Added dietary cobalt or vitamin B_{12}, or injecting vitamin B_{12} does not improve performance or indicators of ketosis in pre- and post-partum Holstein-Friesian dairy cows

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Short title: Cobalt and vitamin B_{12} metabolism in dairy cows

Abstract

Vitamin B_{12} is synthesised in the rumen from cobalt and has a major role in metabolism in the peripaturient period, although few studies have evaluated the effect of the dietary inclusion of cobalt (Co), vitamin B_{12} or injecting vitamin B_{12} on the metabolism, health and performance of high yielding dairy cows. Fifty-six Holstein-Friesian dairy cows received one of four treatments from 8 weeks prior to calving to 8 weeks post calving: C, no added Co; DC, additional 0.2 mg Co/kg DM; DB, additional 0.68 mg vitamin B_{12}/kg DM; IB, intra-muscular injection of vitamin B_{12} to supply 0.71 mg/cow/day pre-partum and 1.42 mg/cow/day post-partum. The basal and lactation rations both contained 0.21 mg Co/kg DM. Cows were weighed and condition scored at drying off, 4 weeks prior to calving, within 24 h of calving and at 2, 4 and 8 weeks post-calving, with blood samples collected at drying off, 2 weeks pre-calving, calving and 2, 4 and 8 weeks post-calving. Liver biopsy samples were collected from all animals at drying off and 4 weeks post-calving. Live weight changed with time, but there was no effect of treatment ($P>0.05$), whereas cows receiving IB had the lowest mean body condition score and DB the highest ($P<0.05$). There was no effect of treatment on post-partum DM intake, milk yield or milk fat concentration ($P>0.05$) with mean values of 21.6 kg/day, 39.6 kg/day and 40.4 g/kg respectively. Cows receiving IB had a higher plasma vitamin B_{12} concentration than those receiving any of the other treatments ($P<0.001$), but there was no effect ($P>0.05$) of treatment on homocysteine or succinate concentrations, although mean plasma methylmalonic acid concentrations were lower ($P=0.019$) for cows receiving IB than for Control cows. Plasma β-hydroxybutyrate concentrations increased sharply at calving followed by a decline, but there was no effect of treatment. Similarly, there was no effect ($P>0.05$) of treatment on plasma non-esterified fatty acids or glucose. Whole tract digestibility of DM and fibre measured at week 7 of lactation were similar between treatments, and

there was little effect of treatment on the milk fatty acid profile except for C15:0, which was lower in cows receiving DC than IB (P<0.05). It is concluded that a basal dietary concentration of 0.21 mg Co/kg DM is sufficient to meet the requirements of high yielding dairy cows during the transition period, and there is little benefit from additional Co or vitamin B_{12}.

Key words: dairy cow, digestibility, liver metabolism, milk, minerals

Implications

The microbes in the rumen of dairy cows require cobalt to synthesise vitamin B_{12} which the cows then requires for efficient energy and protein metabolism, particularly during the transition from pregnancy to early lactation. This study investigated the effect of feeding different levels of dietary cobalt and supplementing the diet or injecting vitamin B_{12}, and found little benefit from providing additional amounts on cow performance, health or milk quality. This information can be used to more accurately formulate diets for dairy cows to improve health and performance and reduce diet costs.

Introduction

During the transition period from late gestation to early lactation it is usual for dairy cows to experience an imbalance between dietary energy intake and nutrient demand for milk production (Lean et al., 2013; Raboisson *et al.,* 2014). This results in varying degrees of negative energy balance and consequently mobilisation of body adipose tissue (Raboisson *et al.,* 2014). The inability of the liver to metabolise mobilised fat can result in sub-clinical or clinical ketosis, normally defined when plasma β-hydroxybutyrate (3-OHB) concentrations are ≥ 1.2 mmol/L (McArt *et al.,* 2013). The prevalence of sub-clinical ketosis (SCK) has been reported to be approximately 22% in European dairy herds (Suthar *et al.,* 2013), whilst in North America a rate of 43%

has been reported (McArt *et al.,* 2012). Cows with SCK have been reported to have an increased odds of developing clinical ketosis, displaced abomasum and metritis of 9.5, 5.0 and 1.5 respectively (Suthar *et al.,* 2013), and a decreased probability of pregnancy (Raboisson *et al.,* 2014).

Vitamin B_{12} has a major role in the metabolism of lipid by the liver and subsequently plays a central role in controlling SCK. One of the two main functions of vitamin B_{12} in the dairy cow is its action as a co-factor in the transformation of methylmalonyl CoA to succinyl CoA which is then used in the Krebs cycle within the liver for the synthesis of glucose from propionate (McDowell 2000). Insufficient tissue supply of vitamin B_{12} therefore results in an accumulation of methylmalonic acid (MMA) and ketones in the blood. The second role of vitamin B_{12} is its requirement as a co-factor for the synthesis of methionine via the transfer of a methyl group from 5-methyl-tetrahydrofolate to homocysteine (McDowell 2000). Methionine is generally regarded as one of the first limiting amino acids in milk protein synthesis (NRC, 2001) and plays a key role in the synthesis of *S*-adenosylmethionine as a methyl donor (McDowell 2000), and consequently has a major impact on milk production.

Within the rumen, elemental cobalt (Co) is used by bacteria to synthesise vitamin B_{12}, and it has traditionally been considered that the rumen bacteria are able to synthesise sufficient amounts to meet the cows requirements over the peri-parturient period and throughout lactation, provided there is a sufficient dietary supply of Co (NRC, 2001). The current recommended level of dietary Co for dairy cows, based on NRC (2001), is 0.11 mg/kg DM, but vitamin B_{12} synthesis in the rumen has been shown to increase linearly between 0.1 and 1.0 mg Co per kg DM (Tiffany *et al.,* 2003; 2006), and growing beef cattle are known to respond to dietary Co levels up to 0.25 mg/kg DM (Stangl *et al.,* 2000). In the well-fed dairy cow, serum vitamin B_{12} concentrations decline during the dry period and throughout early lactation (Kincaid

and Socha, 2007), but crucially, production and liver-health related responses to dietary Co spanning current recommended levels have not been determined in the transition cow, highlighting an area where investigation is required. Additionally, whilst elemental Co is nontoxic, dietary Co sources have recently been re-classified under EU legislation (EU 601/2013 amended 2014) as carcinogenic, so that mineral premixes have been set to contain a maximum of 0.34 mg Co/kg DM. Rumen protected forms of vitamin B_{12} are available, but given that vitamin B_{12} is important in rumen function, it is questionable if rumen protected sources of vitamin B_{12} alone are desirable substitutes for dietary Co. Consequently, there is a need to re-evaluate dairy cow Co requirements and the benefits of vitamin B_{12} supplementation over the peri-parturient period in higher yielding dairy cows. The objectives of the study were to determine the effects of the dietary addition of Co, vitamin B_{12} or the injection of vitamin B_{12} in late gestation/early lactation on dairy cow metabolism, intake and performance.

