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# Mineral status, metabolism and performance of dairy heifers receiving a combined trace element bolus and out-wintered on perennial ryegrass, kale or fodder beet.

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ABSTRACT

The effects of a cobalt (Co), copper (Cu), selenium (Se), and iodine (I) trace-mineral ruminal bolus on the mineral status and performance of out-wintered, pregnant dairy heifers was investigated. Nine commercial farms grazing pasture (G), kale (K), or fodder beet (F) were used (n=3 per forage), with forty heifers on each farm randomly allocated to not receive (B-) or receive (B+) two combined mineral boluses. Mean plasma Co concentrations were 0.021 and 0.041 μmol/L in B- and B+ respectively ($p < 0.001$), with serum vitamin $B_{12}$ also higher in heifers receiving B+ than B- ($p < 0.001$). Mean plasma Se concentration was 0.50 and 0.82 μmol/L in B- and B+ respectively, with heifers that received B+ also having a higher ($p < 0.05$) mean blood GSH-Px concentration (30 and 76 U/mL haematocrit in B- and B+ respectively). Providing a mineral bolus did not affect plasma Cu concentration in heifers receiving G or F ($p < 0.05$), but was higher in KB+ compared to KB- ($p < 0.05$) at the middle and end of the out-wintering period. Heifers receiving KB- also had a lower haemoglobin and red blood cell count, but a higher mean corpuscular volume than KB+ at the end of the out-wintering period. Animals receiving B- had a higher plasma thyroxine concentration ($p < 0.05$). Neither the bolus nor forage type affected body weight ($p > 0.05$), however condition score was higher ($p < 0.05$) in B+ at the end of the study. It is concluded that the provision of a trace mineral bolus increased plasma concentrations of the minerals supplied, with the greatest benefits in animals grazing kale, but these increases were not translated into improved performance.

*Keywords:* brassica, dairy heifer, forages, wintering, minerals, vitamin $B_{12}$

## 1. Introduction

Rearing heifers outside during the winter period is of interest to dairy farmers as one of the largest expenses of milk production is the cost of replacement animals (Boulton et al., 2015a), which increases substantially with the number of days that heifers are housed (Boulton et al., 2015b). Seasonal, spring-calving herds have amongst the lowest heifer rearing costs in temperate regions such as the UK (Boulton et al., 2015b), and commonly out-winter their replacements (Atkins et al., 2014). In the case of heifers older than one year, a typical out-wintering system would graze kale (*Brassica oleracea*), fodder beet (*Beta vulgaris*), or autumn-saved perennial ryegrass pasture (deferred grazing) *in situ*, with approximately one-third of the diet comprised of baled grass silage (Atkins et al., 2014).

Out-wintering systems for replacement dairy heifers present particular trace-mineral nutrition challenges which may impact animal health and productivity. For instance, kale has been reported to be deficient in copper (Cu), cobalt (Co) and iodine (I) (Grace et al., 2010). Brassicas' also contain glucosinolates, which hydrolyse in the rumen to produce goitrogens, interfering with I absorption and inhibiting thyroxine synthesis (Barry et al., 1981). Kale is also high in sulphur (S), a Cu metabolism antagonist (Suttle, 2010), with much of the S in kale contained in the anti-nutritional factor S-methylcysteine sulphoxide, which causes damage to red blood cells and can lead to haemolytic anaemia (Barry, 2013). In addition, soil particles adhere to pasture during winter and may typically contribute around 10% of dry matter intake (DMI), potentially inhibiting mineral and element availability (Suttle et al., 1975), an effect that may be greater with fodder beet grazed *in situ*. Fodder beet is also low in trace minerals such as Co and selenium (Se; Atkins et al., 2018), although there is comparatively little published data regarding the trace-mineral content of this forage. In addition, cold conditions can increase thyroid activity (Tucker et al., 2007), and out-wintered animals may benefit from additional I.

