British Journal of Nutrition (2019), 121, 121–129
doi:10.1017/S0007114518003069

The effect of maternal iron deficiency on zinc and copper levels and on genes of zinc and copper metabolism during pregnancy in the rat

Sarah C. Cottin[1], Guenievre Roussel[2], Lorraine Gambling[3], Helen E. Hayes[3], Valerie J. Currie[3] and Harry J. McArdle[3]*

[1]*Division of Food and Drink, School of Science Engineering and Technology, Abertay University, Dundee DD1 1HG, UK*
[2]*Laboratoire de Neurosciences Cognitives, Aix-Marseille Université, 13331 Marseille, France*
[3]*Rowett Institute of Nutrition and Health, University of Aberdeen, Foresterhill House, Ashgrove Road West, Aberdeen AB25 2ZD, UK*

(Submitted 4 May 2018 – Final revision received 9 September 2018 – Accepted 17 September 2018 – First published online 28 November 2018)

Abstract

Fe deficiency is relatively common in pregnancy and has both short- and long-term consequences. However, little is known about the effect on the metabolism of other micronutrients. A total of fifty-four female rats were fed control (50 mg Fe/kg) or Fe-deficient diets (7·5 mg/kg) before and during pregnancy. Maternal liver, placenta and fetal liver were collected at day 21 of pregnancy for Cu and Zn analysis and to measure expression of the major genes of Cu and Zn metabolism. Cu levels increased in the maternal liver ($P=0·002$) and placenta ($P=0·018$) of Fe-deficient rats. Zn increased ($P<0·0001$) and Cu decreased ($P=0·006$) in the fetal liver. Hepatic expression of the Cu chaperones antioxidant 1 Cu chaperone ($P=0·042$) and cytochrome *c* oxidase Cu chaperone (*COX17*, $P=0·020$) decreased in the Fe-deficient dams, while the expression of the genes of Zn metabolism was unaltered. In the placenta, Fe deficiency reduced the expression of the chaperone for superoxide dismutase 1, Cu chaperone for superoxide dismutase ($P=0·030$), ceruloplasmin ($P=0·042$) and Zn transport genes, ZRT/IRT-like protein 4 (*ZIP4*, $P=0·047$) and Zn transporter 1 (*ZnT1*, $P=0·012$). In fetal liver, Fe deficiency increased *COX17* ($P=0·020$), ZRT/IRT-like protein 14 ($P=0·036$) and *ZnT1* ($P=0·0003$) and decreased *ZIP4* ($P=0·004$). The results demonstrate that Fe deficiency during pregnancy has opposite effects on Cu and Zn levels in the fetal liver. This may, in turn, alter metabolism of these nutrients, with consequences for development in the fetus and the neonate.

Key words: Placenta: Liver: Iron status: Copper: Pregnancy

Fe deficiency is the most common nutrient deficiency worldwide and is particularly prevalent in children, women of child bearing age and pregnant women. Even in the UK, it is estimated that 23 % of women aged 19–64 years and nearly half of teenage girls do not reach their lower reference nutrient intake for Fe[1]. During development, the fetus relies entirely on maternal supply for Fe, Zn and Cu, which are critical for development and health. Crosstalks between these three trace elements have been established decades ago[2–4], but the effect of Fe deficiency on the metabolism of Cu and Zn is still not fully understood, particularly during pregnancy. The ability to carry out work establishing these links is, of course, limited, but micronutrient metabolism is well-conserved in mammals, and the rat has been shown to be a very good model for pregnancy and nutrition in humans[3].

Generally, Fe deficiency in mammals results in increased Cu levels in the blood, gut and liver[5–7]. Circulating ceruloplasmin (CP), produced by the liver, and its gut homologue hephaestin are Cu-dependent ferroxidases and appear as an evident link between Fe and Cu metabolism. Accordingly, Fe deficiency in rats induces an increase in serum Cu associated with an increase in *CP* levels[5]. Interestingly, Fe-deficient rats have higher liver Cu levels that correlate with CP expression and its ferroxidase activity, while hepatic *CP* mRNA remains unchanged, suggesting that Cu loading is associated with an increased metallation of the protein[8]. Fe deficiency also increases the expression of the Menkes Cu ATPase (*ATP7a*) in the duodenum[6,9], suggesting an increased export of Cu from the enterocyte to the circulation, while Wilson Cu ATPase (*ATP7b*) expression in the liver is unaltered[6]. In addition, Cu and Fe may compete for divalent metal transporter 1 (*DMT1*) for their uptake in the gut. Although Cu influx through *DMT1* in the enterocyte is minor compared with Cu transporter 1 (*CTR1*) in replete conditions, it is of particular importance during Fe deficiency due to its up-regulation and the lack of Fe in the lumen, which favours Cu uptake[10].

Abbreviations: ATOX1, antioxidant 1 copper chaperone; CCS, copper chaperone for superoxide dismutase; COX17, cytochrome c oxidase copper chaperone; CP, ceruloplasmin; ZIP, ZRT/IRT-like protein; ZnT, zinc transporter.