Materials and methods

Animals and treatments

Fifty-six Holstein-Friesian dairy cows (12 primiparous and 44 multiparous) commenced the study 57 (SE ± 0.9) days pre-partum, and remained on study until 56 days post-partum. Cows were blocked according to parity (1^{st}, 2^{nd} and 3^{rd}+), and previous 305 day milk yield, and randomly allocated to one of four dietary treatments. Pregnant, non-lactating cows were housed in a free stall-building from the end of lactation (approximately 8 weeks prior to calving), with *ad libitum* access to the same basal dry cow diet which was fed as a total mixed ration (TMR; Table 1) via a barrier with individual head locks. Approximately 3 days prior to calving the cows were transferred to a straw bedded calving yard where they remained until approximately 24 h post-calving. For approximately the first 7 to 10 days post-calving cows were

132 housed in a loose yard where they had access to a lactation TMR that contained no
133 added Co via a feed barrier with individual head locks. Cows were then transferred to
134 free-stall housing containing individual feeders. All cows had continual access to
135 water.

136 Prior to calving the cows received one of four dietary treatments: C = no added
137 Co; DC = an additional 0.2 mg Co/kg DM (supplied as cobalt carbonate; Anima,
138 Krakow, Poland); DB = 0.68 mg added vitamin B_{12}/kg DM (Impextraco, Heist-op-den
139 Burg, Belgium) or IB = injected vitamin B_{12} (5 ml of 1000 µg vitamin B_{12}/ml resulting in
140 0.71 mg/day; AnimalCare, York, UK; intra muscular every 7 days). The additional level
141 of 0.2 mg Co/kg DM in DC was chosen to provide a daily Co supply approximately
142 twice that of C, but to be within the permitted EU legal limit of 0.34 mg/kg DM. The
143 0.68 mg vitamin B_{12}/kg DM provided in DB was calculated to provide a similar
144 predicted tissue supply of vitamin B_{12} as DC assuming a 4 % conversion of Co to
145 cobalamin, a loss of 80 % of supplementary vitamin B_{12} in the rumen, and 10 %
146 absorption of vitamin B_{12} at the duodenum (Stemme *et al.,* 2008; Girard *et al.,* 2009;
147 Akins *et al.,* 2013). The level of injected vitamin B_{12} was chosen to provide a supra-
148 nutritional tissue supply based on Girard and Matte (2005a), and therefore act as a
149 positive control. All animals were individually supplemented with 200 g/day of ground
150 wheat which acted as a carrier for treatments DC and DB by restraining each cow
151 using the individual head-lock gates until the daily allowance had been consumed, and
152 assuming a DM intake of 12 kg/cow/day (NRC, 2001).

153 From calving until approximately 8-10 days post-calving all cows were fed a TMR
154 (Table 1) containing no additional Co or vitamin B_{12}. During this period all animals
155 were supplemented with 200 g/day of ground wheat as a carrier to provide treatments
156 DC and DB, assuming a DM intake of approximately 20 kg/day (NRC 2001), with IB
157 receiving 10 ml of 1000 µg vitamin B_{12}/ml injected every 7 days, resulting in 1.42

158 mg/day. For the remainder of the study the supplements were included into the TMR,
159 and intake monitored using roughage intake feeders (Hokofarm, Marknesse, The
160 Netherlands).
161 *Experimental routine*
162 The cows were weighed and condition scored (Ferguson *et al.,* 1994) at drying off, 4
163 weeks post-drying off, within 24 h of calving, and at weeks 2, 4 and 8 post-calving.
164 Samples of the TMR and individual forages were collected weekly: the forage sample
165 was oven dried and the quantity of lucerne and maize silage adjusted to achieve the
166 desired ratio, and the TMR sample frozen at -20°C prior to analysis. Blood samples
167 were collected by jugular venepuncture at 1000 h on the day of drying off, 6 weeks
168 post-drying (approximately 2 weeks prior to calving), within 24 h of calving, and 2, 4
169 and 8 weeks post calving. Liver samples were collected by biopsy through the 11^{th}
170 intercostal space from all animals at drying off and at 4 weeks post-calving; samples
171 were snap frozen in liquid N and stored at -80°C until subsequent analysis. Cows were
172 milked twice daily at approximately 0600 and 1700 h, with yield recorded at each
173 milking, and samples taken weekly on consecutive am and pm milkings for subsequent
174 analysis. Additional milk samples were collected during week 7 of lactation for the
175 determination of the fatty acid (FA) profile. Whole tract digestibility was estimated
176 during week 7 of lactation by collecting faecal samples from each cow over 5 days at
177 approximately 0800 and 1400 h (Van Keulen and Young, 1977), and stored at -20°C
178 for subsequent analysis.
179 *Chemical analysis*
180 Weekly TMR samples were bulked within month and analysed according to AOAC
181 (2012) for DM (934.01) and CP (988.05), whilst NDF and ADF were determined
182 according to Van Soest *et al.* (1991). The determination of NDF was conducted without
183 sodium sulphite, with alpha-amylase and was corrected for ash. Samples were also