Achieving adequate live weight gain in heifers is generally considered an important factor affecting both current and lifetime performance (Hoffman, 1997; Le Cozler et al., 2011; Roche et al., 2015). Sub-optimal trace-mineral nutrition during the heifer rearing period can impact on subsequent productivity with, for example, low dietary concentration of Co or Cu restricting average daily gain (ADG) in growing cattle (Mills et al., 1976; Schwarz et al., 2000), and Cu, Co, I and Se supply are all important for immunity and fertility (Corah, 1996; Panousis et al., 2001; Stabel et al., 1993). Recent surveys have indicated that in housed, winter-fed dairy cows, minerals are generally supplied well in excess of requirements (Sinclair and Atkins, 2015). In contrast, grazed animals present less control over mineral nutrition (McDowell, 1996). Offering free-choice mineral licks can result in in variable mineral intake between animals (Valk and Kogut, 1998), whilst administering a reticulorumen trace-mineral bolus offers the opportunity to deliver a consistent dose of selected trace-minerals throughout the grazing period (Kendall et al., 2001). Despite mineral bolus use being common commercial practice on many UK dairy farms (Sinclair and Atkins, 2015), the benefits of trace-mineral supplementation on the mineral status, metabolism and performance in lower-input, out-wintered heifer rearing systems are unclear. The aim of the current study was to investigate the effects of a trace-mineral bolus on blood mineral status and performance in replacement heifers out-wintered on pasture, kale or fodder beet, in commercial, spring-calving pasture based dairy farms.

## 2. Material and methods

### *2.1. Animals, treatments and management*

Nine spring-calving, pasture-based dairy farms in the UK that were due to out-winter heifers in 2012/2013 were used. The farms were selected to be representative of out-wintering farms based on a survey of commercial practice (Atkins et al., 2014). Three of the farms grazed kale (K), three fodder beet (F), and three pastures which were composed predominately of perennial ryegrass (G). The farms were located in the counties of Shropshire, Staffordshire and Hampshire for K; Dumfries and Shropshire (x2) for F; and Derbyshire, Shropshire and Somerset for G. The heifers were all Friesian/Jersey crosses, due to calve at 24 months of age from February 2013, and were destined for a grazed grass production system. A sub-set of 40 primiparous heifers were randomly selected on each study farm, resulting in a total of 360 heifers recruited onto the study. Within each farm, the 40 heifers were paired according to body weight (BW) and body condition score (BCS; Mulvany, 1977) and randomly allocated to one of two treatments; un-supplemented (B-) or supplemented with trace-mineral boluses (B+). The B+ heifers received two reticulorumen trace-mineral boluses (CoSeICure, Telsol Ltd, Leeds, UK) according to the manufacturer's recommendations at the start of the study. Each bolus contained Cu (13.4 g); Co (0.5 g); Se (0.15 g, as sodium selenite) and I (1.0 g, as calcium iodate). No other mineral supplementation was available during the study period. The heifers on each farm were kept together throughout the winter and were managed within larger groups that included non-trial heifers and received supplementary forage in the form of big bale perennial ryegrass silage.