* **Corresponding author:** Professor H. J. McArdle, email h.mcardle@abdn.ac.uk

The effect of Fe deficiency on Zn status is believed to be less important than on Cu status. Fe deficiency appears to reduce Zn requirements in rats, increasing Zn levels in several organs such as liver, spleen, brain or kidney[11,12] while decreasing apparent Zn absorption[13] and plasma Zn[14]. Noteworthy, marginal Fe deficiency (9 and 13 mg Fe/kg diet) did not affect Zn status in rat, while it was sufficient to induce Cu accumulation in the liver[15]. Because Fe and Zn have similar mechanisms of absorption and transport in the gut, they were originally thought to compete with each other, notably through *DMT1*. Accordingly, Fe treatment reduces the transport of Zn in Caco-2 cells although to a lesser extent than other divalent cations[16]. However, *DMT1*'s affinity for Zn is much lower than that for Fe, and its contribution to Zn transport and uptake is probably minimal[17].

Two families of Zn transporters (ZnT) have been identified, ZRT/IRT-like protein (ZIP) and ZnT, generally involved in influx and efflux of the cells, respectively. ZIP stands for Zn-regulated transporter, Fe-regulated transporter-like protein, and is composed of 14 members in mammals. They are characterised by their ability to transport Zn, but several of them have also been reported to mediate the uptake of Fe as well as Mn[18]. In rats, Fe overload increases the expression of several ZIP in the liver, namely, *ZIP5* (up), *ZIP6, ZIP7* and *ZIP10* (down), while *ZIP14*, the most abundant ZIP in the liver, is upregulated by Fe deficiency[19]. On the other hand, the ZnT family comprises 10 members so far and contribute to Zn homoeostasis by exporting Zn out of the cell or sequestrating Zn into cellular compartments when Zn levels are low. While there is evidence for direct action of Fe on ZIP, which may lead to alterations in Zn metabolism, interactions between Fe and ZnT have not been identified.

Metabolic crossroads between Fe, Cu and Zn during pregnancy are less known. Maternal Fe deficiency in rat increases Cu levels in the maternal liver, maternal serum as well as the placenta[20,21] along with an increase in serum Cu and CP[22]. In contrast, we showed that Fe deficiency decreased Cu levels in the fetal liver, without affecting the fetal expression of the main genes involved in Cu metabolism[23,24]. In humans, Fe supplementation has been shown to decrease plasma Zn levels during pregnancy and Zn absorption during lactation in humans[25–27]. Whether Fe deficiency, as opposed to overload, affects Zn metabolism in the mother and fetus remains essentially unknown. In this study, we investigated the effect of maternal Fe deficiency on Zn status in pregnant rats and the offspring as well as the implication of major genes of Zn metabolism. We also examined the mechanisms through which Fe deficiency differentially affects Cu metabolism in the mother and fetus and tested the implication of most genes of Cu metabolism in the liver of the mother and fetus as well as in the placenta.

Methods

Animal procedures

Experiments were performed using female Rowett hooded rats from three different studies of identical design. The protocol and animal procedures have recently been described in detail[28] and were approved by the Rowett Institute Ethics Committees and conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. Briefly, 54 weanling females were fed a control diet (AIN93, containing 860 mg/kg Zn and 170 mg/kg Cu) for two weeks and were then randomised into two groups. In all, 30 remained on the control diet (Fe = 50 mg/kg) for four weeks until mating and 24 switched to an Fe-deficient (Fe = 7·5 mg/kg) diet for the same duration. They were mated with males of the same strain and remained on the same diet until they were killed at day 21 of gestation. Dams were killed by stunning and cervical dislocation and the fetuses by decapitation. Animals were housed under 12-h light–12-h dark cycle with a constant temperature (22°C). No adverse effects were recorded as a result of this dietary intervention.

Livers from the dams and fetuses, as well as the placentas, were collected and immediately frozen in liquid N_2. They were kept in metal-free vials and stored at –80°C until analysis. Whenever possible, placentas and fetal livers from the three male median weights in each litter were pooled together and ground in liquid N_2. Ground tissues from maternal liver, pooled placentas and pooled fetal livers were used for Fe, Cu and Zn content (control *n* 24, Fe deficient *n* 30) and gene analysis (control *n* 21, Fe deficient *n* 24). It should be noted that control and Fe-deficient samples from each of the three animal studies were analysed together, and results from each experiment were then pooled together for data analysis.

Assessment of copper and zinc status

Total Cu and Zn were measured in maternal liver, placenta and fetal liver by inductively coupled plasma (ICP)-MS as described previously[28]. Samples were digested in 2 % HNO_3, 0·5 % HCl and metal concentrations measured using an Agilent 7700X spectrophotometer (Agilent Technologies) equipped with a MicroMist nebuliser, Ni sampler and skimmer cones. Intra-assay CV% were 10·8 % (Fe), 11·1 % (Cu) and 5·2 % (Zn).