184 analysed for major and trace minerals as described by Cope *et al.* (2009). Plasma
185 samples were analysed for urea, glucose, non-esterified fatty acids and 3-
186 OHB (Randox Laboratories, County Antrim, UK; kit catalogue no. TP245, UR221,
187 AB362, GL1611, FA115 and RB 1008 with an intra-assay CV of 3.5, 1.8, 3.0 and 4.7%
188 respectively) using a Cobas Miras Plus auto-analyser (ABX Diagnostics, Bedfordshire,
189 UK). Plasma samples were analysed for minerals as described by Cope et al. (2009).
190 Analysis of plasma vitamin B_{12}, homocysteine, MMA, succinic acid (SA) and hepatic
191 triacylglycerol (TAG) concentration (along with their intra-assay CV) are provided in
192 Supplementary Material S1. For MMA and SA only the two most extreme treatments
193 (C and IB) were analysed. Milk samples were analysed for fat, protein, lactose and
194 somatic cell count by Eurofins UK (Wolverhampton, UK). Fatty acid methyl esters
195 (FAME) in hexane were prepared from milk fat and individual FAME were determined
196 by GLC (Hewlett Packard 6890, Wokingham, UK) fitted with a CP-Sil 88 column (100
197 m x 0.25 mm i.d. x 0.2 μm film) as described previously by Sinclair et al., (2015).
198 Faecal samples were bulked between days and sampling times and analysed for acid
199 insoluble ash (Van Keulen and Young 1977), ash (AOAC, 2012; 942.05), CP, NDF
200 and ADF.
201 *Statistical analysis*
202 Performance and blood parameters were analysed as repeated measures
203 analysis of variance, using Genstat 17.1 (VSN Int. Ltd., Oxford, UK). The performance
204 in the week prior to allocation was used as a co-variate where appropriate:
205 $$Y_{ijk} = \mu + B_i + C_j + T_k + C_j.T_l + \varepsilon_{ijk}$$
206 Where Y_{ijk} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; C_j =
207 effect of treatment; T_k = effect of time; $C_j.T_k$ = interaction between treatment and time,
208 and ε_{ijk} = residual error. Milk SCC and hepatic TAG concentrations were log
209 transformed prior to analysis to homogenise variance. A subsequent analysis of TAG

levels substituted parity for treatment. Milk FA concentration and diet digestibility were analysed as a randomised block design by analysis of variance as:

$$Y_{ij} = \mu + B_i + C_j + \varepsilon_{ij}$$

Where Y_{ij} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; C_j = effect of treatment, and ε_{ij} = residual error. Results are presented as treatment means with a SED, with post-hoc analysis using Tukey's test at a 5% level of significance.

Results

Diet analysis, intake and animal performance.

The chemical composition of the basal dry-cow diet was similar to that predicted, whereas the background concentration of Co was higher in both the dry cow and lactation diets at 0.21 mg/kg DM (Table 2). The four lactation diets had a similar mean DM, OM, CP, NDF and ADF concentration of 469 g DM/kg, 940, 168, 368 and 231 g/kg DM respectively. The four diets also had a similar concentration of macro and trace elements. In contrast, the treatments with no added Co (C, and IB) had a dietary concentration of approximately 0.21 mg Co/kg DM, whilst DC had almost double the dietary concentration of Co, with DB being 0.02 mg Co/kg DM higher than the Control.

There was an increase in DM intake ($P<0.001$) from 18.6 kg/d during week 2 of lactation to 23.7 kg/d during week 8, with a mean intake of 21.6 kg/d over the study period, but there was no effect ($P>0.05$) of dietary treatment or treatment x time interaction (Table 3). Similarly, milk yield increased ($P<0.001$) from 30.3 kg/d in week 1 to 43.6 kg/d in week 8 of lactation, but there was no effect ($P>0.05$) of treatment or time x treatment interaction on milk performance or composition, with mean values of 39.6 kg/day, 40.4 g/kg and 33.1 g/kg for milk yield, fat and protein concentration respectively. There was an effect of time ($P<0.001$) on live weight, which increased

between drying off and 4 weeks pre-calving, before decreasing immediately post-calving but there was no effect (P>0.05) of treatment, or time x treatment interaction. There was also an effect of time on BCS (P<0.001), which declined post-calving, and treatment, with cows receiving IB having the lowest and DB the highest BCS (P<0.05).

Plasma metabolite and mineral concentrations, and hepatic triacylglycerol content

Plasma glucose concentration decreased from 8 weeks pre-calving to calving, then increased to week 8 post calving (P<0.001), but there was no effect (P>0.05) of dietary treatment on weekly or mean concentration (Table 4 and Supplementary Figure 1a). In contrast, plasma 3-OHB concentrations increased pre-calving, with a sharp increase at calving followed by a decrease at week 2 of lactation (Supplementary Figure 1b), but there was no effect (P>0.05) of dietary treatment. There was an effect of time on plasma NEFA concentration (Supplementary Figure 1c; P<0.001), which decreased pre-calving, before increasing post-calving, but there was no effect (P>0.05) of treatment. Plasma urea concentrations declined rapidly at calving (Supplementary Figure 1d; P<0.001) but were not affected (P>0.05) by treatment. There was no effect (P>0.05) of dietary treatment on mean plasma mineral concentration, except Co, which was higher (P<0.05) in cows receiving DC or IB than C or DB, and Zn, which was higher in cows receiving DC than IB (P<0.05).

There was a treatment x time interaction (P<0.001) for plasma vitamin B_{12} concentration, which decreased post calving in all treatments (Figure 1a), and overall was higher (P<0.001) in cows receiving IB than any of the other treatment groups (246 vs 192, 195 and 190 pmol/L for IB vs C, DC and DB respectively). There was no treatment effect on plasma concentrations of homocysteine, which decreased (P<0.001) following calving (Figure 1b). In contrast, plasma concentrations of both MMA (Figure 1c) and SA (Figure 1d) increased (P<0.001) following parturition, with an interaction between treatment and time for plasma MMA concentrations (P=0.033);

MMA being lower in IB than Control cows post-calving. There was no treatment effect on hepatic TAG concentrations pre- or post-partum (P>0.05), however, TAG concentrations were higher (P<0.001) in samples collected during the early post-partum period than during late gestation.

Diet digestibility and milk fatty acid profile

There was no effect (P>0.05) of dietary treatment on the digestibility of any of the parameters measured (P>0.05; Table 5). There were few differences in the FA profile of milk from cows fed any of the treatments, except C15:0 which was lower (P<0.05) in cows receiving supplementary Co (DC) compared to injected vitamin B_{12} (IB; Table 6). The desaturase index calculated using *cis*-9, *trans*-11 CLA was lower (P<0.01) in cows receiving IB compared to C or DC, but there was no other effect of dietary treatment.

Discussion

To attempt to provide a low dietary Co concentration in the current study, feed ingredients were selected to be low in Co, with the mineral/vitamin supplement formulated to contain no Co. Despite this, both the dry cow and lactation control diets contained 0.21 mg Co/kg DM, above the recommended dietary requirement of 0.11 mg/kg DM stated by NRC (2001), but considerably lower than a number of similar studies that have attempted to provide low dietary Co diets (Akins *et al.,* 2013; Girard *et al.,* 2009). The addition of 0.2 mg Co/kg DM in DC resulted in a dietary concentration approximately twice that of C, but was well within the permitted EU legal limit of 0.34 mg added Co/kg DM, or the 1.14 mg Co/kg DM limit in the total diet.

