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Vitamin A deficiency during the perinatal period induces changes in vitamin A metabolism in the offspring. ***The regulation of intestinal vitamin A metabolism via ISX occurs only in male rats severely vitamin A-deficient.***

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Abstract

Purposes: 1) To test the hypothesis of the existence of a perinatal vitamin A (VA) programming of VA metabolism and to better understand the intestinal regulation of VA metabolism. **Methods**: Offspring from rats reared on a control (C) or a VA-deficient (D) diet from 6 weeks before mating until offspring weaning, i.e. 7 weeks after mating, were themselves reared on a C or D diet for 19 weeks, resulting in the following groups: C-C (parents fed C - offspring fed C), D-C, C-D and D-D. VA concentrations were measured in plasma and liver. β-carotene bioavailability and its intestinal conversion rate to VA, as well as vitamin D and E bioavailability, were assessed after gavages with these vitamins. Expression of genes involved in VA metabolism and transport was measured in intestine and liver. **Results:** C-D and D-D had no detectable retinyl esters in their liver. Retinolemia, hepatic retinol concentrations and postprandial plasma retinol response to β-carotene gavage were higher in D-C than in C-C. Intestinal expression of *Isx* was abolished in C-D and D-D and this was concomitant with a higher expression of *Bco1*, *Scarb1*, *Cd36* and *Lrat* in males receiving a D diet as compared to those receiving a C diet. β-Carotene, vitamin D and E bioavailabilities were lower in offspring receiving a D diet as compared to those receiving a C diet. **Conclusions**: A VA-deficient diet during the perinatal period modifies the metabolism of this vitamin in the offspring. *Isx*-mediated regulation of *Bco1* and *Scarb1* expression exists only in males severely deficient in this vitamin. Severe VA deficiency impairs β-carotene and vitamin D and E bioavailability.

Keywords: β-carotene, retinol, retinyl palmitate, bioavailability, cholecalciferol, tocopherol.

Statements and Declarations:

None of the authors reported a potential conflict of interest.

1) Introduction

Vitamin A (VA) deficiency is still a public health problem in many developing countries [1,2]. A range of strategies to fight against this deficiency are available [3], from the distribution of VA supplements to the provision, in countries where it is approved, of genetically modified organisms artificially enriched in β-carotene [4,5]. It has even been recently suggested that the consumption of insects enriched in proVA carotenoids, following their rearing on proVA carotenoid-containing plant by-products, could be a sustainable strategy to help fighting against this deficiency [6].

The pathophysiological consequences of VA deficiency are multiple and can be dramatic if not corrected [7]. Beside the many metabolic effects due in particular to the regulation by this vitamin of the expression of hundreds of genes [8], it can also cause changes in its own metabolism. As a matter of fact, it was observed in rats that VA deficiency increases intestinal β-carotene cleavage activity [9]. It was also observed that VA deficiency reduces intestinal β-carotene uptake by brush border membrane vesicles [10]. J. von Lintig and colleagues have extensively studied the factors involved in this regulation [11-13] ENREF 13. They showed that when retinoic acid concentration is high, the intestinal transcription factor ISX (intestine specific homeobox) represses the expression of both *BCO1* (beta-carotene oxygenase 1), the main enzyme that cleaves β-carotene in the intestine [14-18], and *SCARB1* (scavenger receptor class B member 1), which encodes for SR-BI, an apical membrane protein involved in β-carotene uptake by enterocytes [19,20]. When VA status, and hence retinoic acid concentration, is low, it is assumed that ISX does not repress *BCO1* and *SCARB1* anymore. The consequent increase in their expression leads to a higher β-carotene absorption and conversion rate by the small intestine [12,21]. However, the results of two studies contradicted this mechanism [22,23]. Lemke et al. observed a decrease in β-carotene conversion efficiency

following VA supplementation together with an increase in the absorption of β-carotene[22] while Goswami et al. [10] observed a decrease in β-carotene bioavailability in case of VA deficiency. We therefore decided to reinvestigate this regulation in a recent study which aimed to assess the effect of dietary VA content on intestinal and hepatic metabolism of VA in adult rats [24]. The results of this study first allowed us to hypothesize that this regulatory mechanism may only be effective when the VA status is very low. They also allowed us to assume that this mechanism is only present in male rats. We wanted to verify these two exciting hypotheses very quickly and we therefore decided to take advantage of this study in the offspring of the rats of the previous study [24] to verify them. Moreover, the fact that VA status can regulate the intestinal expression of *SCARB1*, which encodes for the protein SR-BI, in the intestine led us to wonder whether VA status could also affect the bioavailability of other molecules whose absorption involves SR-BI, such as vitamin D and E [25]. This hypothesis is further supported by the fact that the regulation of the intestinal expression of SR-BI via ISX affected the concentrations of vitamin E in mouse tissues [13]. Moreover, supplementation of rats with retinoic acid decreased the absorption efficiency of vitamin E [26].

VA deficiency not only affects the health of the deficient individuals but also affects the health of their offspring. Surprisingly, although it has been suggested that VA metabolism is modified during pregnancy [27], the consequences of VA deficiency during the perinatal period on VA metabolism in the offspring has never been addressed. Given the importance of VA in many metabolic pathways, we hypothesize, by drawing a parallel with what has been suggested for energy metabolism, i.e. the thrifty phenotype hypothesis put forth by Hales and Barker [28], that VA deficiency in parents during the perinatal period could induce an adaptation of VA metabolism in the offspring, increasing its chance of survival in a VA-poor environment.

In summary, many questions regarding the effect of the VA content of the diet, and consequently of the VA status, on VA metabolism remain unanswered. The protocol for this

study was developed to determine whether there is a prenatal programming induced by VA deficiency during the perinatal period and if so, how it affects the metabolism of this vitamin in the offspring. For this, we measured the hepatic and plasma concentrations of VA, the bioavailability and the efficiency of conversion of beta-carotene into VA, and the expression of genes involved in hepatic and/or intestinal VA metabolism (**Supplemental table 1**). The data obtained also provide information on 1) the intestinal regulatory mechanism of VA metabolism mediated by ISX, 2) sex differences in VA metabolism [24,29-31], and 3) the consequences of VA deficiency on the bioavailability of other fat-soluble vitamins that share absorption pathways with β-carotene.