RNA extraction and real-time RT-quantitative PCR

In all, 20–30 mg of frozen placenta, maternal and fetal liver ground tissue were homogenised after grinding, using a T25 Ultra-Turrax (IKA (England) Ltd). RNA was isolated with the RNeasy Mini Kit (Qiagen), and 200 ng was reverse transcribed on a PTC-100 Thermal Cycler (Bio-Rad Laboratories) using the Applied Biosystems TaqMan RT reagent kit (Life Technologies). Complementary DNA fragments were amplified by real-time quantitative PCR (7700 Sequence Detection System; Life Technologies) using the primers (TaqMan; ThermoFisher Scientific) described in Table 1. The most relevant genes of Cu[24] and Zn metabolism were selected. Specifically, *ZIP-1* is ubiquitously expressed and was shown to bind Fe with high affinity[29]. *ZIP-4* is crucial during development and for the absorption of maternal Zn[30]. *ZIP8* and *ZIP14* are closely related and have been shown to mediate Fe transport in addition to Zn[31,32]. *ZnT1* (ubiquitous), *ZnT4* and *ZnT5* are sensitive to maternal Zn status and may play an important role in fetal Zn supply from the maternal diet[33,34]. The gene expression was normalised to 18s rRNA (HS99999901_s1) in all samples. This was chosen

Table 1. List of genes analysed by PCR using TaqMan® Gene Expression Assays (ThermoFisher Scientific)

Gene	Protein	Assay ID
ATOX1	Antioxidant 1 Cu chaperone	Rn00584459_m1
COX17	Cytochrome *c* oxidase Cu chaperone	Rn00585530_m1
CCS	Cu chaperone for superoxide dismutase	Rn00584772_m1
CP	Ceruloplasmin	Rn00561049_m1
ATP7A	ATPase Cu transporting alpha (Menkes)	Rn00583815_m1
ATP7B	ATPase Cu transporting beta (Wilson)	Rn00560862_m1
SLC31A1/CTR1	Cu transporter 1	Rn00683634_m1
SLC39A1/ZIP1	ZRT/IRT-like protein 1	Rn01458936_g1
SLC39A4/ZIP4	ZRT/IRT-like protein 4	Rn01505595_g1
SLC39A8/ZIP8	ZRT/IRT-like protein 8	Rn01748352_m1
SLC39A14/ZIP14	ZRT/IRT-like protein 14	Rn01468336_m1
SLC30A1/ZnT1	Zn Transporter 1	Rn00575737_m1
SLC30A4/ZnT4	Zn Transporter 4	Rn00597094_m1
SLC30A5/ZnT5	Zn Transporter 5	Rn01493867_m1

although the levels of expression were higher than the test genes because it was the only gene that was consistently unaffected by the nutritional treatments (G Roussel and HJ McArdle, unpublished results).

The experiment was performed and data interpreted according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines[35]: 18s RNA expression did not significantly vary between the Fe-deficient and control groups in all tissues (Mann–Whitney test, data not shown). Linear amplification was observed for 18s and all genes of interest across their range of expression.

Statistical analysis

Fe, Cu and Zn content are expressed in μg/g dry tissue. The material used in this article were obtained from three separate experiments. To minimise variations between the three animal studies, as well as between each PCR run, the expression of Cu and Zn genes was expressed as percentage of the average gene expression of the control group for each of the three experiments. The normality of the distribution and equality of variances were analysed with GraphPad Prism (version 5.04). Data were analysed with IBM SPSS statistics 21.0 software using unpaired *t* tests when data were normally distributed and otherwise using non-parametric tests (Mann–Whitney *U*).

Results

Copper and zinc levels

The effect of maternal Fe deficiency on growth, placental:fetal ratio, placenta and liver weights, haematocrit and Fe levels in this study has been recently reported[28]. Specifically, Fe levels were decreased by 152·6 μg/g (approximately –30 %), 604·0 μg/g (approximately –60 %) and 369·4 μg/g dry tissue (approximately –75 %), in the placenta, fetal and maternal liver, respectively[28]. Maternal liver and placenta weight were not affected by Fe deficiency, while fetal weight and fetal liver weight were significantly decreased by approximately 13 % and 25 %, respectively[28]. Maternal Fe deficiency increased Cu levels by 1·44 μg/g dry tissue (95 % CI 0·34, 2·54; $P=0·002$) and 3·45 μg/g (95 % CI –0·42, 7·32; $P=0·018$) in maternal liver and placenta, respectively. In contrast, Fe deficiency decreased Cu levels in the fetal liver by 9·64 μg/g (95 % CI –15·85, –3·42; $P=0·006$) (Fig. 1(a), Mann–Whitney), while Zn levels were increased by 81·6 μg/g (95 % CI 50·0, 113·1; $P<0·0001$). Zn levels in maternal liver and placenta were not significantly altered by maternal Fe deficiency (Fig. 1(b), *t* test). Changes in absolute levels of Fe, Zn and Cu followed a similar pattern (Table 2) apart from the absolute Zn levels that were reduced by 4·08 μg (95 % CI –1·64, –6·52; $P=0·003$) in the fetal liver.