Performance and dry matter intake

The peri-parturient period in dairy cows is characterised by a phase of negative energy balance as cows transition from late gestation into early lactation, with associated

288 homeorhetic and homeostatic regulation of metabolic functions (McArt *et al.,* 2013). In
289 particular, there is a substantial increase in demand for glucose for foetal growth in
290 late pregnancy, and for the synthesis of lactose for milk production by the mammary
291 gland in early lactation (Lean *et al.,* 2013). Vitamin B_{12}, through its role as a co-enzyme
292 for *methylmalonyl-CoA mutase* (EC:5.4.99.2), is crucial for the synthesis of glucose
293 from propionate (McDowell, 2000), and a deficiency in this vitamin can be translated
294 into a reduction in milk synthesis (NRC, 2001). Milk secretion also requires a
295 considerable supply of amino acids, with methionine generally being regarded as one
296 of the first rate limiting AA in most dairy cow rations (NRC, 2001), and vitamin B_{12} has
297 a key role in the activity of *methionine synthase* (EC:2.1.1.13) which regenerates
298 homocysteine to methionine (McDowell 2000). In the current study, additional dietary
299 Co or vitamin B_{12} or parenteral supply of vitamin B_{12} did not influence milk production
300 or composition in early lactation. Similarly, Kincaid and Socha (2007) reported no
301 effect of dietary Co concentration (0.15, 0.89 and 1.71 mg/kg DM) on early lactation
302 milk yield or composition. In contrast, Juchem *et al.,* (2012) reported a decrease in
303 milk fat concentration, but not fat yield, when rumen protected B-vitamins were
304 included in dairy cow rations, although the combination of vitamins used precludes
305 any conclusion to be drawn on the relative efficacy of vitamin B_{12}. Duplessis *et al.,*
306 (2017) also reported no effect of weekly injections of 10 mg of vitamin B_{12} on milk
307 performance or composition over the transition period. Weekly intramuscular
308 injections of vitamin B_{12}, however, resulted in a significant increase in energy corrected
309 milk yield of early lactation primiparous dairy cows supplemented with folic acid and
310 methionine in the study of Girard and Matte (2005a), due to a combination of an
311 increase in milk yield and milk fat concentration, despite dietary Co levels being stated
312 as adequate. In contrast, NRC (2001) concluded that there was no evidence of a
313 performance response to vitamin B_{12} injections in dairy cows when fed adequate (i.e.

314 above 0.11 mg/kg DM) amounts of Co. Results from the current study support that the
315 recent EU restriction on the amount of supplementary Co that can be added to the diet
316 should not impair dairy cow performance or health. There may, however, be some
317 value to higher dietary levels of Co, or rumen protected vitamin B_{12} to increase the
318 vitamin B_{12} content in milk for human consumption as suggested by Girard and Matte
319 (2005b), particularly in situations where foods are fortified with folic acid.

320 Regulation of lipogenesis and lipolysis in the transition period involves a
321 complex interaction between neural, hormonal and paracrine stimuli, along with
322 immune cell migration that changes the structure and cellular distribution of adipose
323 tissue (Contreras *et al.,* 2017). In the current study, both live weight and body condition
324 score were characterised by an increase pre-partum, followed by a rapid decline post-
325 partum, reaching a nadir at approximately 4 weeks post-calving. This rapid decline in
326 body condition and live weight is greater than that reported in other studies that have
327 investigated the effect of diet on body energy reserves where the nadir in energy
328 balance is typically not reached until approximately week 8 to 10 post-partum
329 (Duplessis *et al.,* 2017). Parenteral administration of vitamin B_{12} resulted in the lowest
330 mean BCS post-partum, but this was not reflected in an increase in milk or milk fat
331 yield. Duplessis *et al.* (2017) reported no benefit to injections of vitamin B_{12} on body
332 condition over the transition period, although dietary levels of Co (1.45 and 1.05 mg/kg
333 DM for the dry period and lactation respectively) were substantially higher than that
334 used here.

335 Cows fed any of the dietary treatments in the current study continued to
336 increase their DM intake throughout the first 8 weeks of lactation, but there was no
337 effect of dietary Co level or vitamin B_{12} on intake. Several other studies have also failed
338 to demonstrate a benefit to Co supplementation or parenteral administration of vitamin
339 B_{12} on DM intake either pre-partum or during lactation (Akins *et al.,* 2013; Girard and

340 Matte, 2005a). Indeed, supra-nutritional levels of Co have been demonstrated to result
341 in a reduction in DM intake in some studies, particularly in early lactation (Kincaid and
342 Socha, 2007).
343 *Blood metabolites, minerals and hepatic triacylglycerol content*
344 Poor adaptation to negative energy balance through the transition period is associated
345 with an elevation in plasma NEFA and 3-OHB concentrations (McArt *et al.,* 2013).
346 Whilst there is much debate regarding definitive cut-point concentrations of these
347 metabolites for milk production and disease outcomes, NEFA values of 0.3 to 0.5
348 mmol/l pre-partum and 0.7 to 1.0 mmol/l post partum, and 3-OHB concentrations of
349 1.2 mmol/l, have been suggested as a good combination between sensitivity and
350 specificity (McArt *et al.,* 2013). Based on these assumptions, mean plasma NEFA in
351 the current study were high at drying off and again at calving, whereas mean plasma
352 3-OHB concentrations were always well within accepted limits. Plasma glucose
353 concentrations were also affected by day of sampling in the current study, decreasing
354 sharply at 2 weeks post-calving before returning to pre-partum levels by week 4. There
355 was, however, no effect of dietary treatment on any of these metabolites pre or post-
356 partum, and it is apparent that vitamin B_{12} was not limiting for cows. A number of other
357 studies have also reported no effect of dietary Co level (Akins *et al.,* 2013; Kincaid and
358 Socha, 2007), dietary supplementation with vitamin B_{12} (Graulet *et al.,* 2007) or
359 following parenteral administration of vitamin B_{12} (Akins et al., 2013; Duplessis et al.,
360 2017) on serum NEFA concentration.
361 A threshold for plasma vitamin B_{12} of 150 µmol/l has been suggested, above
362 which there is little further benefit to milk performance in dairy cows (Duplessis *et al.,*
363 2017). Plasma vitamin B_{12} in the current study was above this threshold in cows fed
364 any of the dietary treatments, and were approximately 28% higher in cows receiving
365 weekly injections of vitamin B_{12}. The high demands for metabolism and secretion into