2) Material and methods

Chemicals

Ethanol, *n*-hexane, isopropanol, trichloromethane and HPLC grade dichloromethane, methanol, acetonitrile and water were purchased from Carlo Erba reagents (Val de Reuil, France). β-carotene was from Carotenature GmbH (Müsingen, Switzerland). Ovolife IF 50 phospholipids, which contained 50% w/w phospholipids derived from egg yolk and a maltodextrin excipient, were from Lecico, Inc. (Hamburg, Germany). *RRR*-α-tocopherol, retinol, retinyl palmitate, cholecalciferol, tocol, triolein, sodium chloride, sodium citrate, tris hydroxide, bovine serum albumin and protease inhibitor cocktail were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Sevoflurane was from Baxter (Lublin, Poland). TRIzol reagent was from Euromedex (Souffelweyersheim, France). Dithiothreitol was from Thermo Fisher Scientific (Les Ulis, France). Phosphate buffered saline (PBS) was from Life Technologies (Illkirch, France).

Animals

Institutional guidelines for the care and use of animals were followed and all experimental procedures were approved by the local animal care and use committee (agreement number D 13-055-20). Rats were housed under standard conditions of light (12-h light/dark cycle; lights on at 8 am) and temperature (22-24°C) with free access to tap water and the different VA diet. Rats were the offspring of Sprague Dawley RjHan:SD rats from a previous study which aimed to assess the effect of dietary VA content on intestinal and hepatic metabolism of VA in adult rats [24]. Ten-week-old female and male rats were fed either a diet with usual VA content, i.e. 2300 IU/kg, thereafter called the control (C) diet, or a diet low in VA, i.e. 400 IU/kg, thereafter called the deficient (D) diet (Test Diet Limited, London, UK).

These diets, which differed only in their VA content, were started 6 weeks before mating, in both females and males, and were continued throughout the pregnancy and the lactation period. In order to avoid a diet-induced postnatal programming [32], each litter was culled to 12 pups (6 females and 6 males) on postnatal day 3. The offspring was weaned at postnatal day 21 and was then fed either the D diet or the C diet for 19 weeks, i.e. all along their growing period. Consequently, there were 4 groups of offspring (n=24 with 12 males and 12 females): i) C-C, i.e. rats whose parents received the C diet during the perinatal period and that received the C diet after weaning, ii) D-C, i.e. rats whose parents received the D diet during the perinatal period and that received the C diet after weaning, iii) C-D, i.e. rats whose parents received the C diet during the perinatal period and that received the D diet after weaning and iv) D-D, i.e. rats whose parents received the D diet during the perinatal period and that received the D diet after weaning.

Bioavailability measurement

After 20 weeks on the diets, half of the rats in each group, i.e. 6 males and 6 females, were force-fed micronutrient-rich lipid emulsions on two occasions, separated by at least 3 weeks, as previously described [24]. The first gavage was aimed at measuring vitamin D and E bioavailability while the second one was aimed at measuring β-carotene bioavailability and intestinal conversion into VA. Micronutrient doses were chosen in order to obtain postprandial plasma concentrations greater than our limits of quantification by HPLC analysis following a preliminary experiment. The preparation of the β-carotene-rich emulsion, which provided 3 mg β-carotene/gavage, was described in a recently published paper [24]. The preparation of the emulsion rich in both cholecalciferol and α-tocopherol was as follows: 0.66 mL of an aqueous solution containing 0.9% NaCl and 1.4% bovine serum albumin was first deposited on 0.33 mL triolein, into which 1 mg cholecalciferol and 10 mg α-tocopherol had been incorporated. The

mixture was then vortexed for 6 min and sonicated for 10 min (Branson 3510 MT, 40 kHz; Branson Ultrasonics, Danbury, CT, USA) to obtain the emulsion intended for the gavage of one rat. This procedure allowed us to obtain emulsions which remained apparently, i.e. to the naked eye, stable for the duration of the gavage experiment (approximately 1 hour to force-feed an average of 6 rats).

Blood samples (about 500 µL) were collected from a tail nick into tubes containing 50 µL of a 0.109 M sodium citrate solution at fast and during the postprandial period (1.5 h, 3 h, 4.5 h). The last postprandial sample, taken 6 h after gavage, was obtained by cardiac puncture under deep sevoflurane anesthesia using a syringe filled with 0.109 M sodium citrate (1/10 of the blood volume to be sampled). Blood was immediately centrifuged at 4,000 *g* for 10 min at 4°C and the resulting plasma was immediately frozen at -80°C.

Liver and intestine sample collection

After 20 weeks on the diets, the remaining half of the rats, i.e. 6 females and 6 males/group, were euthanized at fast to obtain tissues (liver and intestine). Liver and small intestinal samples were collected as previously described [24] and stored at -80°C.

Plasma and tissue analysis

The molecules of interest were first extracted in an organic phase as previously described [24]. The organic phase was left to evaporate under nitrogen until obtaining a dried extract. All dried extracts were dissolved in 200 µL HPLC mobile phase (see below). A volume of 50–180 µL was used for HPLC analysis.

All compounds were separated using a 250 x 4.6 mm RP C18, 5 µm Zorbax Eclipse XDB-C18 column (Agilent Technologies, Santa Clara, CA, USA) preceded by a guard column, maintained at a temperature of 35°C. The mobile phase consisted of acetonitrile-

dichloromethane-methanol (70:20:10; vol:vol:vol), using an isocratic elution and a flow rate of 1.8 mL/min. The HPLC system comprised a separation module and a photodiode array detector (Shimadzu, Marne-la-Vallée, France). Compounds were detected at their maximum absorption wavelengths, i.e. 265 nm for cholecalciferol, 292 nm for α-tocopherol and tocol (the internal standard), 325 nm for retinol and retinyl esters, and 450 nm for β-carotene. Retinol, β-carotene, α-tocopherol, tocol, cholecalciferol and retinyl palmitate were identified by retention times and absorption spectra coincident with authentic (>95% pure) standards. Retinyl stearate, retinyl oleate and retinyl linoleate were identified by spectral analysis and quantified by comparing peak areas with standard reference curves of retinyl palmitate, correcting by their molecular extinction coefficient relative to that of retinyl palmitate. Quantifications were performed using Chromeleon software (version 6.8).

Measurement of gene expression in liver and duodenum samples

The expression levels of several genes coding for proteins known to be involved in the metabolism and transport of VA [33] were measured as previously described [24].