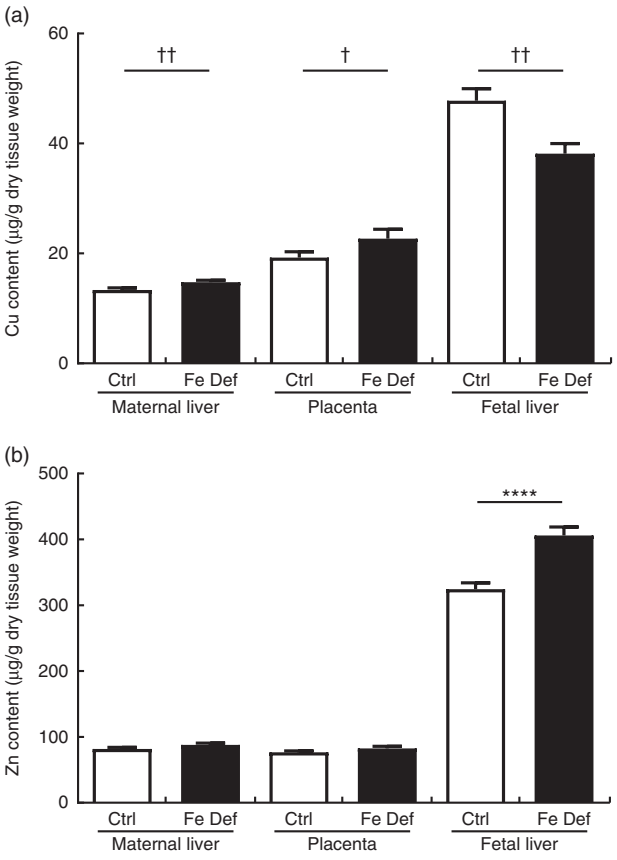

Fig. 1. Effect of maternal iron deficiency on copper (a) and zinc (b) levels in maternal liver, placenta and fetal liver 21 d after mating (n 54). Values are mean percentage of control, with their standard errors represented by vertical bars. Results are significantly different between the control (Ctrl, n 24) and iron-deficient (Fe Def, n 30) groups: **** $P<0·0001$ (independent t test); † $P<0·05$, †† $P<0·01$ (Mann–Whitney test).

Zinc and copper gene expression

Maternal Fe deficiency decreased the hepatic expression of the Cu chaperones antioxidant 1 Cu chaperone (*ATOX1*) by –10·5 % (95 % CI –20·7, –0·4; $P=0·042$) and cytochrome *c* oxidase Cu chaperone (*COX17*) by –13·8 % (95 % CI –25·3, –2·3; $P=0·020$) in the dams. In contrast, the hepatic expression of *COX17* was increased by 15·0 % (95 % CI 2·5, 27·5; $P=0·020$) in

Table 2. Organ weight, water content and absolute iron, copper and zinc content in the maternal liver, placenta and fetal liver of control and iron-deficient rats at day 21 of gestation
(Mean values with their standard errors)

	Control		Fe deficient	
	Mean	SEM	Mean	SEM
Maternal liver				
Organ weight (g)‡	10·94	0·39	11·60	0·35
Water content (%)§	70·04	0·26	71·22**	0·24
Absolute Fe content (mg)‡	1·96	0·29	0·70††	0·12
Absolute Cu content (μg)‡	44·77	1·20	51·10*	1·48
Absolute Zn content (μg)‡	305·9	8·2	326·8	8·5
Placenta				
Organ weight (g)‖	0·512	0·017	0·551	0·009
Water content (%)§	87·83	0·24	88·10	0·27
Absolute Fe content (mg)‖	39·07	1·88	28·47***	1·66
Absolute Cu content (μg)‖	1·17	0·10	1·52	0·09
Absolute Zn content (μg)‖	5·52	0·20	5·60	0·19
Fetal liver				
Organ weight (g)‡	0·313	0·009	0·234***	0·009
Water content (%)§	78·23	0·21	79·26*	0·18
Absolute Fe content (mg)‡	80·37	2·38	20·08†††	1·14
Absolute Cu content (μg)‡	3·62	0·27	1·90***	0·15
Absolute Zn content (μg)‡	23·37	0·87	19·27**	0·85

Mean values were significantly different compared with control: * $P<0·05$, ** $P<0·01$, *** $P<0·001$ (independent *t* test).
Mean values were significantly different compared with control: †† $P<0·01$, ††† $P<0·001$ (Mann–Whitney *U* test).
‡ *n* 16 (eight control, eight Fe deficient).
§ *n* 54 (twenty-four control, thirty Fe deficient).
‖ *n* 38.

the fetus. In the placenta, Fe deficiency led to a decrease in Cu chaperone for superoxide dismutase (*CCS*), the chaperone that delivers Cu to superoxide dismutase (SOD)1, by –9·5 % (95 % CI –18·0, –1·0; $P=0·030$) and *CP* by –15·0 % (95 % CI –29·4, –0·5; $P=0·042$) expression (Fig. 2).

The maternal hepatic expression of the genes related to Zn metabolism was not affected by Fe deficiency. In the placenta, *ZIP4* was reduced by –23·7 % (95 % CI –47·0, –0·4; $P=0·047$) and *ZnT1* by –12·3 % (95 % CI 21·8, –2·8; $P=0·012$) in the Fe-deficient group compared with control (Fig. 3). In fetal liver, Fe deficiency also decreased *ZIP4* by –17·0 % (95 % CI –28·1, –5·8; $P=0·004$), while *ZIP14* and *ZnT1* were increased by 10·0 % (95 % CI 0·7, 19·3; $P=0·036$) and by 18·7 % (95 % CI 9·0, 28·4; $P=0·0003$), respectively (Fig. 3).

The effect of maternal Fe deficiency on Cu and Zn metabolism observed in the current study is summarised in Table 3.