366 milk (Kincaid and Socha 2007) may predispose higher yielding dairy cows to vitamin
367 B_{12} deficiency and can be exhibited in an increase in plasma concentrations of key
368 intermediaries in energy and amino acid metabolism. The dependency of *methionine*
369 *synthase* and *methylmalonyl-CoA mutase* on vitamin B_{12} results in plasma
370 concentrations of homocysteine and MMA acid being good indicators of vitamin B_{12}
371 adequacy (Stangl *et al.,* 2000). These authors reported a significant decline in both
372 plasma homocysteine and MMA when dietary Co concentrations were increased
373 above 0.2 mg/kg DM in growing beef cattle. However, in the current study there was
374 little effect of supplementary Co, vitamin B_{12} or parental administration of vitamin B_{12},
375 and supports the performance and blood metabolite results that the basal dietary Co
376 concentration of 0.21 mg/kg DM was sufficient to meet requirements in late pregnancy
377 and early lactation. Despite the lack of an effect of treatment on hepatic TAG
378 concentrations in the current study there was a 3-fold increase in concentration post-
379 compared to pre-partum (mean values of 19 and 6 mg/g liver respectively), although
380 mean values were still within the 1-5% liver TAG concentration that is associated with
381 mild fatty liver (Bobe *et al.,* 2004).

382 *Whole tract digestibility*

383 In addition to the requirement for the metabolism of glucose and methionine in the
384 dairy cow, vitamin B_{12} has been demonstrated to be an essential growth factor for
385 efficient rumen microbial metabolism, with ruminal synthesis of vitamin B_{12} and
386 subsequent flow to the duodenum being significantly related to the rate of microbial
387 protein synthesis in the rumen (Castagnino *et al.,* 2016). This has been suggested to
388 improve fibre digestion in the rumen (Lopez Guisa and Satter, 1992), and increase the
389 ruminal production of propionate in some studies (Tiffany *et al.,* 2003), but not others
390 (Stemme *et al.,* 2008). Increasing dietary Co concentration from sub-optimal levels
391 has been shown to increase whole tract digestibility quadratically in sheep, being

392 highest at a dietary level of 0.6 mg/kg DM (Wang *et al.,* 2007). Similarly, Kadim *et al.*
393 (2003) reported that the parental provision of vitamin B_{12} increased diet digestibility in
394 goats, which was speculated to be due to an increase in intestinal absorption of
395 nutrients or microbial production in the rumen. In the current study digestibility co-
396 efficients for DM, N, OM and fibre fractions were similar to that reported by others
397 when feeding similar diets (Sinclair *et al.,* 2015), but there was no effect of dietary
398 treatment, and it can be concluded that there was sufficient vitamin B_{12} available for
399 ruminal metabolism.
400 *Milk fatty acids*
401 The concentration of saturated FA in milk may be reduced via the action of steroyl-
402 CoA desaturase (SCD) on both *de novo* and absorbed FA such as C14:0, C16:0 and
403 C18:0, and is responsible for the majority of the *cis*-9, *trans*-11 CLA from its action on
404 C18:1 *trans*-11 (Shingfield *et al.,* 2013). Several studies have investigated the impact
405 of the dietary inclusion, ruminal infusion or intravenous injection of Co on SCD activity,
406 and reported a dose dependent decrease in activity by up to 72% (Leskinen *et al.,*
407 2016). Infusing Co does not impact on gene expression of SCD, and its effects would
408 therefore appear to be at a post-transcriptional level (Karlengen *et al.,* 2012). However,
409 in previous studies that have reported a reduction in SCD activity supra-nutritional
410 levels of Co have been fed. For example, Karlengen *et al.* (2012) reported no effect
411 on the milk desaturase index in cows when fed approximately 0.2 or 19 mg Co/kg DM,
412 but it was reduced when 270 mg/kg DM was infused into the rumen. In the current
413 study, there was little evidence of an effect of adding approximately 0.2 mg Co/kg DM,
414 or additional vitamin B_{12} administered *per os,* on SCD activity. In contrast, SCD activity
415 was reduced when supra-nutritional levels of vitamin B_{12} were injected, although few
416 other studies have examined the effect of vitamin B_{12} on the activity of this enzyme.
417

Conclusions

A dietary Co concentration of 0.21 mg/kg DM provides sufficient Co for the synthesis of vitamin B_{12} required for the metabolism, performance and diet digestibility of high-yielding dairy cows. Recent restrictions on the inclusion of Co in the diet in the European Union are therefore unlikely to have an impact on intake, performance or health over the periparturient period, and there is little justification for the use of supplementary sources of vitamin B_{12}.

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Declaration of interest

None

Ethics Statement

The procedures involving animals were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 (amended 2012), and were approved by the Harper Adams University Local Ethical Review.

Software and data repository resources

None.

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Table 1 *Basal dry cow and lactation total mixed ration composition*

	Dry cow[1]	Lactation
Ingredient, g/kg DM		
Chopped wheat straw	471	---
Maize silage	218	389
Lucerne silage	87	111
Wheat	109	79
Molassed sugar beet feed	---	79
Soy hulls	---	66
Molasses	3	7
Protected fat	---	16
Rapeseed meal	31	91
Corn gluten feed	26	76
Hipro soybean meal	19	55
Palm kernel meal	9	25
Feed grade urea	10	1
Dry cow minerals[2]	13	---
Lactation minerals[3]	---	5
Magnesium chloride	4	---

[1]Water was added at 0.43 kg/kg DM

[2]Dry cow minerals g/kg: Ca 20; P 60; Mg 200; Na 5; mg/kg I 400; Se 30; Cu 750; Mn 4 000; Zn 6 000; iu/kg Vit A 800 000; Vit D 200 000; Vit E 10 000; Biotin 75 mg/kg.

[3]Lactation minerals g/kg: Ca 180; P 25; Mg 80; Na 120; mg/kg I 400; Se 40; Cu 1 400; Mn 4 000; Zn 6 000; iu/kg Vit A 1 000 000; Vit D 300 000; Vit E 4 500; Biotin 135 mg/kg.