*2.2. Experimental routine and measurements*

The farms were visited on three occasions during the out-wintering period; start (late October to early November 2012), middle (mid-December 2012), and at the end of the out-wintering period (late January to early February 2013). The initial visit coincided with the beginning of the out-wintering regime on each farm (± one week), and the final visit with the end of the out-wintering feeding regime, prior to the onset of calving. On each visit, samples of pasture or forage crop and supplementary forage were collected and stored at -20°C prior to subsequent analysis. Pasture samples were collected from 10 random positions in the subsequent weeks grazing area at a height of approximately 4 cm. Kale samples were collected by cutting 10 random plants from the subsequent weeks grazing area at approximately 5-10 cm above ground level, just above the woody base. The plants were then chopped into approximately 3 to 5 cm pieces, mixed, quartered, and mixed again to obtain a 1 kg sub-sample. Fodder beet samples were collected by pulling 10 random plants from the subsequent weeks grazing area. The leaves of each plant were separated from the bulb and the weight of leaf and bulb recorded. Leaves were then chopped and mixed, quartered and a sub sample collected. Loose soil was washed from the bulb which was then cut into approximately 2 cm cubes, mixed, quartered, and a 1 kg sub-sample obtained. Samples of big bale silage were collected from the bales being fed on the day of the visit. Crop and pasture yield, pre and post grazing, were assessed on each occasion by quadrat cut (10 x 1 $m^2$ for K and F, and 10 x 0.1 $m^2$ for G), as described by Atkins et al. (2018), and the area grazed, number of heifers, and silage fed to calculate the proportion of crop and silage in the diet of each farm.

Beginning at approximately 1000 h on each visit body weight (BW) of the heifers was recorded using electronic weigh-cells (Trutest, Auckland, New Zealand), and body condition score (BCS) recorded. On visits 1 and 3, a hair length sample was collected as described by Boyle et al. (2008). On each of the 3 visits blood samples were collected from 12 pairs of study heifers via the coccygeal vein into tubes (Becton Dickinson Vacutainer Systems, Plymouth, UK) containing $K_2$EDTA, lithium heparin or without an anti-coagulant, and immediately stored on ice until centrifuged at 1300 g and 4°C for 10 minutes. Plasma and serum were decanted and stored at -20°C until subsequent analysis. In addition, a sub-sample of whole blood was stored at -20°C prior to subsequent analysis. The farms recorded calving date and calving-ease score as: 1. No assistance/ calved unaided, 2. Farmer assistance – normal presentation, 3. Farmer assistance – mal presentation, 4. Vet assistance.

*2.3. Chemical analysis*

Forage samples were analysed for dry matter (DM) according to AOAC (2012; 934.01) prior to milling through a 1 mm screen (Cyclone Mill, Retsch, Haan, Germany). Dried, milled samples of fodder beet leaf and bulb were then bulked in proportion to the measured amount to create a representative sample of the whole crop. Crude protein (CP) concentration of the forages was determined by combustion using a LECO FP 528 N analyser (Leco Corporation, St. Joseph, MI) according to AOAC (2012; 990.03), neutral detergent fibre (NDF) content was determined according to Van Soest et al. (1991), and water-soluble carbohydrate (WSC) content as described by Thomas (1977). In addition, forage samples were digested using the DigiPREP digestion system (Qmx Laboratories, Essex, UK), for analysis by inductively coupled plasma mass spectrometry (ICP-MS; Thermo Fisher Scientific Inc., Hemel Hempstead, UK), as described by Sinclair and Atkins (2015). Briefly, 0·5 g duplicates of dried, milled sample were accurately weighed into a DigiTUBE and 1 mL of concentrated HCl and 6 mL of concentrated $HNO_3$ added. The tubes were then heated over 30 min to 45 °C in a DigiPREP heating block and held for 1 min before being increased over 25 min to 65 °C, held for another 5 min, then increased over 15 min to 100 °C and refluxed for 40 min. The digested samples were then diluted to 50 mL with purified water. On the same day as analysis, a reagent blank of 2% $HNO_3$, 1% methanol and 0.1% Triton X (Sigma-Aldrich Ltd., Gillingham, UK) was prepared using purified water, with Gallium (Ga) as an internal standard. Calibration standards were prepared within an expected range of the analytes for content of calcium (Ca), magnesium (Mg), phosphorous (P), potassium, sodium (Na), S , Co , Se, Cu, iron (Fe), zinc (Zn), manganese (Mn), and molybdenum (Mo). A 2.5 mL aliquot of digested sample was diluted with 2.5

mL of reagent blank before analysis by ICP-MS following the instrument passing a performance report program. Certified EU reference samples of hay (BCR-129) and dairy concentrate (BCR-708) were routinely extracted and analysed, and limit of detection and limit of quantification calculated from the blank (Table 1). Forage I content was determined by alkali digestion followed by ICP-MS based on Fecher et al. (1998), and conducted at Sciantec Analytical, Yorkshire, UK. Estimated chemical composition of the total diet was calculated from the proportion of forage and big bale silage on each farm.

