Short communication: Relationships among plasma and milk vitamin B₁₂, plasma free fatty acids, and blood β-hydroxybutyrate concentrations in early lactation dairy cows

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ABSTRACT

This study was undertaken to evaluate the relationship between plasma and milk concentrations of vitamin B₁₂ as well as the relationship between plasma or milk concentrations of vitamin B₁₂ and plasma concentration of free fatty acids (FFA) or blood concentration of β-hydroxybutyrate (BHB) of early lactating Ayrshire (AY) and Holstein (HO) cows. A total of 44 dairy herds (7 AY and 37 HO herds) and 62 AY (21 in first, 19 in second, and 22 in third and more lactations) and 228 HO (51 in first, 74 in second, and 103 in third and more lactation) cows between 3 and 40 d in milk were involved in the study. Hand-stripped milk samples and blood samples were taken 6 h after the morning milking. Milk and plasma samples were analyzed for vitamin B₁₂ concentration and plasma samples were analyzed for FFA concentration. A handheld device was used for blood BHB concentration determination. Thresholds for elevated plasma FFA concentration and hyperketonemia were set at ≥ 0.70 and ≥ 1.2 mmol/L, respectively. Vitamin B₁₂ concentration in milk of AY primiparous cows [2,557 (1,995–3,276) pg/mL] was lower than in milk from HO primiparous cows [3,876 (3,356–4,478) pg/mL], whereas no difference was observed among other parities and breeds. Regardless of breeds, plasma concentration of vitamin B₁₂ of first and second parities was lower than plasma concentration of third and more lactation cows. Milk vitamin B₁₂ concentration was positively correlated with plasma vitamin B₁₂ concentration, but the relationship was stronger for AY (ρ averaging 0.63) than for HO cows (ρ averaging 0.36). For AY and HO breeds, a significant relationship

between milk or plasma vitamin B₁₂ concentrations and plasma FFA concentration (ρ between 0.29 and 0.59) was observed. Moreover, cows with elevated plasma FFA concentration had greater milk and plasma vitamin B₁₂ concentrations than cows with normal plasma FFA concentration. No relationship between vitamin B₁₂ concentration in milk or plasma and blood BHB concentration and hyperketonemia was noted. In summary, milk is not well correlated with plasma vitamin B₁₂ concentration for HO. It could be hypothesized that elevated plasma concentration of FFA could have a negative effect on the use of vitamin B₁₂ by cow cells, which increases the concentration of the vitamin in plasma and its secretion in milk.

Key words: dairy cow, vitamin B₁₂, milk, free fatty acid, β-hydroxybutyrate

Short Communication

In ruminants, vitamin B₁₂ acts as a coenzyme for methylmalonyl-CoA mutase (Enzyme Commission number = 5.4.99.2), which transforms methylmalonyl-CoA into succinyl-CoA (Scott, 1999), a step allowing propionate to enter the Krebs cycle. In dairy cows, propionate is the precursor for up to 60% of glucose released from the liver (Larsen and Kristensen, 2013; Duplessis et al., 2017a). In early lactation, when energy supply is lower than energy demand for milk production, dairy cows enter into a state of negative energy balance. They adapt by mobilizing body fat reserve (Goff and Horst, 1997), increasing plasma concentration of free fatty acids (FFA). When FFA oxidation by the Krebs cycle is incomplete, plasma concentration of ketone bodies such as BHB will increase as a consequence (Goff and Horst, 1997). Several studies reported that a combined supplement of vitamin B₁₂ and folic acid altered energy partitioning in early lactation (Graulet et al., 2007; Preynat et al., 2009; Duplessis et al., 2017b) either by increasing milk production without affecting

Received January 22, 2018.

Accepted May 14, 2018.

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plasma FFA concentration or by decreasing plasma FFA concentration with no effect on milk production. Nevertheless, under the current state of knowledge, it is not known if a relationship exists between plasma or milk vitamin B₁₂ concentrations and plasma FFA or blood BHB concentrations of cows without vitamin supplementation. However, older studies reported lower plasma concentration of vitamin B₁₂ for cows with elevated concentration of ketones in urine and blood (Corse and Elliot, 1970; Korpela and Mykkänen, 1983).