Calculations

Vitamin D and E bioavailability was estimated by measuring the areas under the curves (AUC) of their postprandial plasma concentrations (0-6 h) following force-feeding. Regarding cholecalciferol, it is well established that its fasting plasma concentration is negligible, as also observed in our study (data not shown). Moreover, when cholecalciferol is ingested, it is practically not metabolized in the upper small intestine before its absorption by intestinal cells [34]. Finally, it is assumed that most cholecalciferol absorbed by the intestinal cell is incorporated as such in chylomicrons, which are then secreted in the lymph allowing cholecalciferol to join the general circulation during the postprandial period [35]. Thus,

measuring the AUC of the plasma cholecalciferol concentration during the postprandial period provides us with a good estimate of its bioavailability. Regarding vitamin E, the only difference with cholecalciferol is that its plasma concentration at fast is not null. Thus, to assess its bioavailability, it is necessary to calculate its incremental AUC, i.e. the AUC of the increase in its postprandial plasma concentration compared to its fasting plasma concentration.

β-Carotene bioavailability, β-carotene conversion rate to VA and VA status were estimated as previously described [24]. Briefly, β-carotene bioavailability was estimated by summing the β-carotene and the retinyl palmitate responses because, under these experimental conditions, it is assumed that the postprandial plasma retinyl palmitate originates only from the intestinal conversion of β-carotene into VA. Concerning β-Carotene conversion rate to VA, it was calculated as the percentage of bioavailable β-carotene found in the form of retinyl palmitate in the plasma during the postprandial period, i.e. retinyl palmitate response / (β-carotene response + retinyl palmitate response). Concerning VA status, it was estimated using 3 biomarkers that were used in the study on the parents of these animals [24]. The rationale for the use of these biomarkers, and the interpretation of variations in the values of these biomarkers, are explained in detail in the first part of the discussion of this previous study.

Statistical analysis

Results are expressed as means ± SEM. Departures from normality were assessed using Q-Q plots of standardized residuals and homogeneity of variances was tested by Levene's test. In case of departure from normality or homoscedasticity, data were log-transformed. In a first approach, differences in measured variables were analyzed by 3-way ANOVA using a full factorial design with parent diet, offspring diet and sex of the animals as fixed factors. For post hoc pairwise comparisons, the Benjamini-Hochberg procedure was used, controlling the false

discovery rate at 0.05. Values of $p<0.05$ were considered significant. Statistical analyses were performed using SPSS 20 (SPSS Inc., Chicago, IL, USA).

3) Results

The protocol included 4 groups of rats to evaluate 1) the effect of the VA content of the diet given after weaning (offspring diet effect), 2) the effect of the VA content of the diet consumed by the parents (parent diet effect), and 3) the effect of the sex of the offspring (sex effect). This is summarized in **Supplemental figure 1**.

Plasma and liver VA concentrations.

First of all, it is interesting to mention that the average liver weight of male rats was significantly higher than that of female rats, 17.4 ± 0.5 g vs 10.3 ± 0.2 g. In addition, while female liver weights were not significantly different between the different groups, they were significantly lower in the male fed a D diet after weaning, i.e. 16.5 ± 0.9 g for the C-D and 14.7 ± 0.9 g for the D-D, as compared to male fed a C diet after weaning, i.e. 19.9 ± 0.8 g for the C-C and 18.2 ± 0.7 g for the D-C (data not shown).

Plasma retinol concentrations were measured at fast in the different offspring groups (**Figure 1**). Offspring on the D diet, i.e. C-D and D-D, exhibited markedly lower retinolemia (-84% and -86%, respectively) compared to offspring on the C diet, i.e. C-C and D-C ($p<0.05$). Moreover, we also observed a significant effect of the parent diet: among offspring that received a C diet (C-C and D-C), that whose parents received a D diet, i.e. D-C, had higher retinolemia (+70% for females and +41% for males) than that whose parents received a C diet, i.e. C-C. This effect of the parent diet was also observed for female offspring that received a D diet. Indeed, females whose parents received a D diet (D-D) had higher retinolemia (+51%) than females whose parents received a C diet (C-D). Finally, there was a marked sex effect among offspring that received a C diet (C-C and D-C): males displayed significantly higher

retinolemia (+69% and +40%, respectively) compared to females. This sex difference was not found in the C-D and D-D groups.

Concerning hepatic VA concentrations, both retinol and retinyl esters were measured (**Figure 2**). We observed again a significant effect of the offspring diet: hepatic VA concentration, i.e. the sum of retinol and retinyl esters, was significantly lower in the offspring that received the D diet compared to that which received the C diet (**Figure 2a**, 80% lower in C-D vs C-C and 78% lower in D-D vs D-C). This was mostly due to the absence of retinyl esters in the offspring that received the D diet (**Figure 2b**). Indeed, although hepatic retinol concentrations were systematically lower in the offspring that received the D diet compared to that which received the C diet, this was not significant, except when comparing D-D vs D-C females (-31%) (**Figure 2c**). Concerning the different species of retinyl esters in the C-C and D-C groups, the main one was retinyl palmitate (representing at least 55% of all retinyl esters), followed by retinyl stearate (**Table 1**).

The effect of the parents' diet on offspring hepatic VA concentrations, i.e. D-C vs C-C and D-D vs C-D, was not significant, but the concentrations of hepatic VA, retinyl esters and retinol were systematically higher in the offspring groups from parents that received the D diet than in those from parents that received the C diet. For example, D-C females had 41% higher hepatic retinol concentration compared to C-C females, and D-C male had 31% higher hepatic retinol concentration compared to C-C males ($p=0.09$).

Finally, VA, retinyl ester and free retinol hepatic concentrations were systematically lower in males than in females, although this was only significant in the D-C group for free retinol (30% lower in males), likely because of a lack of statistical power for this parameter.

285 *Postprandial plasma β-carotene and VA responses following force-feeding with β-carotene.*

286 As expected, and unlike retinol, neither β-carotene nor retinyl palmitate were detected
287 in plasma prior to gavage. **Figure 3a** shows the postprandial β-carotene response, i.e. the 0-6 h
288 AUC of its postprandial concentration, expressed in μmol/L*h. There was a significant effect
289 of the offspring diet: groups that received a D diet after weaning exhibited lower responses than
290 those that received a C diet after weaning, i.e. C-D vs C-C (-78%) and D-D vs D-C (-59%).
291 Concerning the effect of the parent diet, it is very interesting to note that the D-C offspring had
292 a lower response than the C-C offspring (-64%, almost significant at p=0.059). Finally, there
293 was a striking sex effect, with females exhibiting a higher response than males regardless of the
294 diet (mean of all the dietary groups: +528% in females vs males).