Discussion

In this article, we examined the interactions between Fe, Cu and Zn during pregnancy in the rat. The study has some limitations in that the data can only be extrapolated to humans to a limited extent. However, given how well-conserved micronutrient metabolism is between species, it is likely that the same results would be observed in humans with Fe deficiency. We restricted our measurements to liver and placenta, as these are the major organs involved in micronutrient metabolism[3]. Fe deficiency altered Cu but not Zn concentrations in the maternal liver and placenta, confirming that the metabolism of Cu is more sensitive to Fe deficiency than that of Zn[15]. However, maternal Fe deficiency had an important impact on both Cu and Zn levels in the fetal liver, which were reduced by approximately 15 % and augmented by approximately 25 %, respectively.

Effect of maternal iron deficiency on copper metabolism

Maternal Fe deficiency leads to the hepatic accumulation of Cu in the dams, while Cu levels are decreased in the fetal liver, as described previously by our group, as well as others[20,21,23]. Interestingly, Fe deficiency decreased the expression of the chaperones *ATOX1* and *COX17* in the maternal liver, contrasting with our previous findings, where no change in *ATOX1* mRNA levels was observed[23]. It is important to note that in our previous experiment, *COX17* was not measured and *ATOX1* was only measured in six samples. Further, the previous study used Northern blotting to detect expression levels which is less sensitive than using RT-PCR. The present study included 45 samples, which allows us to detect smaller changes with greater sensitivity.

ATOX1 does not seem to be involved in Cu uptake but is a Cu chaperone that interacts with *ATP7A* and *ATP7B* and delivers Cu to these pumps and hence to trans-Golgi network[36] or possibly the efflux pathway of the placenta[37]. Moreover, *ATOX1* appears essential to peri-natal Cu homoeostasis[38] for synthesis of CP or export from the cell into the portal circulation (*ATP7A* in gut) or into the bile for excretion (*ATP7B*). Understandably, the inhibition of *ATOX1* expression reduces the trafficking of ATPases[39], thus reducing Cu export and causing its accumulation in the cell[40]. Therefore, the small but significant reduction in *ATOX1* expression in the Fe-deficient liver could be responsible – at least partially – for the increase in Cu levels.

In addition, the hepatic expression of *COX17*, a chaperone delivering Cu to the cytochrome oxidase complex in the respiratory chain, was also decreased. The impairment of mitochondrial function and the reduction in cytochrome *c* activity (which requires both Fe and Cu to function) is well-established in Fe-deficient tissues, including in the liver[41,42],

