     | ↑         | ↑↑      | ↔       | ↔       |
| Glycemic control | ↑ (Gly)   | ↓ (OGT) | ↔       | ↓ (OGT) |
| Insulin          | ↔         | ↑       | ↔       | ↔       |
| Leptin           | ↔         | ↔       | ↔       | ↑       |
| Adiponectin      | ↔         | ↓       | ↔       | ↔       |

↑, increase; ↓, decrease; ↔, no change detected; 3M, 3 months of age; 13M, 13 month of age; Gly, glycemia; OGT, oral glucose tolerance.

adjust food intake to maintain constant energy intake (Bellinger *et al.* 2004), or in failure to respond to a hypophagia-inducing stimulus (Porto *et al.* 2009).

This study, however, is the first, to our knowledge, to obtain evidence that excess maternal micronutrient intake has an effect on feeding behavior of female progeny.

We observed that still in female offspring of HFA dams, high birth weight and hyperglycemia at 3 months of age appear as early markers of later-in-life overt metabolic dysfunction. Also, the increased pancreas weight observed at birth in offspring of HFA-exposed dams, indicative of pancreas hypertrophy or hyperplasia, may be associated with such complications. Indeed it is known that maternal diabetes, when mild, induces hypertrophy of the endocrine pancreas and hyperplasia of the B-cells in rat fetuses (Van Assche *et al.* 2001) and, when severe, it induces increased mass of the endocrine pancreas in adulthood (Aerts *et al.* 1997, Van Assche *et al.* 2001), in both cases associated with a deregulation of metabolic control in adulthood (Van Assche *et al.* 2001).

The decrease in glycemic control observed in female progeny of HFA mothers may directly result from some degree of insulin resistance herein manifested as high insulin levels in female offspring of HFA-exposed dams compared with controls. In addition, considering that adiponectin improves insulin sensitivity (Gulcelik *et al.* 2009, Groeneveld *et al.* 2012) by promoting glucose utilization (Yamauchi *et al.* 2002), it is likely that the observed decrease in adiponectin levels may also be involved in the impairment of glycemic control as also suggested elsewhere (Liao *et al.* 2005). These observations are in perfect agreement with those of Yajnik *et al.* (2008) who have previously shown that high concentrations of folate in maternal erythrocytes were associated with insulin resistance in the offspring.

Another interesting finding relates to the observed increase in heart weight, indicative of cardiac hypertrophy, in at 3 and 13 months of age in female offspring of dams

exposed to HFA. As cardiac hypertrophy is an important feature of diabetic cardiomyopathy (DCM) (Nunes *et al.* 2013), we speculate that perigestational exposure to HFA may program female progeny to develop DCM, increasing the risk of heart failure (Boudina & Abel 2010, Nunes *et al.* 2013). Strikingly, hyperglycemia (Murarka & Movahed 2010), hyperinsulinemia (Boudina & Abel 2010, Murarka & Movahed 2010), and adiponectin deficiency (Guo *et al.* 2013), all manifested in HFA female offspring, have been suggested to contribute to cardiac hypertrophy (Guo *et al.* 2013).

Feeding with high levels of fructose was applied to the offspring, at 10 months of age, as a means of assessing the effect of exposure to HFA on their susceptibility to developing metabolic dysfunction in response to a metabolic insult. Female progeny of HFA mothers, but not those of control mothers, failed to buffer body weight and fat mass gain after feeding with fructose, although both seemed to compensate caloric intake, provided by fructose, by consuming lower amounts of food. Thus, it is likely that perigestational exposure to HFA renders female progeny more susceptible to developing metabolic unbalance upon a metabolic insult. This was not the case with C or HFA male offspring that were able to avoid body weight and fat mass gain after feeding with fructose.

The results of a study by Huang *et al.* (2014) strongly corroborate our findings by indicating that the administration of HFA (in the same dose used in this study) to pregnant mice worsens the metabolic response to high-fat-diet stimulus in adult offspring.

Our results indicate that there is a sex-specific response to perigestational HFA. Indeed, in this study, male offspring seem to be metabolically more resistant to HFA exposure, as they show lesser and later metabolic alterations when compared with female offspring of dams exposed to HFA (Table 2). Also male progeny of dams exposed to HFA only slightly increased the intake of food, to a much lower degree than that observed in females, which was not enough to induce an increase in weight gain, as observed for female progeny of dams exposed to HFA. While the observed hyperleptinemia in male offspring of dams exposed to HFA may represent some degree of leptin resistance, and may eventually predict an increased risk of diabetes (Schmidt *et al.* 2006), it may also exert some anorexigenic effect (Shan & Yeo 2011) and thus protect male offspring from overt hyperphagia and from concomitant excessive weight gain, upon early-life HFA exposure.