295 Regarding the retinyl palmitate response, **Figure 3b** shows that there was a significant
296 effect of the offspring diet, but only in males. Indeed, males on the D diet had a significantly
297 decreased response as compared to males on the C diet (C-D vs C-C (-78%) and D-D vs D-C (-
298 65%)). Finally, there was a significant difference between males and females in the groups of
299 offspring that received a D diet but not in groups that received a C diet (-69% in C-D males vs
300 C-D females and -66% in D-D males vs D-D females).

301 Finally, with regard to the retinol response (**Figure 3c**), there was a significant effect of
302 the offspring diet, but only in females. Indeed, females that received a D diet displayed a higher
303 response compared to females that received a C diet, i.e. C-D vs C-C (+167%) and D-D vs D-C
304 (+170%). In addition, we also observed a significant effect of the parent diet, in females on the
305 D diet (D-D vs C-D, +100%) and in males on the C diet (D-C vs C-C, +65%). Finally, contrary
306 to what was observed for retinyl palmitate, there was a significant difference between males
307 and females that received a C diet (+143% in males of the C-C and D-D groups as compared to
308 females of these groups), and not in the offspring that received a D diet.

309

β-Carotene bioavailability and conversion rate to VA.

With regard to β-carotene bioavailability (**Figure 4a**), there was a significant effect of the offspring diet: in rats from parents that received the C diet, offspring that received the D diet, i.e. C-D, exhibited lower β-carotene bioavailability (-64%) compared to offspring that received the C diet, i.e. C-C. In rats from parents that received the D diet, this effect was only seen in males (-63%; p=0.071). There was no significant effect of the parent diet on this phenotype. Finally, β-carotene bioavailability was always higher in females than in males (+120% in females as compared to males).

β-Carotene conversion rates to VA are shown in **Figure 4b**. Regarding the effect of the offspring diet, only a marginally significant increase (+78%; p=0.052) could be seen between females that received the D diet as compared to females that received the C diet and whose parents were fed the C diet (C-D vs C-C). There was an effect of the parent diet only in females on the C diet: those whose parents had received the D diet exhibited higher β-carotene conversion rates to VA (D-C vs C-C, +96%). Finally, β-carotene conversion rates to VA were generally higher in males than in females (mean of all male groups +40% higher than the mean of all female groups), with fairly elevated values observed in males (mean rate of conversion of all male groups: 90%).

α-Tocopherol and cholecalciferol bioavailability.

Results are shown in **Figures 5a and 5b**. As expected, α-tocopherol but not cholecalciferol was detected in plasma prior to gavage. It is remarkable to note that similar trends emerge from these two figures. There was a significant effect of the offspring diet, or marginally significant in the case of α-tocopherol (p=0.075), but only in rats whose parents were fed the D diet: offspring that received the D diet exhibited lower α-tocopherol (-47% for the C-D and D-D groups as compared to the C-C and D-C groups) and cholecalciferol

bioavailability (+35% for the C-D and D-D groups as compared to the C-C and D-C groups) than offspring that received the C diet. There was no significant effect of the parent diet or of sex on these phenotypes.

Expression of genes involved in VA metabolism.

The expression levels of several genes coding for proteins known to be involved in the hepatic metabolism and transport of VA were measured in rats that were not force-fed (**Figure 6**). A significant effect of the offspring diet was observed for 3 genes: *Lrat* (lecithin retinol acyltransferase), *Rbp2* (retinol binding protein 2) and *Pnpla3* (patatin like phospholipase domain containing 3). There was a complete inhibition of *Lrat* expression in the offspring fed the D diet compared to that fed the C diet, i.e. C-D and D-D vs C-C and D-C. There was also a significant decrease of the expression of *Rbp2* in males that received the D diet after weaning (C-D and D-D) compared to males that received the C diet after weaning (C-C and D-C). Finally, *Pnpla3* was not expressed in males that received a C diet after weaning (C-C and D-C) whereas it was expressed in those that had a D diet after weaning (C-D and D-D). There was no significant effect of the parent diet on gene expression levels, except for *Pnpla3*, whose expression level in females from parents on the D diet (D-C and D-D) was lower than that of females from parents on the C diet (C-C and C-D). We also observed a significant effect of sex on the expression levels of several genes, namely *Pnpla3*, *Rbp2*, *Rbp4* (retinol binding protein 4), *Ttr* (transthyretin) and, to a lesser extent, *Dgat2* (diacylglycerol O-acyltransferase 2) and *Lrat*.

Concerning the expression levels of genes coding for proteins involved in intestinal metabolism and transport of VA, results are presented in **Figure 7**. Firstly, there was a strong effect of the offspring diet on the expression levels of *Isx*. Indeed, its expression was completely abolished in offspring fed the D diet compared to offspring fed the C diet, both in

females and in males. An effect of the offspring diet was also observed for 4 other genes (*Lrat*, *Scarb1*, *Cd36* (CD36 molecule) and *Bco1* (almost significant)) whose expression levels were increased in males that had a D diet after weaning compared to those that had a C diet after weaning. No such effect was observed in females. There was no significant effect of the parent diet on gene expression levels, except for *Rbp2* whose expression levels in D-C males were lower than those of C-C males. In males only, and once again with the exception of *Isx* and *Rbp1* (retinol binding protein 1), the expressions of the studied genes were always lower in the D-C groups than in the C-C groups, suggesting an effect of the parent diet, although this was only significant for *Rbp2*, likely because of an insufficient statistical power. Finally, the expression of all genes, except *Isx* and *Rbp1*, was lower in males of the D-C group than in females of the same group (significant for *Scarb1*, *Cd36* and *Lrat*, and almost significant for *Bco1*). Conversely, the expression of all genes, except again *Isx* and *Rbp1*, was higher in males of the C-D group than in females of the same group (significant for *Cd36* and *Lrat*).

VA status, bioavailability of β-carotene and vitamin D and E, and β-carotene conversion rate in parents and offspring.

As the rats in this study were the offspring of rats from a previous study [24], where the dietary VA content also varied and in which we also measured the bioavailability of β-carotene and vitamin D and E (data not shown on these vitamins in the previous study), we gathered the results of all these groups to assess more precisely the relationships between the VA status and the studied phenotypes. The VA status for all groups is presented in **Table 2**.