Table 2 *Chemical and mineral composition of dry cow and lactation diets that contained no added cobalt (C), added dietary Co (DC), added dietary vitamin B_{12} (DB) or injected vitamin B_{12} (IB)*

Nutrient	Dry cow[1]	C and IB	DC	DB
DM, g/kg	474	471	466	468
Organic matter, g/kg DM	932	941	939	937
Ash, g/kg DM	67.8	59.2	60.9	63.1
CP, g/kg DM	139	168	169	165
NDF, g/kg DM	561	364	359	386
ADF, g/kg DM	359	230	226	238
Hemicellulose, g/kg DM	202	134	133	148
Fat, g/kg DM	16.7	33.5	34.4	31.7
Fatty acids, g/100 g FA				
C16:0	25.1	25.9	24.1	25.9
C18:0	nd[2]	2.10	1.99	2.10
C18:2 *cis*-9, *cis*-12	28.5	27.5	27.1	27.5
C18:3 *cis*-9, *cis*-12, *cis*-15	3.15	2.69	2.44	2.69
Macro minerals, g/kg DM				
Ca	5.82	7.84	7.71	7.87
P	2.92	4.03	4.15	4.05
Mg	4.25	2.69	2.71	2.74
Na	1.32	1.66	1.66	1.66
K	12.8	15.7	15.8	15.9
S	1.77	2.50	2.50	2.32
Micro minerals, mg/kg DM				
Co	0.21	0.21	0.36	0.23
Mn	76.0	58.9	57.2	59.4
Fe	279	281	274	285
Zn	102	67.1	64.0	65.6
Mo	1.34	1.16	1.20	1.48

[1]Dry cow treatments achieved by individually providing either no added Co (C), 0.2 mg Co/kg DM (DC), 0.68 mg vitamin B_{12}/kg DM (DB) or injected vitamin B_{12} (IB).
[2]nd = not detected

Table 3 *Intake (post calving), milk performance and body condition (mean value pre- and post-calving) of dairy cows offered diets that contained no added cobalt (C), added dietary Co (DC), added dietary vitamin B_{12} (DB) or injected vitamin B_{12} (IB)*

	Treatment					*P*-value[1]	
	C	DC	DB	IB	SED	Treatment	Time
DM intake (kg/day)	21.5	21.3	21.7	21.8	0.78	0.918	<0.001
Milk yield (kg/day)	38.3	39.5	40.8	39.6	1.58	0.480	<0.001
Milk fat (g/kg)	40.6	40.8	40.6	39.6	1.92	0.922	<0.001
Milk protein (g/kg)	32.9	33.2	33.3	33.1	0.67	0.936	<0.001
Milk lactose (g/kg)	45.0	45.0	45.4	45.0	0.37	0.668	<0.001
Milk fat (kg/d)	1.54	1.59	1.63	1.55	0.921	0.715	0.035
Milk protein (kg/d)	1.24	1.28	1.34	1.30	0.046	0.235	<0.001
Milk lactose (kg/d)	1.72	1.75	1.86	1.79	0.073	0.256	<0.001
SCC (log_{10}/ml)[2]	1.74	1.59	1.67	1.83	0.152	0.452	<0.001
Body condition	3.00[ab]	3.08[ab]	3.14[b]	2.94[a]	0.069	0.042	<0.001
Live weight (kg)	665	657	683	651	22.0	0.504	<0.001

[1]There were no (*P*>0.05) Treatment x Time interactions

[2]Scc = somatic cell count

[a,b]Means within a row with a different superscript differ (*P* <0.05).

Table 4 *Mean plasma metabolite and mineral concentration, and hepatic triacylglycerol concentrations in dairy cows offered diets that contained no added cobalt (C), added dietary Co (DC), added dietary vitamin B_{12} (DB) or injected vitamin B_{12} (IB)*

	Treatment					*P*-value[1]	
	C	DC	DB	IB	SED	Treatment	Time
Metabolites[2]							
Glucose (mmol/l)	3.52	3.39	3.48	3.54	0.107	0.480	<0.001
3-OHB (mmol/l)	0.48	0.52	0.51	0.51	0.030	0.598	<0.001
NEFA (mmol/l)	0.40	0.40	0.41	0.44	0.054	0.848	<0.001
Urea (mmol/l)	4.36	4.24	4.29	4.27	0.214	0.953	<0.001
Minerals							
P (mmol/l)	3.01	3.18	3.19	3.19	0.141	0.519	<0.001
Mg (mmol/l)	0.86	0.79	0.90	0.73	0.133	0.582	0.611
Fe (µmol/l)	37.5	35.9	39.7	36.8	2.05	0.294	<0.001
Zn (µmol/l)	12.4^{ab}	13.5^{b}	12.8^{ab}	11.6^{a}	0.59	0.023	<0.001
Cu (µmol/l)	14.5	15.1	14.9	15.0	0.70	0.863	<0.001
Co (µmol/l[2])	0.014 ±0.0022	0.018 ±0.0022	0.015 ±0.0022	0.019 ±0.0022		<0.001	0.045
Se (µmol/l)	1.04	1.03	1.04	1.04	0.031	0.965	<0.001
Mo (µmol/l)	0.25	0.23	0.24	0.25	0.025	0.817	<0.001
Hepatic triacylglycerol, mg/g fresh[3]							
Pre-partum	5.81 ±2.349	5.85 ±2.349	5.56 ±2.349	7.23 ±2.349		0.616	--
Post-partum	16.1 ±2.651	18.4 ±2.651	22.0 ±2.651	19.3 ±2.651		0.862	--

[1]There were no (*P*>0.05) Treatment x Time interactions
[2]3-OHB = ß-hydroxybutyrate, NEFA = non-esterified fatty acids
[3]Data were not normally distributed and were analysed by firstly converting to natural log. The antiln least square means along with the interval of confidence at 95% are presented.
[a,b,c]Means within a row with a different superscript differ (*P* <0.05).

Table 5 *Diet digestibility (kg/kg) of DM, organic matter (OM), nitrogen (N) and fibre in dairy cows offered diets that contained no added cobalt (C), added dietary Co (DC), added dietary vitamin B_{12} (DB) or injected vitamin B_{12} (IB)*

	Treatment					
	C	DC	DB	IB	SED	*P*-value
DM	0.71	0.72	0.73	0.73	0.016	0.630
OM	0.73	0.74	0.74	0.75	0.015	0.631
N	0.66	0.67	0.66	0.69	0.019	0.240
NDF	0.54	0.53	0.58	0.56	0.029	0.257
ADF	0.45	0.45	0.50	0.48	0.031	0.234
Hemicellulose[1]	0.68	0.65	0.73	0.70	0.031	0.136

[1]Calculated as NDF-ADF

Table 6 *Milk fatty acid profile during week 7 of lactation in dairy cows offered diets that contained no added cobalt (C), added dietary Co (DC), added dietary vitamin B_{12} (DB) or injected vitamin B_{12} (IB)*