Vitamin B₁₂ concentrations in plasma and milk have been shown to vary greatly among dairy cows and among studies (Graulet et al., 2007; Preynat et al., 2009; Duplessis et al., 2016, 2017a). However, to our knowledge, the relationship between vitamin B₁₂ concentrations in milk and plasma has never been established in samples collected simultaneously from cows receiving no vitamin B₁₂ supplement. Thus, the first objective of this study was to evaluate if vitamin B₁₂ concentration in milk could be correlated with vitamin B₁₂ concentration in plasma of Ayrshire (AY) and Holstein (HO) cows in early lactation under commercial conditions. Another aim was to determine if a relationship existed between plasma or milk concentration of vitamin B₁₂ and plasma concentration of FFA or blood concentration of BHB. Additionally, the experiment determined plasma and milk concentrations of vitamin B₁₂ across 2 breeds, AY and HO. Vitamin B₁₂ concentration in AY milk and plasma is not well documented.

All procedures of this experiment were approved by the Animal Care Committee from McGill University, Sainte-Anne-de-Bellevue, QC, Canada, following the guidelines of the Canadian Council on Animal Care (2009). A total of 44 dairy herds (7 AY and 37 HO herds) and 62 AY (21 in first, 19 in second, and 22 in third and more lactations) and 228 HO (51 in first, 74 in second, and 103 in third and more lactations) cows between 3 and 40 DIM were involved in the study. All cows were milked twice daily and housed in tie-stall barns, except for one herd that housed cows in a freestall barn. To participate, herds had to record milk production through the DHIA and to have AY or HO cows as main breed. Producers were contacted by phone and participation was on a voluntary basis.

Herds were visited once between May and August 2016. Estimated BW was taken by measuring heart girth circumference using a calibrated tape (Yan et al., 2009). A milk sample with bronopol was taken during the morning milking using an in-line milk meter to obtain its composition and milk yield was recorded. Hand-stripped milk samples were collected 6 h after milking, transported on ice, and stored at -20°C until analysis of vitamin B₁₂ concentration. Blood samples from the tail vein were immediately taken after hand-

stripped milk sample collection using both EDTA as anticoagulant and serum separator Vacutainer tubes (Becton Dickinson and Company, Franklin Lakes, NJ). A handheld device (FreeStyle Precision Neo, Abbott Diabetes Care Inc., Mississauga, ON, Canada) was used to determine blood BHB concentration immediately after blood collection with tubes without anticoagulant. Blood samples taken with EDTA tubes were put on ice for transportation and were centrifuged within 2 h after collection for 12 min at $3,000 \times g$ at 4°C. Plasma samples were then frozen at -20°C until analysis.

Milk composition (fat, protein, and lactose) was analyzed using mid-infrared reflectance spectrometry (MilkoScan FT 6000, Foss, Hillerød, Denmark) by Valacta (Dairy Production Center of Expertise, Québec and Atlantic Provinces, Sainte-Anne-de-Bellevue, QC, Canada). A commercial kit was used for analysis of plasma concentration of FFA [HR Series NEFA-HR(2), Wako Chemicals USA Inc., Richmond, VA] in duplicate. Plasma and milk concentrations of vitamin B₁₂ were analyzed in duplicate by radioassay as previously described by Duplessis et al. (2015) using a commercial kit (SimulTRAC B₁₂/Folate-S, MP Biomedicals, Santa Ana, CA). The interassay CV were 4.3 and 2.7% for plasma and milk vitamin B₁₂ concentration analyses, respectively.

For analysis purpose, cows were divided according to parity as follows: (1) first, (2) second, and (3) third parity and greater, and DIM were divided into 4 categories: (1) below 10 DIM, (2) between 11 and 20 DIM, (3) between 21 and 30 DIM, and (4) between 31 and 40 DIM. Proc MIXED of SAS (version 9.4, SAS Institute Inc., Cary, NC) was used to analyze estimated BW, morning milk yield and components, plasma and milk vitamin B₁₂ concentrations, plasma FFA concentration, and blood BHB concentration according to parity, breed, DIM, as well as parity $\times$ breed interaction as fixed effects. Residuals were studied for normality. Plasma and milk vitamin B₁₂ concentrations, plasma FFA concentration, and blood BHB concentration violated this assumption, and data were then log-transformed. Least squares means and 95% confidence interval results from log-transformed dependent variable models were back-transformed and presented afterward as geometric means and 95% confidence interval. Results from untransformed data were reported as least squares means and 95% confidence interval. The threshold for elevated plasma concentration of FFA was set at 0.70 mmol/L as previously determined by Ospina et al. (2013). Hyperketonemia (HYK) was defined as plasma BHB concentration ≥ 1.2 mmol/L (Macmillan et al., 2017). Proc MIXED of SAS was used to evaluate vitamin B₁₂ concentrations in plasma and milk according to the threshold of elevated plasma concentrations of FFA or