Blood plasma samples were analysed for Co, Se, Cu, Zn, Fe and Mn by ICP-MS as described by Cope et al. (2009). Briefly, 1 mL of lithium heparin plasma was pipetted into auto-sampler tubes and diluted with 4 mL of reagent blank consisting of 1% $HNO_3$, 1% methanol, 0.1% Triton X and Ga internal standard before analysis. Plasma samples were analysed in duplicate, with a calibration standard and blank routinely analysed amongst samples to assess instrument performance. Fresh whole blood samples were analysed using a Vet Animal Blood Counter (Woodley Equipment Company Ltd., Bolton, UK) to determine white blood cells (WBC), red blood cells (RBC), haemoglobin (Hb), haematocrit (Hct), platelets (Plt), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). The performance of the Vet Animal Blood Counter was assessed at each use, using a bovine whole blood reference sample (Woodley Haematology Control, WD1154), with inter-assay CV% of 2.4, 3.7, 2.8, 4.1, 8.7, 0.6, 2.9 and 3.2 for WBC, RBC, Hb, Hct, Plt, MCV, MCH and MCHC respectively. Sub-samples of whole blood samples were analysed for glutathione peroxidase (GSH-Px; intra-assay CV% 1.2) and superoxide dismutase (SOD; intra-assay CV% 2.3) (Randox Laboratories, County Antrim, UK; kit catalogue no. RS505 and SD125, respectively), using a Cobas Miras Plus auto analyser (ABX Diagnostics, Bedfordshire, UK). Samples of blood serum were analysed for β-hydroxybutyrate (3-OHB) and urea (Randox Laboratories, County Antrim, UK; kit catalogue no. RB1008 and UR221; intra-assay CV% 3.5 and 2.8 respectively). Blood serum from the initial and final visit were also analysed for vitamin $B_{12}$ (LOD = 33 pmol/L, and CV for low, med and high quality controls were 13.1, 9.3 and 7.9% respectively) and thyroxine ($T_4$; LOD = 3.9 nmol/L, and CV for low, med and high quality controls were 8.7, 6.0 and 5.8% respectively) using the ADIVA Centaur CP at the Animal and Plant Health Agency Scientific Laboratory, Shrewsbury, Shropshire, UK.

### *2.4. Statistical analysis*

Data were analysed using R (R Core Team, 2016) and the lme4 package (Bates et al., 2015). Continuous variables were fitted by REML with fixed effects of bolus (B+ and B-), forage type (G, K and F), sampling time (start, middle and end of the out-wintering period) and their interaction, and random effects of heifer pair, nested within farm. Tukey's test was performed *post hoc* where necessary. The majority of heifers calved with an ease score of either 1 (no assistance) or 2 (farmer assistance; normal presentation), therefore, calving ease data were reclassified into either calved without assistance (score 1) or assisted calving (scores 2, 3 and 4) and analysed by logistic regression in the lme4 package of R, with the Wald statistic used to assess significance.

## 3. Results

### *3.1. Feed analysis and blood mineral concentration*

The DM content of the three grazed forages was similar (Table 2), with a mean value of 151 g/kg DM. Crude protein content tended to be lowest ($p < 0.1$) in the fodder beet and highest in the kale. In contrast, water soluble carbohydrate was highest ($p < 0.05$) in the fodder beet and lowest in the grass, with kale being intermediate, whereas NDF concentration was lowest ($p < 0.05$) in the fodder beet and highest in the grass. The chemical composition of the supplementary forage was similar ($p > 0.05$) between farms, although on one farm that fed fodder beet the forage DM content was high. The proportion of silage in the total diet was 0.81 in G, 0.25 in K and 0.50 in F (s.e.d. 0.102, $p = 0.004$), and subsequently the diet of G were highest ($p < 0.05$) in NDF and lowest ($p < 0.05$) in WSC, whilst NDF was lowest ($p < 0.05$) in the diet of K and WSC highest ($p < 0.05$) in the diet of F.