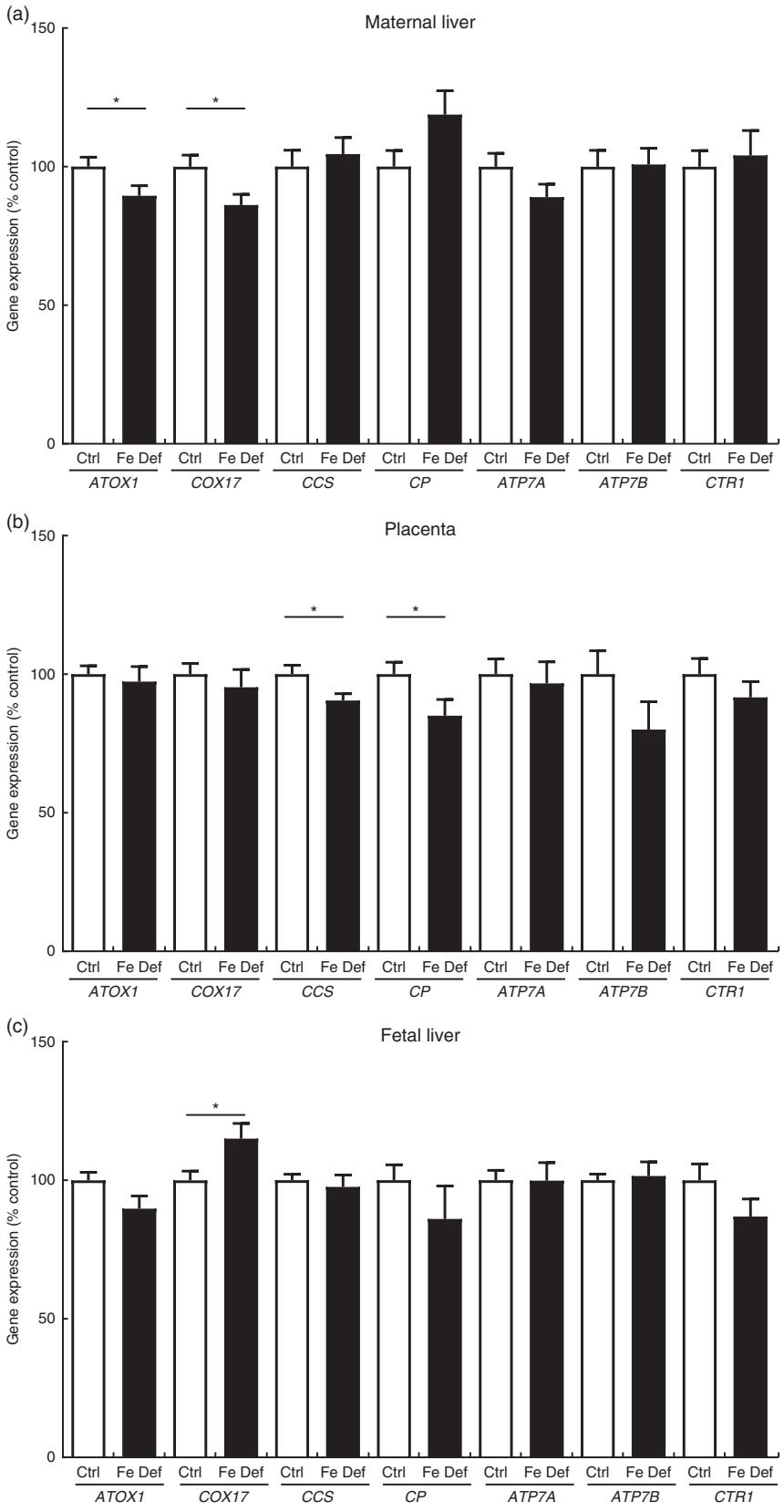

Fig. 2. Effect of maternal iron deficiency on the expression of genes related to copper metabolism (*n* 45) 21 d after mating: (a) maternal liver; (b) placenta; (c) fetal liver. Values are mean percentage of control, with their standard errors represented by vertical bars. * Results are significantly different between the control (Ctrl, *n* 21) and iron-deficient (Fe Def, *n* 24) groups ($P < 0{\cdot}05$; Mann–Whitney test). *ATOX1*, antioxidant 1 copper chaperone; *COX17*, cytochrome *c* oxidase chaperone; *ATP7A*, ATPase copper transporting alpha (Menkes); *ATP7B*, ATPase copper transporting beta (Wilson); *CCS*, copper chaperone for superoxide dismutase; *CP*, ceruloplasmin; *CTR1*, copper transporter 1.

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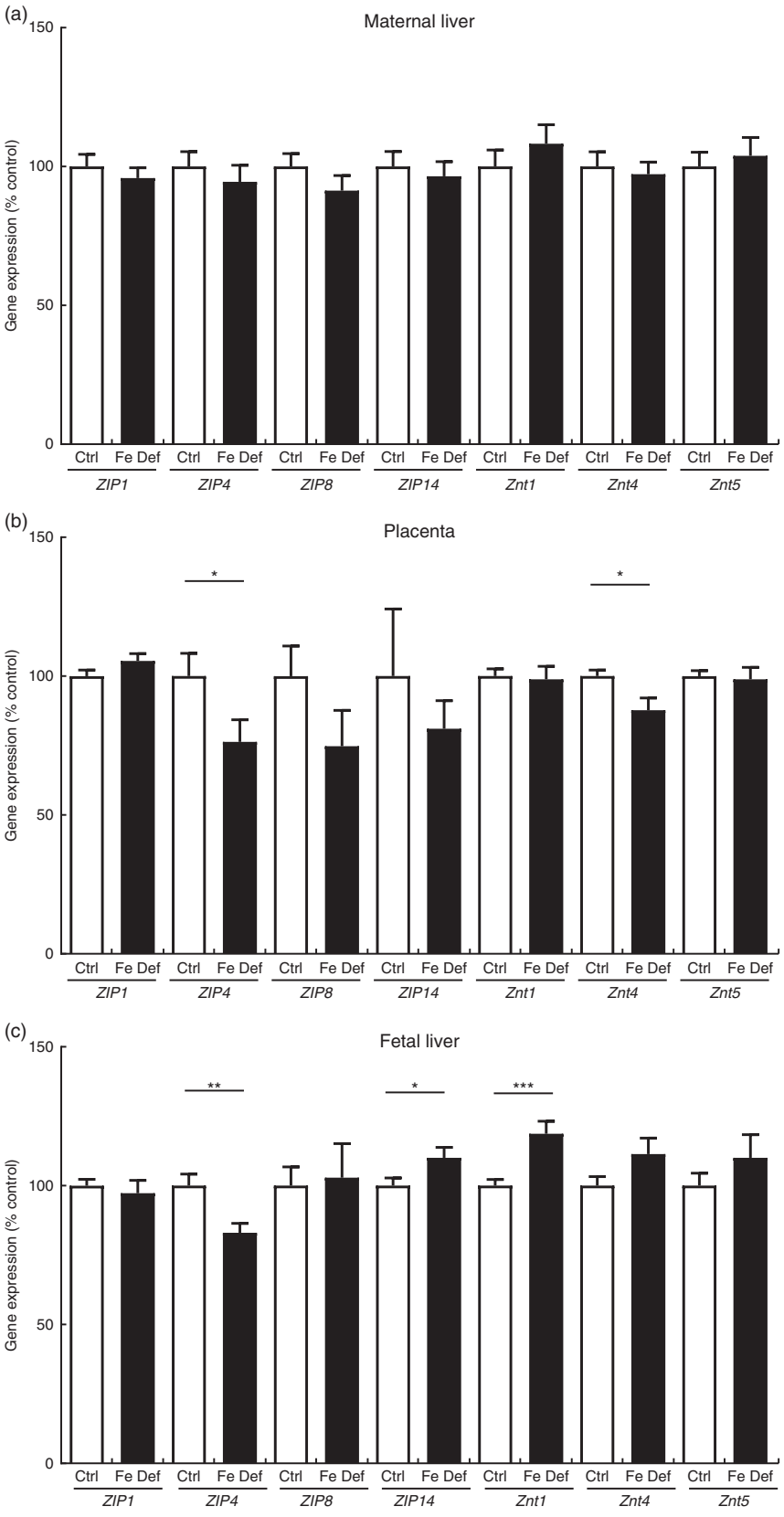