HYK, DIM, as well as their interaction as fixed effects. Dependent variables were log-transformed and results were handled as described above. Herd was considered as random effect in both models described above. Indeed, the Bayesian information criterion (**BIC**) of models with herd as a random effect was smaller than the BIC of models without herd as a random effect (BIC differences between 3 and 29). A Tukey's honest significant difference test was performed when parity results reached significance. Moreover, the SLICE option of the LSMEANS statement was used when an interaction was significant to help interpretation. Proc CORR of SAS was used to calculate Spearman correlation coefficients by parity between vitamin B₁₂ concentration in plasma and milk, between vitamin B₁₂ concentration in milk and morning milk yield or components, and between vitamin B₁₂ concentration in plasma or milk and plasma FFA or blood BHB concentrations, separately for AY and HO. Significance was considered when *P*-value reached 0.05 or less.

Morning milk yield and milk components as well as milk and plasma concentrations of vitamin B₁₂, plasma FFA, and blood BHB concentrations according to breed and parity are presented in Table 1. Ayrshire cows were lighter and produced less milk than HO cows ($P \leq 0.02$, Table 1). Body weight as well as milk yield increased with parity as reported by Valacta (2017).

In the current experiment, vitamin B₁₂ concentration in milk varied greatly among individuals as frequently reported (Rutten et al., 2013; Duplessis et al., 2016). Indeed, it ranged from 645 to 7,666, 1,258 to 8,363, and 1,042 to 16,026 pg/mL for first, second, and third and more parity AY cows, respectively, whereas it varied from 830 to 12,724, 658 to 19,801, and 1,184 to 17,646 pg/mL for first, second, and third and more lactation HO cows, respectively. The greater range in the present study than previously reported could be due to the fact that some cows were sampled as soon as at 3 DIM and Duplessis et al. (2015) showed that vitamin B₁₂ concentration in colostrum could be greater by about 7-fold compared with vitamin B₁₂ concentration in milk at 11 DIM. It is noteworthy that out of 12 cows having a milk concentration of vitamin B₁₂ greater than 10,000 pg/mL, 11 cows were less than 10 DIM. Regardless of breed, vitamin B₁₂ concentration in milk decreased from 9,837 at 3 DIM to 2,746 pg/mL at 40 DIM. Milk of AY primiparous cows had a lower vitamin B₁₂ concentration than milk of HO primiparous cows ($P = 0.004$), whereas no difference was noted for other parities and breeds ($P \geq 0.76$; breed $\times$ parity interaction, $P = 0.007$). To our knowledge, this study is the first one reporting vitamin B₁₂ concentration in milk of several AY cows. Indeed, Collins et al. (1953) reported no difference between milk vitamin B₁₂ concentration of 2 AY

and 12 HO cows. Difference in vitamin B₁₂ concentration in milk between other breeds has already been noted; Miller et al. (1966) and Duplessis et al. (2016) observed that milk of HO cows had greater vitamin B₁₂ concentration than milk of Jersey cows. Variability among individuals was also observed regarding plasma vitamin B₁₂ concentration; it ranged from 78 to 299, 87 to 373, and 102 to 454 pg/mL for first, second, and third and more lactation AY cows, respectively, and from 104 to 714, 90 to 875, and 129 to 960 pg/mL for first, second, and third and more parity HO cows, respectively. As reported by Girard and Matte (1999), plasma concentration of vitamin B₁₂ was higher below 10 DIM than between 11 and 40 DIM ($P < 0.0001$). Plasma vitamin B₁₂ concentration of HO cows was higher than AY cows among all parities ($P = 0.0002$). Regardless of breed, no significant difference was noted between first and second lactation ($P = 0.07$), but plasma vitamin B₁₂ concentration of third and more lactations was greater by 45 and 22% than plasma concentration of first and second lactations, respectively ($P \leq 0.01$). Girard and Matte (1999) reported a difference in plasma concentration of vitamin B₁₂ between first and second and more lactations. Plasma concentrations of FFA and BHB differed according to parity ($P \leq 0.01$; Table 1).