Calcium concentration was highest in kale, and lowest in fodder beet, with an intermediate concentration in grass ($p < 0.05$), whereas P and potassium concentration were similar ($p > 0.05$) across all forages (Table 2). The concentration of Na and Mg were higher ($p < 0.05$) in fodder beet compared with grass or kale. In contrast, kale had a higher ($p < 0.05$) concentration of S than either grass or fodder beet. Grass had a higher concentration ($p < 0.05$) of both Mn and Co compared to kale or fodder beet, while Fe and Cu concentrations were lower ($p < 0.05$) in kale than either grazed grass or fodder beet. The concentration of Zn also tended ($p < 0.1$) to be lowest in kale, while Se tended ($p < 0.1$) to be highest in grass compared to the other 2 forages. Fodder beet had a lower concentration ($p < 0.05$) of Mo compared to grass or kale. The mineral content of the supplementary forage was similar across farms ($p > 0.05$). Total diet mineral intake differed between forage source ($p < 0.05$) for Ca, being highest in K and lowest in F; Co, where G was highest and K lowest; Cu, where K was lower than G or F; Fe, where G was highest and K lowest; and Mo, where G and K were higher than F.

Supplementation with mineral boluses increased plasma concentrations of Co, Se and Cu ($p < 0.05$; Table 3). Mean plasma Co was 0.021 and 0.041 μmol/L in B- and B+ respectively, with the concentration being higher at the middle and end of the out-wintering period in B+ compared to the beginning ($p < 0.05$). Mean plasma Se concentration was 0.50 and 0.82 μmol/L in B- and B+ respectively, with heifers that received a trace mineral bolus having higher concentrations at the middle and end of out-wintering compared to the beginning ($p < 0.05$). In heifers receiving GB-, plasma Se concentration was higher at the end of the out-wintering period compared to animals receiving FB- ($p < 0.05$), but similar to those receiving KB-. Mean plasma Cu concentration was 11.3 and 14.5 μmol/L in B- and B+ respectively, and the provision of a mineral bolus did not affect plasma concentrations in heifers receiving G or F ($p > 0.05$), but in heifers receiving K, plasma Cu was higher ($p < 0.05$) in animals receiving a bolus (KB+) at the middle and end of the out-wintering period compared to those that did not receive a bolus (KB-). There was no effect of providing a mineral bolus on plasma Mn, Fe, Zn or Mo concentration ($p < 0.05$), which averaged 0.06, 62.3 12.6 and 0.48 μmol/L respectively. Plasma Fe concentration was lower ($p < 0.05$) at the end of the out-wintering compared to the beginning in heifers receiving G or F, while in heifers receiving K, plasma Zn was lower ($p < 0.05$) at the middle compared to the beginning. Plasma Zn concentration was also lower ($p < 0.05$) in heifers receiving G at the middle of the out-wintering period, while in heifers fed F, Zn was lower ($p < 0.05$) at the end. In heifers receiving G, plasma Mo concentration was higher ($p < 0.05$) at the middle and end of the out-wintering period compared to the beginning. However, in heifers receiving K, plasma Mo was lower ($p < 0.05$) at the end of out-wintering compared to the beginning, while plasma Mo was similar ($p > 0.05$) over the out-wintering period in heifers receiving F.