4) Discussion

VA status of the different groups of rats

The main objective of this study was to assess the effect of the VA status of rats, whose parents themselves had different VA status, on VA metabolism and on β-carotene, vitamin D and E bioavailability. It was therefore particularly important to properly assess this status in the different groups of rats. The data collected in this study provide us with three biomarkers of VA status, namely hepatic VA concentration, fasting plasma retinol concentration, and postprandial plasma retinol response following gavage with β-carotene [24]. The first two (**Figures 1** and **2**) show that the offspring on a D diet, i.e. C-D and D-D, had a lower VA status than that on the C diet, i.e. C-C and D-C. Moreover, since hepatic retinyl esters constitute the main body VA reserves, we can even consider that the offspring on the D diet had no VA reserves at all. In females, the results of the third biomarker, i.e. postprandial plasma retinol response (**Figure 3c**, *see* [24] for detailed interpretation), are perfectly in agreement with those of the other two biomarkers. Indeed, female offspring that received a D diet had higher postprandial plasma retinol responses than female offspring that received a C diet.

A VA-deficient diet during the perinatal period appears to improve hepatic VA storage capacity, as well as the ability to mobilize hepatic VA stores, in the offspring.

In order to assess whether VA deficiency of parents during the perinatal period may affect VA metabolism in their offspring, we first compared VA metabolism in offspring that received the same diet after weaning but that differed by the diet their parents received, i.e. D-C vs C-C and D-D vs C-D. These comparisons highlighted several differences that support the hypothesis of a prenatal programming of VA metabolism. Firstly, the offspring whose parents received a D diet exhibited a markedly higher fasting retinolaemia as compared to the offspring

whose parents received a C diet (except for males receiving a D diet). Secondly, concerning hepatic VA metabolism, the concentrations of free retinol were higher (close to significance) in the D-C vs the C-C groups and in the D-D vs the D-C groups. It is also worth mentioning that the concentrations of VA, i.e. the sum of retinyl esters and free retinol, and of retinyl esters alone were always higher in D-C than in C-C, although this was not significant. Taken together, these data suggest that, for the same diet after weaning, the hepatic accumulation of VA, or at least that of free retinol, was greater in rats from parents D than in rats from parents C. This may reflect a mechanism to improve the hepatic storage of VA in an environment deficient in this vitamin. Another interesting parameter of hepatic VA metabolism is the postprandial plasma retinol response following the absorption of a large amount of VA (a huge dose of β-carotene in this study), which reflects the ability of the liver to release retinol to peripheral organs. This parameter was also influenced by the parent diet. Indeed, although this was not always significant, this response was always higher in the offspring of parents D than in the offspring of parents C, i.e. D-C vs C-C and D-D vs C-D, and this in both sexes. This observation was consistent with the effects of the parents' diet on fasting retinolemia and on hepatic concentrations of free retinol. Indeed, the offspring from parents that received a VA-deficient diet had more free-retinol in their liver, their liver secreted more retinol in the plasma following a large dietary intake of VA, and their fasting blood concentration of retinol was higher than that of the offspring from parents that received a VA-sufficient diet. This may also reflect a mechanism to bring enough VA from its storage organ to peripheral tissues in a VA-depleted environment.

A VA-deficient diet during the perinatal period appears to improve offspring's ability to convert β-carotene to VA.

In order to determine whether VA deficiency of parents during the perinatal period also affects intestinal VA metabolism in the offspring, we investigated whether the bioavailability of β-carotene and its conversion rate to VA were different between rats from parents with different VA diets, i.e. the D-C vs the C-C and the D-D vs the C-D. We observed a lower postprandial β-carotene response in the D-C vs the C-C ($p=0.059$, **Figure 3a**), which is consistent but only in females, with a higher observed β-carotene conversion rate ($p<0.05$, **Figure 4b**). This suggests the female offspring from D parents had increased its ability to cleave newly absorbed β-carotene in an environment depleted in VA. The fact that this phenomenon was not observed in males is likely due to the fact that the conversion rate was already close to its maximum in the C-C group, i.e. more than 80%, and therefore could not increase significantly even if there was a stimulating effect due to the VA-deficient diet of the parents.

Regulation of intestinal VA metabolism via ISX only appears to work in cases of severe VA deficiency and only in males.

The second main objective of this study was to explore the intestinal regulation mechanism of β-carotene absorption and conversion to VA as a function of the VA status. The current proposed mechanism [11] is as follows: when retinoic acid concentration decreases in the intestinal cell, which is supposed to happen in the event of a drop in dietary VA intake, the expression of *ISX* decreases. This leads to an increase in the expression of *BCO1* and *SCARB1*, which is supposed to result in an increase in β-carotene absorption rate and conversion to VA by the enterocyte. Our previous study in rat mothers did not allow us to confirm that the decrease in *ISX* expression led to an increase in *BCO1* and *SCARB1* expressions, and we have proposed two hypotheses to explain this apparent contradiction [24]. The first one was that the amplitude of dietary VA intakes, and consequently the variation in *Isx* expression levels, was

not sufficiently large to induce significant different expression levels of *Bco1* and *Scarb1*. The second one was that this regulation mechanism does not exist in females (we measured gene expression in females while all other studies were performed in male rats). In the present study, the very low VA status observed in the D offspring, i.e. C-D and D-D, was associated with an almost complete inhibition of *Isx* expression, which is in full agreement with the current paradigm [11,13]. Furthermore, in males, the inhibition of *Isx* expression was also associated with a higher expression level of *Scarb1* and *Bco1* [11,13]. Nevertheless, in females, the inhibition of *Isx* expression was not associated with the expected increase of *Bco1* and *Scarb1* expression levels, which agrees with our previous hypothesis that this regulatory mechanism only exists in males [24]. The present study does not allow us to explain why *Isx* modulates *Bco1* and *Scarb1* expression in males but not in females, and we can only speculate that it could be related to hormonal differences between females and males. Moreover, *Isx* inhibition in males was also associated with a significant increase in the expression levels of *Lrat* and *Cd36*, which suggests that these two genes may also be under the control of *Isx* in males. This is consistent with recent data showing that LRAT is involved in the ISX-mediated regulation of intestinal VA metabolism [36].

Unfortunately, the results from both this study and our previous study on parents, which compiled data on β-carotene bioavailability and conversion efficiency, in rats of both sexes with different VA status (**Table 2**), fail to confirm the current paradigm stating that β-carotene bioavailability increases when VA status decreases. On the contrary, the lower the VA status, the lower the bioavailability of β-carotene. Regarding the conversion rate, it did increase when the VA status decreased (**Table 2**), but this was only significant in females, probably because the conversion rate in males was already very high, around 83%, for the group with the highest VA status.