The relationship between vitamin B₁₂ concentration in milk and morning milk yield and components varied between breeds and parities (Table 2). Results suggest that vitamin B₁₂ concentration in milk was negatively correlated with morning milk yield and milk lactose concentration whereas a positive relationship was observed between vitamin B₁₂ concentration in milk and milk protein concentration for HO cows regardless of parity ($P \leq 0.07$). A positive relationship between vitamin B₁₂ concentration in milk and milk fat concentration was also noted for second and more parity HO cows ($P \leq 0.002$) as previously observed by Miller et al. (1966). However, Spearman correlation coefficients of the current study indicated weak to moderate relationships (ρ between -0.21 and 0.51). For AY and HO cows, milk concentration of vitamin B₁₂ was negatively associated with DIM across parities (ρ between -0.59 and -0.30). These results are in line with the fact that vitamin B₁₂ concentration in milk decreased from 3 to 40 DIM in the current study. Regarding plasma vitamin B₁₂ concentration, there was a negative relationship with DIM for AY first and AY and HO third and greater lactation cows.

Milk concentration of vitamin B₁₂ was positively correlated with plasma concentration of vitamin B₁₂, especially for AY cows in which the Spearman correlation coefficient averaged 0.63 (Table 2). Regarding HO cows across parities, the Spearman correlation coefficient was lower than for AY cows ($\rho \leq 0.42$). Several

Table 1. Milk yield and components from morning milking, milk and plasma vitamin B₁₂, plasma free fatty acids, and blood BHB concentrations according to breed and parity¹

Item	Ayrshire			Holstein			P-value
	First	Second	Third and more	First	Second	Third and more	
Cows (n)	21	19	22	51	74	103	
BW ^{3,4} (kg)	581 (550–612) ^c	603 (571–635) ^b	639 (609–669) ^a	644 (626–663) ^c	693 (677–709) ^b	746 (732–760) ^a	0.11
Morning milk ⁴ yield (kg)	12.8 (10.4–15.1) ^b	17.5 (15.2–19.9) ^a	17.4 (15.2–19.7) ^a	14.6 (13.3–15.9) ^b	20.1 (19.0–21.3) ^a	20.3 (19.2–21.3) ^a	0.76
Milk component (%)							
Fat ^{4,5}	3.96 (3.30–4.75)	4.18 (3.47–5.04)	4.27 (3.57–5.12)	3.99 (3.59–4.43)	3.71 (3.39–4.07)	3.95 (3.63–4.30)	0.51
Protein ⁴	3.19 (2.99–3.38)	3.29 (3.08–3.49)	3.21 (3.01–3.40)	3.12 (3.00–3.25)	3.13 (3.03–3.23)	3.26 (3.17–3.35)	0.35
Lactose ⁴	4.67 (4.53–4.81) ^a	4.53 (4.39–4.68) ^{ab}	4.55 (4.41–4.69) ^b	4.61 (4.53–4.70) ^a	4.59 (4.52–4.66) ^{ab}	4.48 (4.41–4.54) ^b	0.32
Vitamin B ₁₂ (pg/mL)							
Milk ^{4,5}	2,557 (1,995–3,276) ^b	3,925 (3,042–5,065) ^a	4,689 (3,680–5,974) ^a	3,876 (3,356–4,478) ^b	3,993 (3,526–4,523) ^a	4,504 (4,016–5,051) ^a	0.007
Plasma ^{4,5}	126 (102–157) ^b	156 (124–195) ^b	182 (147–225) ^a	185 (163–211) ^b	210 (188–234) ^b	269 (244–297) ^a	0.78
Free fatty acids ^{4,5} (mmol/L)	0.26 (0.20–0.35) ^{ab}	0.21 (0.15–0.28) ^b	0.34 (0.26–0.45) ^a	0.28 (0.23–0.33) ^{ab}	0.24 (0.20–0.27) ^b	0.26 (0.23–0.30) ^a	0.11
BHB ^{4,5} (mmol/L)	0.86 (0.64–1.16) ^b	1.05 (0.77–1.44) ^{ab}	1.37 (1.03–1.84) ^a	0.86 (0.72–1.02) ^b	0.92 (0.79–1.07) ^{ab}	1.07 (0.94–1.23) ^a	0.46

^{a-c}Means in the same row with different superscripts differ regarding parity effect; $P \leq 0.05$.

¹Results are reported as LSM or geometric mean when indicated and 95% CI.

²Breed × parity interaction.

³Estimated using a tape measuring heart girth circumference (Yan et al., 2009).