Fig. 3. Effect of maternal iron deficiency on the expression of genes related to zinc metabolism (*n* 45) 21 d after mating: (a) maternal liver; (b) placenta; (c) fetal liver. Values are mean percentage of control, with their standard errors represented by vertical bars. Results are significantly different between the control (Ctrl, *n* 21) and iron-deficient (Fe Def, *n* 24) groups: * $P<0{\cdot}05$, ** $P<0{\cdot}01$, *** $P<0{\cdot}001$ (Mann–Whitney test). *ZIP1*, ZRT/IRT-like protein 1; *ZIP4*, ZRT/IRT-like protein 4; *ZIP8*, ZRT/IRT-like protein; *ZIP14*, ZRT/IRT-like protein 14; *ZnT1*, zinc transporter 1; *ZnT4*, zinc transporter 4; *ZnT5*, zinc transporter 5.

Table 3. Summary of the effect of maternal iron deficiency on copper and zinc metabolism in maternal liver, placenta and fetal liver at day 21 of pregnancy*

Gene	Maternal liver	Placenta	Fetal liver
Cu†	↑↑	↑	↓
ATOX1‡	↓	=	=
COX17‡	↓	=	↑
CCS‡	=	↓	=
CP‡	=	↓	=
ATP7A‡	=	=	=
ATP7B‡	=	=	=
CTR1‡	=	=	=
Zn†	=	=	↑↑
ZIP1‡	=	=	=
ZIP4‡	=	↓	↓
ZIP8‡	=	=	=
ZIP14‡	=	=	↑
ZnT1‡	=	=	↑
ZnT4‡	=	↓	=
ZnT5‡	=	=	=

ATOX1, antioxidant 1 Cu chaperone; *COX17*, cytochrome *c* oxidase Cu chaperone; *CCS*, Cu chaperone for superoxide dismutase; *CP*, ceruloplasmin; *ATP7A*, ATPase Cu transporting alpha (Menkes); *ATP7B*, ATPase Cu transporting beta (Wilson); *CTR1*, Cu transporter 1; *ZIP*, ZRT/IRT-like protein; *ZnT*, Zn transporter.

* The concentrations of Cu, Zn, and the expression of genes of Cu and Zn metabolism were either greatly increased (↑↑), increased (↑), decreased (↓) or unchanged (=) by maternal Fe deficiency.

† *n* 54 (control *n* 24, Fe deficient *n* 30).

‡ *n* 45 (control *n* 21, Fe deficient *n* 24).

although whether this is cause or consequence is not certain at this time. There are little data on the direct effects of Cu on either *ATOX1* or *COX17* expression, so the possibility that these changes are a response to changes in Cu levels, rather than Cu levels being changed by the alterations in gene expression, cannot be excluded in these experiments.

As observed previously[23], maternal Fe deficiency led to the increase in Cu levels in the placenta (approximately 19 %) to a greater extent than the maternal liver (approximately 10 %). This was accompanied by the reduction in *CCS* expression, another chaperone channeling Cu for incorporation into the Cu–Zn superoxide dismutase (SOD) complex, an essential component of the anti-oxidant cell system. Cu has been shown to modulate the degradation of *CCS*[43] and Cu supplementation in humans reduces *CCS* mRNA levels in peripheral mononuclear cells[44]. It is likely that the down-regulation of *CCS* expression observed here is due to the presence of elevated Cu levels in the placenta, rather than a direct consequence of reduced Fe levels. Our findings suggest that maternal Fe deficiency leads to the impairment of SOD activity (closely related to *CCS* expression) and the anti-oxidant defense of the placenta as observed in liver and brain cells upon Cu accumulation[45,46].

CP is involved in Fe export in the liver, oxidising Fe(II) to Fe(III) for incorporation into transferrin, while its homologue zyklopen performs the same function in placenta in mice and humans[47]. Whether the ferroxidase mRNA measured in the placenta is zyklopen, CP or a combination of both remains uncertain, since the sequences are very similar and because the primers are from a commercial supplier. However, it is likely that we measured the mRNA coding for a multi-copper oxidase responsible for Fe export[48]. The effect that the reduced placental expression might have on Fe transport is not clear and contrasts with previous findings[48]. However, it is important to note that CP protein and enzyme levels do not correlate with mRNA levels[49] and that changes in *CP* mRNA expression observed in the present study may therefore have little physiological impact. Maternal Fe deficiency is partly alleviated in the fetus, due to increased expression of transferrin receptors[50,51], which would imply that ferroxidase expression or activity is not rate limiting.

Cu transport across the placenta is less well-understood. Cu appears to be taken up from the maternal circulation through *CTR1* and excreted through ATPases[52,53]. It has been suggested that *ATP7A* is responsible for the efflux of Cu from the basolateral membrane into the fetal circulation, while *ATP7B* is responsible for excreting Cu back into the maternal circulation at the apical side[54]. Whether CP and/or zyklopen are involved in Cu efflux and/or delivery to the fetal liver, as we have previously[54] suggested for transport of Cu from the mother to the placenta[55], and whether this could explain the differences observed in Cu levels between placenta and fetal liver remain to be clarified.