The variations in β-carotene bioavailability that were measured in this study, together with the variations in *Scarb1* and *Cd36* expression levels, raise questions about the importance of proteins encoded by these genes in β-carotene bioavailability. Indeed, the increase in the expression of *Scarb1* and *Cd36* in the males that received a D diet (C-D and D-D) as compared to those that received a C diet (C-C and D-C), did not translate in an increase in β-carotene bioavailability. On the contrary, β-carotene bioavailability collapsed in these rats. Likewise, β-carotene bioavailability was also decreased in the females that received a D diet (C-D and D-D), in comparison to females that received a C diet (C-C and D-C), while *Scarb1* and *Cd36* expression levels were not significantly different between these four groups. The observed decrease in β-carotene absorption efficiency in the event of VA deficiency fully disagrees with the paradigm but is perfectly in agreement with the results of Boileau et al. [10]. Our first hypothesis is that a major mechanism involved in β-carotene bioavailability other than that going through SR-BI and CD36 [20,25] was strongly impaired by VA deficiency. A second hypothesis is that VA deficiency affected the integrity of the intestinal mucosa and its normal functioning. It is indeed well established that VA is involved in the development of epithelia. Further experiments are needed to address these hypotheses. Concerning the effect of VA status on β-carotene conversion rate to VA, the results which showed that VA deficiency completely inhibited the expression of *Isx* and increased the expression of *Bco1* in males (**Figure 7**), are very consistent with the results that showed that the lowest VA status was associated with the highest β-carotene conversion rate (**table 2** which compiles the esults obtained in the parent rats and in the offspring rats). Indeed, this supports the hypothesis that, in male rats, VA deficiency increases β-carotene conversion rate to VA by increasing the expression of *Bco1* via inhibition of *Isx* expression.

Severe VA deficiency seems to decrease not only the bioavailability of β-carotene, but also that of vitamins E and D.

Knowing that the absorption of β-carotene and vitamin D and E is carried out by mechanisms that are partly common, e.g. implication of common apical membrane proteins such as SR-BI and CD36 [37,38], we hypothesized that variations in VA status, which are assumed to modulate the expression of some of these proteins, could also influence the bioavailability of these vitamins. The data obtained in this study, combined with those of the previous study on the parents of these rats (**Table 2**), show that only a very deficient VA status, such as that observed in the offspring fed a D diet (C-D and D-D), significantly impaired the bioavailability of these two vitamins, in both males and females. The available data do not allow us to identify the mechanism but, as aforementioned, it is possible that this deficiency profoundly altered the integrity of the intestinal mucosa and therefore its ability to absorb these two vitamins as well as other nutrients and micronutrients.

Four key observations about VA metabolism that emerge from this study

On the whole, the results of this study highlighted four new observations on the effect of the VA content of the parent and offspring diets on the hepatic and intestinal metabolism of this vitamin in the offspring. The first one is that there may be a prenatal programming of VA metabolism in the offspring when the diet of the parents is deficient in VA. Indeed, the offspring of VA-deficient parents had higher retinolemia, higher hepatic accumulation of VA and higher ability to cleave β-carotene in the intestine. Based on Barker's hypothesis [28], we suggest that this perinatal programming of VA metabolism allows the offspring to increase their chance of survival in an environment that does not provide sufficient VA sources. This perinatal programming is probably due to epigenetic mechanisms which remain to be identified. The second observation is that *Isx* expression is significantly modulated only in the

event of a very strong VA deficiency. In other words, there is apparently no dose-response effect of the VA content of the diet on *Isx* expression. Furthermore, the effect of *Isx* on *Bco1* and *Scarb1* expression apparently only exists in male rats. The third observation is that severe VA deficiency decreases the bioavailability of both β-carotene and vitamin D and E by an unknown mechanism, likely linked to an overall deterioration of the integrity of the intestinal mucosa due to VA deficiency. The fourth observation confirms the significant effect of the sex of the rats on vitamin A metabolism. We acknowledge we did not verify whether variations in gene expression translated into variations in protein concentrations. Nevertheless, we had shown in the study on the parents of these rats that this was the case, at least for the three key proteins measured [24].

Reflections on the consequences that this new knowledge on the metabolism of VA could have within the framework of the strategies of fight against this deficiency.

All these new observations on VA metabolism, if they are confirmed by other studies, in particular clinical ones, could be considered in the fight against VA deficiency. Indeed, if the perinatal programming of VA metabolism is confirmed, it should be ensured that individuals from VA-deficient parents, and that could therefore be adapted to absorb and store VA more efficiently, would not be intoxicated by the high VA doses given as supplements to fight against this deficiency. Also, our results also suggest that individuals severely VA-deficient could display lower absorption efficiency of vitamin D and E and therefore have an insufficient status in these vitamins. The status of these vitamins must therefore be checked in populations very deficient in VA and corrected if necessary. Finally, the effect of sex on vitamin A metabolism, which had already been observed in humans, and which was investigated further in this study and in the previous one on the parents of these rats, suggests that it would be desirable to adapt VA supplementation according to the sex of the individuals.

Table 1: Retinyl ester concentrations (nmol/g tissue) in the liver of the different groups of rats.

	Females		Males	
	C-C[a]	**D-C**[b]	**C-C**	**D-C**
Retinyl palmitate	1.49 ± 0.37 [a]	1.56 ± 0.30 [a]	1.19 ± 0.30 [a]	1.33 ± 0.32 [a]
Retinyl stearate	0.74 ± 0.15 [a]	0.82 ± 0.10 [a]	0.32 ± 0.08 [b]	0.48 ± 0.14 [b]
Retinyl oleate	0.15 ± 0.04 [a]	0.16 ± 0.03 [a]	0.09 ± 0.02 [a]	0.15 ± 0.05 [a]
Retinyl linoleate	0.15 ± 0.03 [a]	0.29 ± 0.06 [b]	0.13 ± 0.04 [a]	0.15 ± 0.03 [a]

[a]**(C-C)**, offspring whose parents received a C diet and that received a C diet; [b]**(D-C)**, offspring whose parents received a D diet and that received a C diet. Note that the **C-D** and **D-D** groups are not shown because, as shown in Figure 2b, no retinyl ester was detected in these groups. Values are means ± SEM. Means with different superscript letters on the same line indicate that they are significantly different ($p<0.05$, ANOVA followed by post-hoc Tukey-Kramer test).