⁴DIM category effect, $P \leq 0.01$.

⁵Geometric mean and 95% CI for log-transformed data computed as e^x .

Table 2. Spearman rank correlation coefficients (*P*-value in parentheses) between milk and plasma concentrations of vitamin B₁₂ (pg/mL), DIM, plasma free fatty acid and blood BHB concentrations (mmol/L), and milk yield (kg) and composition (%) according to breed and parity

Item	Ayrshire			Holstein		
	First	Second	Third and more	First	Second	Third and more
Milk vitamin B ₁₂ concentration						
Morning milk yield	-0.09 (0.68)	-0.10 (0.68)	-0.43 (0.05)	-0.32 (0.02)	-0.21 (0.07)	-0.39 (<0.0001)
Milk component ¹						
Fat	0.48 (0.03)	0.13 (0.60)	0.49 (0.02)	0.11 (0.45)	0.39 (0.0006)	0.37 (0.0002)
Protein	0.16 (0.49)	0.36 (0.12)	0.45 (0.04)	0.33 (0.02)	0.34 (0.003)	0.51 (<0.0001)
Lactose	-0.33 (0.14)	-0.09 (0.73)	-0.28 (0.22)	-0.35 (0.02)	-0.21 (0.07)	-0.33 (0.0009)
DIM ²	-0.47 (0.03)	-0.45 (0.05)	-0.58 (0.005)	-0.30 (0.04)	-0.44 (<0.0001)	-0.59 (<0.0001)
Plasma vitamin B ₁₂	0.66 (0.001)	0.62 (0.005)	0.61 (0.003)	0.42 (0.003)	0.40 (0.0005)	0.27 (0.006)
Free fatty acids	0.24 (0.31)	0.59 (0.008)	0.52 (0.01)	0.01 (0.97)	0.32 (0.005)	0.29 (0.003)
BHB	-0.02 (0.94)	-0.10 (0.67)	-0.11 (0.62)	-0.07 (0.64)	-0.009 (0.94)	-0.09 (0.36)
Plasma vitamin B ₁₂ concentration						
DIM ²	-0.59 (0.005)	-0.14 (0.57)	-0.46 (0.03)	-0.19 (0.18)	-0.14 (0.25)	-0.19 (0.05)
Free fatty acids	0.14 (0.53)	0.20 (0.42)	0.53 (0.01)	0.36 (0.009)	0.36 (0.002)	0.15 (0.13)
BHB	-0.32 (0.16)	-0.39 (0.10)	0.14 (0.52)	0.26 (0.07)	0.19 (0.10)	-0.07 (0.48)

¹Milk components from morning milking only.²DIM on a continuous scale.

factors could explain this coefficient difference between breeds, among them, genetics. Genotype of dairy cows affects milk concentration of vitamin B₁₂ (Rutten et al., 2013; Duplessis et al., 2016). It is possible that the AY population was less heterogeneous due to the smaller number of available sires in AY compared with HO cows (CIAQ, 2018); however, sire records are not available in the current study. Nevertheless, as studied herds had only one breed, effects of management factors, such as nutritional strategies, cannot be ruled out. To our knowledge, this experiment is the first one reporting the link between vitamin B₁₂ concentration in milk and plasma samples taken simultaneously. These results highlight that vitamin B₁₂ concentration in milk is not well correlated with plasma concentration of vitamin B₁₂, especially for HO cows as suggested by the small correlation coefficients (Table 2 and Figure 1). Plasma concentration of vitamin B₁₂ is the difference between what has been absorbed at the ileum and not yet taken up by cow tissues, including mammary cells, whereas the vitamin in milk represents the amount taken by mammary cells and secreted between 2 milkings. Uptake of the vitamin by the mammary gland has been reported to be dependent on the vitamin concentration in plasma but to be greater than the amount secreted in milk (Peeters et al., 1985).

Except for primiparous cows, vitamin B₁₂ concentration in milk was positively correlated with plasma FFA concentration and the relationship was stronger for AY cows ($P \leq 0.01$; Table 2 and Figure 2). Significant relationships between plasma concentrations of vitamin B₁₂ and FFA of third and more parity AY cows and first and second lactation HO cows were also observed

Figure 1. Relationship between milk and plasma concentrations of vitamin B₁₂ of Ayrshire (a; Spearman rank correlation coefficients of 0.66, 0.62, and 0.61 for first, second, and third and more lactations, respectively) and Holstein (b; Spearman rank correlation coefficients of 0.42, 0.40, and 0.27 for first, second, and third and more lactations, respectively) cows among parities.