Effects of maternal iron deficiency on zinc metabolism

The effect of Fe deficiency on Zn metabolism in the pregnant rat is less marked than for Cu but is nonetheless significant. Zn levels in the maternal liver and placenta were not changed but were markedly increased in the fetal liver.

In the maternal liver, the expression of the genes of Zn metabolism tested were unchanged, which is consistent with the lack of effect on Zn levels but contrasts with previous findings in male rats, where Fe deficiency led to an increase in hepatic *ZIP14* expression[19]. It should be noted, however, that the authors also observed a small decrease in Zn levels which was not seen in the present study nor in their other study of a similar design[19]. Taken together, these results imply that Fe deficiency during pregnancy is unlikely to lead to changes in Zn metabolism that are physiologically important in the maternal liver.

While Zn levels were unchanged in the placenta, *ZIP4* and *ZnT4*, genes of Zn cellular uptake and exit, respectively, were both decreased in the placenta. The results do not fully match those seen in mice given a Zn-deficient diet, where *ZnT4* and also *ZIP1*, *ZnT1* and 5 were reduced in placenta[33]. It is possible that there was a mild reduction in Zn supply to and in efflux from the placenta, with a neutral net effect on Zn levels in the placenta. Zn metabolism and transport in the placenta are still not entirely understood, and the role and localisation of *ZIP4* in the placenta are not known. *ZIP4* is crucial for Zn cellular uptake in the small intestine and is up-regulated upon Zn deficiency in mice[56]. It is also abundantly expressed in other tissues involved in nutrient absorption and reabsorption (stomach, colon and kidney)[57]. While *ZIP4* expression could not be compared with intestinal or kidney expression in the present study, it is important to note that it was higher in the placenta compared with maternal liver (approximately 2-fold) and fetal liver (approximately 15-fold, data not shown). This suggests that the changes observed in placental expression could be biologically relevant, and further research is warranted to clarify the role of *ZIP4* in placenta. If it was involved in the

return of Zn from fetal to maternal circulation, then decreased expression could result in increased levels of Zn in the fetal liver. Interestingly, neither *ZIP8* nor *ZIP14*, both of which have previously been implicated in Fe metabolism as well as that of Zn[32], are altered in the placenta or maternal liver, which would argue against the changes in fetal liver Zn levels being a direct consequence of competition with Fe for these transporters.

Fe deficiency leads to an increase in Zn levels in the fetal liver but also a lesser decrease in Fe levels compared with the decrease observed in the maternal liver[58]. Both these changes may be explained, at least partially, by an upregulation of *ZIP14*, which mediates the uptake of Zn, as well as non-transferrin-bound Fe[31], and thus could help protect the fetus against Fe deficiency. It is important to note that this does not rule out other mechanisms, such as an increased expression of transferrin receptors, as we have previously observed in the placenta of rats and women[50,51]. On the other hand, the reduced *ZIP4* expression in the fetal liver is more likely to be a consequence of the increase in Zn levels as has been seen in Zn overload observed in the intestine and visceral yolk sac of mice[56]. In addition, as described earlier in this discussion, *ZIP4* expression in the fetal liver was fairly low compared with the other tissues, indicating that its changes may have little biological relevance.

Contrasting with our results, *ZnT1* hepatic upregulation has been reported in Zn-deficient rats in association with Zn depletion in the liver[59], although this was only observed at the protein level. Whether the increase in *ZnT1* mRNA expression in the fetal liver would lead to an increase in protein levels is uncertain, and whether this could lead to more Zn efflux back into the fetal circulation is unlikely in the present study. The reasons why *ZnT1* upregulation is associated with a marked increase in Zn levels are not known, and the role of *ZnT1* in fetal liver Zn metabolism needs to be clarified.

In summary, this study has shown that Fe deficiency has opposite effects on Cu and Zn levels in the fetal liver, and this is associated with changes in the expression of genes of Cu and Zn metabolism in the placenta as well as in the fetus. It is important to note that some of these effects may not be directly caused by Fe deficiency but rather by changes in the metabolism of the other two nutrients. The results further demonstrate that micronutrient metabolism, especially during pregnancy, is tightly interlinked and underscores the importance of considering all of the micronutrients when trying to alleviate deficiencies in one of them. It would be important to consider that the symptoms caused by deficiencies in one micronutrient (here Fe) may not be caused directly by that deficiency but by other deficiencies or overloads occurring as a consequence of the primary defect.

Acknowledgements

The authors are grateful for the technical assistance of Gill Campbell (ICP-MS analysis).

This work was supported and funded by Scottish Government (Rural and Environment Science and Analytical Services).

H. J. M., L. G. and S. C. C. designed the research; L. G., H. E. H., V. J. C. and S. C. C. conducted the research; S. C. C. analysed the data and wrote the manuscript; G. R. and H. J. M. reviewed the manuscript. H. J. M., G. R. and S. C. C. take responsibility of data interpretation and presentation. All authors read and approved the final manuscript.

The authors declare that there are no conflicts of interest.

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