	C	DC	DB	IB	SED	*P*-value
Fatty acid (g/100g)						
C4:0	4.87	4.78	4.94	4.42	0.322	0.401
C6:0	2.77	2.66	2.62	2.67	0.099	0.356
C8:0	1.56	1.33	1.34	1.60	0.124	0.066
C10:0	3.27	3.01	2.64	3.22	0.170	0.164
C12:0	3.68	3.44	3.32	3.62	0.190	0.242
C14:0	10.7	10.4	10.1	10.6	0.353	0.366
C14:1 *cis*-9	0.87	0.94	0.89	0.90	0.079	0.817
C15:0	0.99[ab]	0.93[a]	0.94[ab]	1.09[b]	0.056	0.025
C16:0	27.5	27.6	27.1	27.9	0.671	0.651
C16:1 *cis*-9	1.42	1.61	1.58	1.50	0.121	0.383
C17:0	0.53	0.55	0.51	0.58	0.028	0.150
C18:0	7.83	7.96	7.57	7.40	0.352	0.393
C18:1 *trans* 6 to 8	0.40	0.41	0.39	0.38	0.047	0.936
C18:1 *trans*-9	0.30	0.36	0.35	0.31	0.057	0.692
C18:1 *trans*-10	0.61	0.54	0.67	0.73	0.104	0.323
C18:1 *trans*-11	0.61	0.66	0.66	0.91	0.116	0.061
C18:1 *trans*-12	0.53	0.60	0.66	0.48	0.091	0.222
C18:1 *cis*-9	18.3	18.8	18.5	17.3	0.926	0.447
C18:2 *cis*-9, cis-12	2.53	2.41	2.40	2.48	0.124	0.686
C18:2 *cis*-9, *trans*-11	0.56	0.62	0.56	0.56	0.069	0.769
C18:2 *trans*-10, *cis*-12	0.03	0.05	0.05	0.05	0.008	0.221
C18:3 *cis*-9, *cis*-12, *cis*-15	0.06	0.10	0.10	0.12	0.036	0.383
C20:0	0.07	0.06	0.06	0.07	0.006	0.801
C20:1 *cis*-9	0.35	0.31	0.30	0.35	0.038	0.428
C20:5 n-3	0.05	0.05	0.06	0.05	0.008	0.647
C22:6 n-3	0.06	0.08	0.08	0.08	0.014	0.259
Other	9.64	9.69	11.37	10.65	1.528	0.625
Summation						
Saturated	64.0	62.9	61.7	63.4	1.37	0.386
Monounsaturated	23.4	24.3	24.0	22.9	1.07	0.579
Polyunsaturated	3.30	3.32	3.30	3.37	0.181	0.984
<16	28.8	27.6	27.2	28.3	0.88	0.284
16:0 and 16:1	29.0	29.4	28.8	29.5	0.70	0.693
>16	32.9	33.6	33.0	31.9	1.33	0.651
Desaturase index						
14:1/(14:0+14:1)	0.07	0.08	0.08	0.08	0.005	0.475
16:1/(16:0+16:1)	0.05	0.06	0.06	0.05	0.004	0.264
18:1/(18:0+18:1)	0.70	0.70	0.71	0.70	0.010	0.861
c-9, t-11 CLA/(18:1 t-11+ c-9, t-11 CLA[1]	0.50[b]	0.51[b]	0.47[ab]	0.40[a]	0.033	0.006

[1]CLA = conjugated linoleic acid.

[a,b]Means within a row with a different superscript differ (P <0.05).

Figure 1 Pre- and post-partum blood concentrations of vitamin B_{12} (a) and secondary metabolites, homocysteine (b), methylmalonic acid (c) and succinate (d). Cows offered diets that contained no added cobalt (Co; C: ■), added dietary Co (DC: ●), added dietary vitamin B_{12} (DB: ○) or injected vitamin B_{12} (IB: ∆). For plasma B_{12}, interaction between Treatment and Time: P=0.007; SED = 22.7. For homocysteine, main effect of Time: P<0.001; SED = 0.265. For methylmalonic acid, interaction between Treatment x Time: P=0.033; SED = 0.031. For succinate, main effect of Time: P<0.001; SED = 0.231.

Supplementary Material S1

Added dietary cobalt or vitamin B_{12}, or injecting vitamin B_{12} does not improve performance or indicators of ketosis in pre- and post-partum Holstein-Friesian dairy cows

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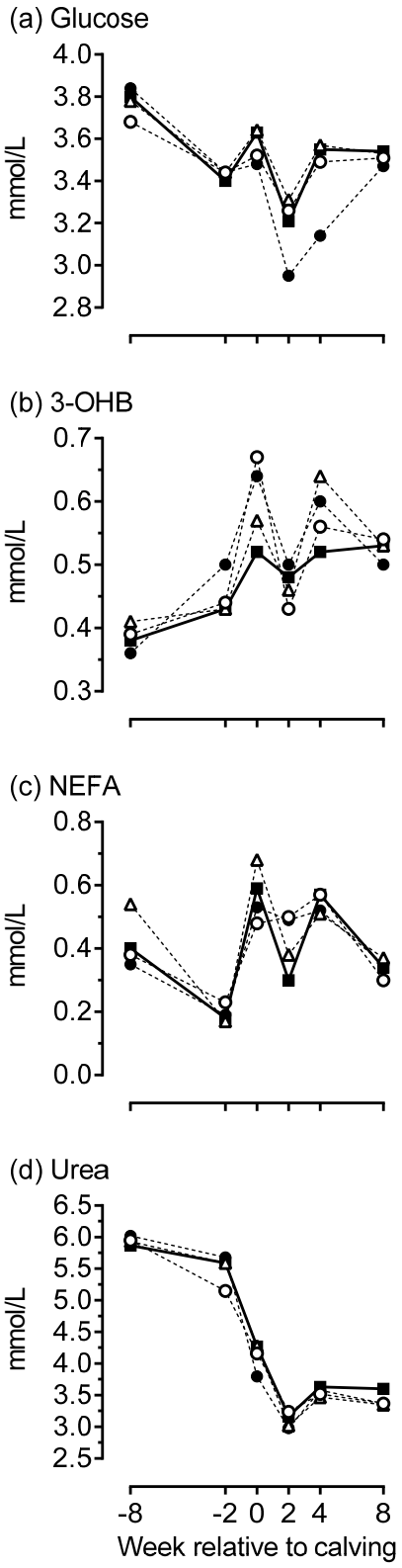
Plasma vitamin B_{12} analysis was conducted using the ADIVA Centaur CP VB12 assay at the AHVLA laboratory, Shrewsbury, Shropshire, UK. The limit of detection was 33 pmol/l, and coefficients of variation (CVs) for low, medium and high quality controls were 12.2, 12.4 and 12.4% respectively. Plasma homocysteine (Hcy) was determined using an Imola Auto analyser (RX imola; Randox Laboratories Ltd., Antrim, U.K.) using a kit supplied by Randox Laboratories (catalogue no. HY4036). The limit of detection was 1.74 µM and CV's for low, medium and high quality controls were 1.03, 0.83 and 0.95% respectively.