($P \leq 0.01$). Moreover, cows with elevated plasma FFA concentration (≥ 0.70 mmol/L) had a greater milk vitamin B₁₂ concentration than cows with normal plasma FFA concentration [6,283 (95% CI: 4,877–8,095) and 3,991 (95% CI: 3,647–4,367) pg/mL for cows having elevated (AY, $n = 3$ and HO, $n = 16$ between 3 and 36 DIM) and normal plasma FFA concentrations, respectively; $P = 0.0004$], throughout DIM categories (plasma FFA concentration level $\times$ DIM interaction, $P = 0.37$). Similar results were obtained regarding plasma concentration of vitamin B₁₂ [295 (95% CI: 230–377) and 208 (95% CI: 192–226) pg/mL for cows with elevated and normal plasma FFA concentrations, respectively; $P = 0.006$], throughout DIM categories (plasma FFA concentration level $\times$ DIM interaction, $P = 0.19$). Excessive FFA often leads to the development of liver steatosis, which impairs metabolism of hepatic cells (Bobe et al., 2004). Obitz and Füll (2014) concluded that, in early lactation dairy cows, plasma concentration of vitamin B₁₂ may be an indicator for evaluating energy and fat metabolism. It could then be hypothesized that elevated plasma FFA concentration had a negative effect on vitamin B₁₂ use by cow cells, especially hepatocytes, and could explain why the vitamin concentration was higher in milk and plasma. More cows with elevated plasma FFA concentrations are needed to confirm those current results, but it could indicate that early lactation cows with elevated plasma FFA are more at risk to lack vitamin B₁₂ for their metabolic functions. No significant relationship was noted between milk or plasma concentration of vitamin B₁₂ and blood concentration of BHB ($P \geq 0.36$). Moreover, milk and plasma concentrations of vitamin B₁₂ were not different between HYK (≥ 1.2 mmol/L; AY, $n = 26$ and HO, $n = 68$) and non-HYK ($P \geq 0.50$). Future studies are needed to evaluate if vitamin B₁₂ concentration in milk could be analyzed with a fast and reliable method and if vitamin B₁₂ concentration in milk could be used as an indicator of elevated plasma concentration of FFA as similarly suggested by Obitz and Füll (2014) with serum concentration of vitamin B₁₂.

In summary, milk and plasma concentrations of vitamin B₁₂ differed between AY and HO cows and parities. Weakly positive relationships were observed between milk and plasma concentrations of vitamin B₁₂ of HO cows, suggesting that milk is not well correlated with vitamin B₁₂ concentration in plasma for this breed. Although cows with elevated plasma FFA concentration represented only 6.6% of the studied population, their vitamin B₁₂ concentrations in milk and plasma were higher than the concentrations of cows with normal plasma FFA concentration. This experiment involved cows in early lactation; therefore, these results need to be confirmed with more cows varying in lactation stage

and plasma FFA concentration using training and test data sets.

ACKNOWLEDGMENTS

Realization of this project would not have been possible without the invaluable participation of the dairy producers. We acknowledge the help of Camille Bergeron (McGill University, Sainte-Anne-de-Bellevue, QC, Canada), Sabrina Plante, Yolaine Lebeuf, Doris Pellerin (Université Laval, Québec, QC, Canada), Jasmijn Brochu (Agriculture et Agroalimentaire Canada, Sherbrooke, QC, Canada), and Valacta (Sainte-Anne-de-Bellevue, QC, Canada). We are grateful to Clinique vétérinaire Etchemin (Saint-Anselme, QC, Canada) and Hôpital vétérinaire Claudia Forget (Varennes, QC, Canada) for allowing us using their centrifuge. This project has been made possible through the support from Programme Innov'Action agroalimentaire from Cultivons l'avenir 2 (project IA115301) concluded be-

Figure 2. Relationship between plasma concentration of free fatty acids and milk concentrations of vitamin B₁₂ of Ayrshire (a) and Holstein (b) cows among DIM. Primiparous cows were deleted from this figure because the Spearman rank correlation coefficients between plasma concentration of free fatty acids and milk concentrations of vitamin B₁₂ were not significant for these cows ($P \geq 0.31$).

tween Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec and Agriculture et Agroalimentaire Canada (Ottawa, ON, Canada).

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