Sex-specific responses to developmental programming have been identified in a large number of studies (see Aiken & Ozanne (2013) for a comprehensive review).

While it is not straightforward to conclude which sex is more sensitive to metabolic dysfunction programming, it is apparent that the type and timing of the exposure to the programer and also the animal strain will affect the outcome (Aiken & Ozanne 2013).

In agreement with the sex dimorphism in metabolic programming indicated by the results of this study, Ravelli *et al.* (1999) have reported that exposure to the Dutch famine during early gestation resulted in worse obesity anthropometric measures in women but not in men at 50 years of age. On the other hand, it has been observed that male offspring of rat dams subjected to methyl-deficient diets during the periconceptional period were programmed for impaired glycemic control (Maloney *et al.* 2011) or for a worse metabolic profile (Sinclair *et al.* 2007) to a greater extent than female offspring.

Although sex hormones must be considered as plausible players in sexual dimorphism, particularly in adult organisms, they may not act alone in a sex-specific response to fetal programming. This is particularly relevant when sex-specific changes are observed at early stages of development, such as the sex-specific HFA-induced increase in body weight herein detected at birth. Indeed, it is well known that there are sex-specific patterns of methylation/demethylation which arise during development which may underlie these sex-specific responses to phenotype programers, such as methyl deficient or excessive diets (Vige *et al.* 2008).

From the maternal follow-up, we could conclude that, in addition to the programming of metabolic dysfunction in the offspring, perigestational supplementation with HFA also causes a deterioration in the maternal (G0) long-term metabolic phenotype, as it induces an increase in maternal Lee index before delivery and programs the mothers for increased weight gain, hyperphagia, and hyperdypsia.

As a whole, we can conclude that exposure to high doses of during the perigestational period fuels later-in-life metabolic dysfunction not only in the mothers but also in their offspring, with the more marked effect on females presenting as a particular concern. Indeed, by programming female offspring, which will be dams in the future, to develop metabolic dysfunction, FA oversupplementation may strongly contribute to a snow-ball effect of an exponential increase in the prevalence of metabolic dysfunction and associated diseases such as obesity and diabetes.

Epigenetic changes in all probability may underlie the phenotypic effects of exposure to HFA during the perigestational period demonstrated in this study. Indeed, periconceptional use of FA by women has been associated

with increased methylation of IGF2 in very young offspring (Steegers-Theunissen *et al.* 2009). In addition, very recently, maternal feeding with high levels of FA during pregnancy has been demonstrated to alter methylation status in the offspring of mice, particularly in white adipose tissue (Huang *et al.* 2014) and in the brain, the latter with a sex dimorphism response (Barua *et al.* 2014).

Many questions still warrant future research efforts. It would be desirable to determine the critical temporal windows during development, and the critical dose of FA that induces the observed programming effects. The dose used in this study (40 mg FA/kg of diet) represents 20 times the recommended dose of FA for pregnancy in the rat. In the case of human beings, a maximum dose of 4 mg FA/day is recommended for prevention of NTD recurrence, which corresponds to 20 times the dose initially recommended for low-risk pregnant women (0.2 mg/day) (de Bree *et al.* 1997), and ten times the current WHO recommended dose (0.4 mg/day). Moreover, in some countries only pills with 5 mg of FA (or more) are available for daily intake, corresponding to 12.5 times the WHO recommended dose (and in addition, some women take multivitamin pills containing also FA and are exposed to a diversity of FA-enriched foodstuffs). So, considering that increased amounts of folates are consumed during pregnancy, not only through intake of folate-fortified foods, but also by clinical administration of FA and/or multivitamin pills, the identification of a safe upper dose is indeed urgent.

Our results, together with previous results from others (Sinclair *et al.* 2007, Engeham *et al.* 2010, Maloney *et al.* 2011), strongly indicate that the effect of FA supplementation during early stages of development on health programming may conform to a U-shaped curve, and thus they may play a fundamental part in the recasting of current public health policies concerning fortification of food with FA and clinical recommendations.

#### Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-14-0448>.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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