560 **Table 2: Bioavailability of β-carotene and vitamin D and E, as well as β-carotene conversion rate to VA, in groups of rats with different**

561 **VA status[1].**

562

Group[2]	Vitamin A status[3] (nmol/g)		β-Carotene bioavailability[4] (µmol/L.h)		β-Carotene conversion rate[5] (%)		Vitamin D response[6] (µmol/L.h)		Vitamin E response[7] (µmol/L.h)	
	females	**males**	**females**	**males**	**females**	**males**	**females**	**males**	**females**	**males**
HVA p	1849 ± 173 ^{a}	1195 ± 181 ^{a}	0.537 ± 0.120 ^{a}	0.047 ± 0.022 ^{a*}	26 ± 6 ^{b}	83 ± 4 ^{b*}	19 ± 3 ^{a}	16 ± 3 ^{a}	144 ± 53 ^{a}	55 ± 24 ^{a*}
MVA p	506 ± 45 ^{b}	506 ± 77 ^{b}	0.210 ± 0.069 ^{b}	0.034 ± 0.014 ^{a*}	64 ± 9 ^{a}	86 ± 5 ^{b}	21 ± 2 ^{a}	17 ± 4 ^{a}	122 ± 21 ^{a}	76 ± 26 ^{a*}
LVA p	55 ± 20 ^{c}	124 ± 37 ^{c*}	0.366 ± 0.092 ^{b}	0.028 ± 0.008 ^{a*}	43 ± 6 ^{b}	88 ± 5 ^{b*}	21 ± 3 ^{a}	17 ± 4 ^{a}	114 ± 39 ^{a}	75 ± 26 ^{a*}
C-C o	2.5 ± 0.6 ^{d}	1.7 ± 0.4 ^{d}	0.183 ± 0.047 ^{b}	0.032 ± 0.008 ^{a*}	37 ± 8 ^{b}	82 ± 3 ^{b*}	14 ± 3 ^{a}	14 ± 2 ^{ab}	42 ± 7 ^{b}	50 ± 12 ^{a}
D-C o	2.9 ± 0.4 ^{d}	2.1 ± 0.5 ^{d}	0.053 ± 0.010 ^{c}	0.023 ± 0.003 ^{a*}	72 ± 4 ^{a}	87 ± 4 ^{b*}	16 ± 1 ^{a}	18 ± 2 ^{a}	54 ± 7 ^{b}	52 ± 7 ^{a}
C-D o	0.0 ± 0.0 ^{e}	0.0 ± 0.0 ^{e}	0.046 ± 0.011 ^{c}	0.002 ± 0.001 ^{b*}	66 ± 6 ^{a}	93 ± 6 ^{ab*}	9 ± 2 ^{b}	11 ± 1 ^{b}	30 ± 5 ^{b}	24 ± 4 ^{b}
D-D o	0.0 ± 0.0 ^{e}	0.0 ± 0.0 ^{e}	0.030 ± 0.010 ^{c}	0.002 ± 0.001 ^{b*}	80 ± 7 ^{a}	97 ± 2 ^{a*}	10 ± 1 ^{b}	10 ± 2 ^{b}	23 ± 3 ^{c}	29 ± 6 ^{b}

563 [1]Pooling of the data obtained in this study and in a previous study dedicated to study VA metabolism in the parents of these rats [24].

[2]The MVA and LVA groups were the parents of the four others. (HVA p): parents that were fed a high VA diet (9858 IU/kg diet); (MVA p): parents that were fed a medium VA diet (2300 IU/kg); (LVA p): parents that were fed a low VA diet (400 IU/kg). Note that the MVA and LVA diets fed to the parents (p) contained the same amount of VA than the control diet (C) and the deficient diet (D) fed to the offspring (o), respectively.

[3]The VA status was estimated by measuring retinyl ester concentrations in the liver, which is assumed to be the best biomarker of VA status [39].

[4]Areas under the curves (AUC) of β-carotene postprandial plasma concentrations of following gavage with β-carotene (μmol/L.h).

[5]Estimated according to the following formula: % conversion = AUC retinyl ester / (AUC β-carotene + AUC retinyl ester) x 100.

[6]AUC of cholecalciferol postprandial plasma concentrations following gavage with cholecalciferol (μmol/L.h).

[7]Incremental AUC of α-tocopherol postprandial plasma concentrations following gavage with α-tocopherol (μmol/L.h).

Values are means ± SEM (n=4-6 depending on the group). Different letters in the same column indicate significant differences ($p<0.05$) between groups. ANOVA followed by post-hoc Tukey-Kramer test. An asterisk associated with a mean in a group of males indicates that this mean is significantly different ($p<0.05$) from that of the corresponding group of females (Student t-test for unpaired values).

576 **Figure legends**

577

578 **Fig. 1** Plasma retinol concentrations. White bars: females (n = 6/group), black bars: males (n =
579 6/group). **(C-C)**, offspring whose parents received a C diet and that received a C diet; (**D-C**),
580 offspring whose parents received a D diet and that received a C diet; (**C-D**), offspring whose
581 parents received a C diet and that received a D diet; (**D-D**), offspring whose parents received a
582 D diet and that received a D diet. Bars represent means ± SEM. Gender effect p=0.007, parent
583 diet effect p<0.0005, offspring diet effect p<0.0005. Statistical differences between groups are
584 indicated in the insert. M&F indicates that there is a significant difference (p<0.05) for males
585 and for females. F indicates that there is a significant difference only in females. An asterisk
586 indicates a significant difference between males and females from a given group. * p<0.05; **
587 p<0.01.

588

589 **Fig. 2** Hepatic VA concentrations. **a:** hepatic VA = retinyl esters + free retinol. **b:** hepatic
590 retinyl esters = retinyl palmitate + retinyl stearate + retinyl oleate + retinyl linoleate. **c:** hepatic
591 free retinol. White bars: females (n = 6/group), black bars: males (n = 6/group). **(C-C)**,
592 offspring whose parents received a C diet and that received a C diet; (**D-C**), offspring whose
593 parents received a D diet and that received a C diet; (**C-D**), offspring whose parents received a
594 C diet and that received a D diet; (**D-D**), offspring whose parents received a D diet and that
595 received a D diet. Bars represent means ± SEM. **a:** Gender effect p=0.004, parent diet effect
596 p=0.023, offspring diet effect p<0.0005. **b:** Gender effect p=0.418, parent diet effect p=0.899,
597 offspring diet effect p<0.0005. **c:** Gender effect p=0.001, parent diet effect p=0.001, offspring
598 diet effect p=0.002. Statistical differences between groups are indicated in the insert. M&F
599 indicates that there is a significant difference (p<0.05) for males and for females. F indicates
600 that there is a significant difference only in females. ns: not significant. For p-values comprised

between 0.05 and 0.10, the p-value is given. The three asterisks indicate a significant difference ($p<0.001$) between males and females from this group.