Plasma methylmalonic acid (MMA) and succinic acid (SA) concentrations were determined by GC with mass spectroscopic detection (GC-MS) following derivatisation and extraction, modified from the method of Kanakkarambil *et al.* (2009). Briefly, 50 µl of plasma and 5 ul of internal standard (4-chlorobutyric acid (CBA) 250 µM) were added to 250 µl 12% BF_3-methanol in a 2.5 ml screw capped glass vial, vortexed for 30 s and heated at 95°C for 15 minutes on a heating block. After cooling, 250 µl distilled water and 250 µl dichloromethane (CH_2Cl_2) was added to the vial and vortexed

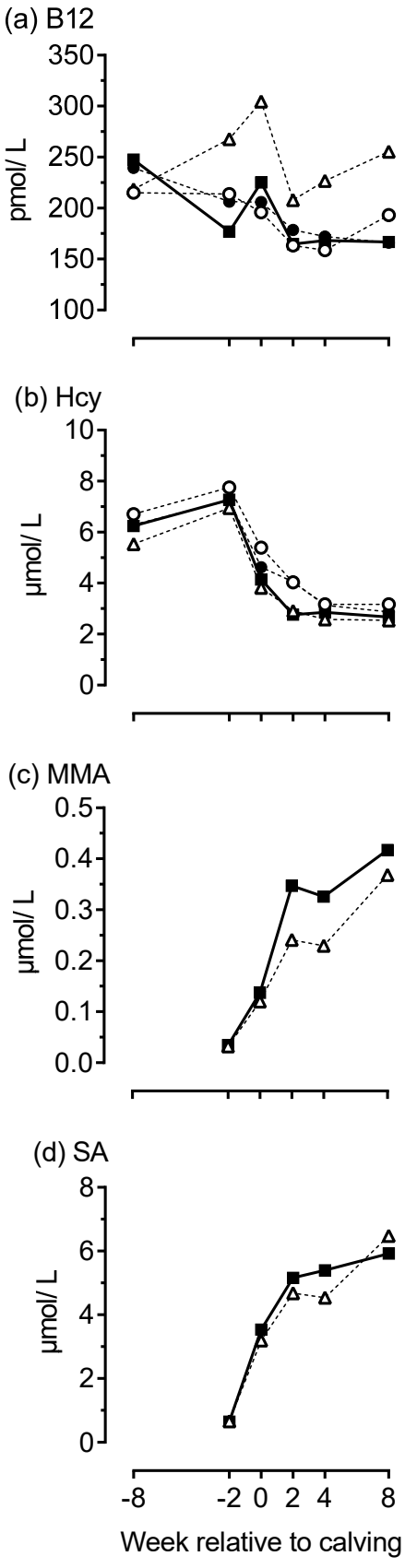
for 30 s. The mixture was then centrifuged for 8 minutes at 2500 g at 4°C to separate the layers. The lower dichloromethane layer was transferred to a screw capped auto-sampler vial with insert for GC-MS analysis. The method used a DB-WAX (crosslinked polyethylene glycol; J&W Scientific Agilent technology) 30 m long column of 0.25mm i.d and 0.15 μm film thickness. The carrier gas was He and flow rate was 1.0 ml/min. Injection mode was splitless and volume was 1 μl for both SCAN mode, for qualification, and SIM mode, for quantification. Injection port temperature was 260°C. The MS selective detector interference temperature was 280°C. The chromatograph was programmed for an initial temperature of 50°C for 2 minutes, increased to 150°C at 8°C per minute, then increased to 220°C at 100°C per minute and held for 5 minutes at the final temperature. The MS was operated in electron impact (EI) ionization mode with the ionization energy of 70eV. SCAN mode measured at m/z: 20-500 and SIM (selected ion-monitoring) ions were set at 105 (for CBA), 115 (for MMA and SA). The same method was used for standards of MMA and SA at concentrations 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10 and 20 μmol/l. Calibration was carried out by comparison of peak areas of CBA with MMA and SA in the standards and the final results expressed in μmol/l plasma. The limit of detection and limit of quantification was 0.156 μmol/l. The CV's for low, medium and high quality controls were 0.44, 1.03, 0.83% for MMA and 1.2, 1.1 and 2.3% for SA respectively.

For hepatic triacylglycerol (TAG) analyses, samples were reduced to powder under liquid N, and known amounts (around 90-120 mg) weighed and homogenised in 1.6 ml of 0.47 M sodium sulphate, followed by the addition of 2 ml of 0.47 M sodium sulphate and 5.4 ml hexane:isopropanol (3:2, v/v). The mixtures were vortexed for 30 s and centrifuged at 2000 g for 5 minutes at room temperature, then 2.5 ml of the top layer transferred to a glass tube and dried under nitrogen. The dried lipid was

reconstituted in 1 ml of hexane and 10 or 60 µl transferred to a second tube and dried under nitrogen. Dried samples were then re-suspended in 120 µl of isopropanol and 50 µl was mixed with 250 µl of colour reagent from Wako LabAssay triglyceride kit (Catalogue No. 290-63701; Alpha Laboratories, Hampshire, UK) for triacylglycerol measurement. Different concentrations of triolein standard (Catalogue No. T7140, Sigma-Aldrich, Gillingham, UK) were processed in parallel with liver samples to generate a standard curve. Different concentrations of glycerol and non-extracted triolein were included in each plate to monitor enzyme activity and completeness of lipolysis by lipoprotein lipase. In addition, a single liver analysed in each plate served as a quality control. The inter-assay CV was 8.4%.



Supplementary Figure 1 Plasma glucose (a), ß-hydroxybutyrate (3-OHB) (b), non esterified fatty acids (NEFA) (c) and urea (d) in pre- and post-partum of dairy cows offered diets that contained no added cobalt (Co) (C: ■), added dietary Co (DC: ●), added dietary B12 (DB: ○) or injected B12 (IB: ∆).


(a) B12
350
300
250
200
150
100
pmol/ L
(b) Hcy
10
8
6
4
2
0
µmol/ L
(c) MMA
0.5
0.4
0.3
0.2
0.1
0.0
µmol/ L
(d) SA
8
6
4
2
0
µmol/ L
-8
-2
0
2
4
8
Week relative to calving