Fig. 3 Postprandial plasma responses of different VA species following force-feeding with β-carotene. **a:** β-carotene responses. **b:** Retinyl palmitate responses. **c:** Retinol responses. White bars: females (n = 6/group), black bars: males (n = 6/group). **(C-C)**, offspring whose parents received a C diet and that received a C diet; (**D-C**), offspring whose parents received a D diet and that received a C diet; (**C-D**), offspring whose parents received a C diet and that received a D diet; (**D-D**), offspring whose parents received a D diet and that received a D diet. Postprandial plasma response means incremental area under the curve (AUC, expressed in μmol/L*h) of the plasma concentrations of the molecule of interest measured at regular time intervals up to 6.5 hours after force-feeding. Bars represent means ± SEM. **a:** Gender effect $p<0.0005$, parent diet effect $p=0.004$, offspring diet effect $p=0.253$. **b:** Gender effect $p=0.02$, parent diet effect $p=0.007$, offspring diet effect $p<0.0005$. **c:** Parent diet effect $p=0.022$, offspring diet effect $p=0.203$. Statistical differences between groups are indicated in the insert. M&F indicates that there is a significant difference ($p<0.05$) for males and for females. M or F alone indicates that there is a significant difference only in males or in females. ns: not significant. For p-values comprised between 0.05 and 0.10, the p-value is given. An asterisk indicates a significant difference between males and females from a given group. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Fig. 4 β-Carotene bioavailability and conversion rate to VA in the intestine following force-feeding with β-carotene. **a:** β-Carotene bioavailability, calculated by summing the β-carotene and the retinyl palmitate responses because, under these experimental conditions, we assumed that postprandial plasma retinyl palmitate originated only from the intestinal metabolism of β-

carotene. **b**: β-Carotene conversion rate to VA, calculated as the percentage of newly absorbed β-carotene found in the form of retinyl palmitate, i.e. retinyl palmitate response / (β-carotene response + retinyl palmitate response). White bars: females (n = 6/group), black bars: males (n = 6/group). **(C-C)**, offspring whose parents received a C diet and that received a C diet; (**D-C**), offspring whose parents received a D diet and that received a C diet; (**C-D**), offspring whose parents received a C diet and that received a D diet; (**D-D**), offspring whose parents received a D diet and that received a D diet. Bars represent means ± SEM. **a:** Gender effect $p<0.0005$, parent diet effect $p=0.99$, offspring diet effect $p<0.0005$. **b:** Gender effect $p<0.0005$, parent diet effect $p=0.001$, offspring diet effect $p=0.001$. Statistical differences between groups are indicated in the insert. M&F indicates that there is a significant difference ($p<0.05$) for males and females. M or F alone indicates that there is a significant difference only in males or in females. ns: not significant. For p-values comprised between 0.05 and 0.10, the p-value is given. An asterisk indicates a significant difference between males and females from a given group. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Fig. 5 Vitamin E and D bioavailability following force-feeding. **a:** α-Tocopherol bioavailability, i.e. incremental area under the curve (AUC) of the plasma concentrations of α-tocopherol measured at regular time intervals up to 6.5 hours after force-feeding with an emulsion containing both α-tocopherol and cholecalciferol. **b:** Cholecalciferol bioavailability, i.e. AUC of the plasma concentrations of cholecalciferol measured at the same time intervals after force-feeding with the same emulsion. White bars: females (n = 6/group), black bars: males (n = 6/group). **(C-C)**, offspring whose parents received a C diet and that received a C diet; (**D-C**), offspring whose parents received a D diet and that received a C diet; (**C-D**), offspring whose parents received a C diet and that received a D diet; (**D-D**), offspring whose parents received a D diet and that received a D diet. Bars represent means ± SEM. **a:** Offspring

diet effect p<0.0005. **b:** Offspring diet effect p<0.0005. Statistical differences between groups are indicated in the insert. M&F indicates that there is a significant difference (p<0.05) for males and for females. ns: not significant. For p-values comprised between 0.05 and 0.10, the p-value is given.

Fig. 6 Expression levels of hepatic genes involved in VA metabolism. White bars: females (n = 6/group), black bars: males (n = 6/group). **(C-C)**, offspring whose parents received a C diet and that received a C diet; (**D-C**), offspring whose parents received a D diet and that received a C diet; (**C-D**), offspring whose parents received a C diet and that received a D diet; (**D-D**), offspring whose parents received a D diet and that received a D diet. The full names of the genes not commented in the text are: *Ces1e* (carboxylesterase 1E), *Bco2* (beta-carotene oxygenase 2) and *Dgat1* (diacylglycerol O-acyltransferase 1). P-values for the effect of each tested factor, e.g. gender, parent diet and offspring diet, on the expression of each gene are shown in supplemental Table 2. Bars represent means of fold changes ± SEM. Statistical differences between groups are indicated in the insert. M&F indicates that there is a significant difference (p<0.05) for males and for females. M or F alone indicates that there is a significant difference only in males or in females. ns: not significant. For p-values comprised between 0.05 and 0.10, the p-value is given. An asterisk indicates a significant difference between males and females from a given group. * p<0.05; ** p<0.01.

Fig. 7 Expression levels of intestinal genes involved in VA metabolism. White bars: females (n = 6/group), black bars: males (n = 6/group). **(C-C)**, offspring whose parents received a C diet and that received a C diet; (**D-C**), offspring whose parents received a D diet and that received a C diet; (**C-D**), offspring whose parents received a C diet and that received a D diet; (**D-D**), offspring whose parents received a D diet and that received a D diet. The full names of the

genes are either in the text at their first occurrence or in the legend of figure 6. Bars represent means of fold changes ± SEM. P-values for the effect of each tested factor, e.g. gender, parent diet and offspring diet, on the expression of each gene are shown in supplemental Table 2. Statistical differences between groups are indicated in the insert. M&F indicates that there is a significant difference ($p<0.05$) for males and for females. M or F alone indicates that there is a significant difference only in males or in females. For p-values comprised between 0.05 and 0.10, the p-value is given. An asterisk indicates a significant difference between males and females from a given group. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

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