*3.2. Blood vitamin, enzyme and metabolite concentrations*

Heifers that received the trace-mineral boluses had a higher ($p < 0.05$) mean serum vitamin $B_{12}$ concentration than those that did not receive the bolus, with mean concentrations of 116 and 128 pmol/L for B- and B+ respectively (Table 4). However, in heifers receiving G, serum vitamin $B_{12}$ concentration did not differ between the start and end of the out-wintering period ($p > 0.05$), while in heifers receiving F, concentrations were lower ($p < 0.05$) at the end of out-wintering compared to the beginning. In contrast, heifers receiving kale along with the mineral boluses (KB+), had a higher ($p < 0.05$) serum vitamin $B_{12}$ concentration at the end of out-wintering compared to KB-. Heifers that received the trace-mineral boluses had a higher ($p < 0.001$) mean blood SOD concentration than un-supplemented heifers at the end of the out-wintering period, with mean concentrations of 2067 and 2338 U/g Hb for B- and B+ respectively. However, there was no main effect ($p > 0.05$) of forage source, although heifers fed KB- had the lowest ($p < 0.05$) SOD at the end of the out-wintering period compared to any of the other treatments. Heifers that received the boluses also had a higher ($p < 0.05$) mean blood GSH-Px concentration than un-supplemented animals, with a mean concentration at the end of out-wintering of 30 and 76 U/mL Hct in B- and B+ respectively. In heifers that received the trace mineral boluses, blood GSH-Px concentration increased ($p < 0.05$) by the end of the out-wintering period irrespective of forage source, but in animals that did not receive a mineral bolus and grazed kale (KB-), blood GSH-Px concentration decreased ($p < 0.05$). The serum concentration of $T_4$ did not differ between treatments at the beginning of the study ($p > 0.05$), with heifers that received B+ having a similar ($p < 0.05$) concentration at the end of the out-wintering period compared to the beginning, while those that received B- had a higher ($p < 0.05$) concentration. Provision of the mineral boluses did not affect serum 3-OHB concentration ($p > 0.05$), with an overall mean of 0.38 mmol/L in B- and B+. However, heifers receiving K had a higher concentration of 3-OHB at the end of out-wintering compared to the beginning ($p < 0.05$), whereas in animals receiving F, serum 3-OHB concentration decreased with time ($p < 0.05$). Serum urea concentration was not affected by providing mineral boluses ($p > 0.05$), with an overall mean of 4.1 mmol/L for B- and B+. In contrast, for heifers receiving any of the forages, serum urea concentration was lower at the end of out-wintering period than the beginning ($p < 0.05$).

### *3.3. Haematology*

Provision of the mineral boluses had no effect ($p > 0.05$) on WBC, with an overall mean count of 8.27 and 8.57 x$10^3$ cells/$mm^3$ in B- and B+ respectively, although there was a trend ($p < 0.1$) for fewer WBC at the end compared to the beginning of the out-wintering period in heifers receiving F along with a bolus (FB+; Table 5). Similarly, the provision of the trace mineral boluses had no effect ($p > 0.05$) on Hb, with an overall mean concentration of 12.2 g/dL blood. However, there was an interaction between time and forage, with Hb being lower ($p < 0.05$) during the middle and end compared to the beginning of the out-wintering period in heifers receiving G or F. In contrast, in heifers fed K, Hb was higher ($p < 0.05$) in the middle compared to the beginning of the out-wintering period. The mineral boluses had no effect ($p > 0.05$) on RBC, with a mean count of 7.73 and 7.79 x 103 cells/$mm^3$ in B- and B+, respectively. However, in heifers receiving G or F, RBC was lower ($p < 0.05$) during the middle and end compared to the beginning of the out-wintering period. In contrast, at the end of out-wintering, heifers receiving KB+ had a higher ($p < 0.05$) RBC concentration than those receiving KB-. The mineral boluses had no effect ($p > 0.05$) on blood Hct volume, with an overall mean of 33.4 and 33.3 % for B- and B+ respectively, but there was an effect of time, with Hct being lower ($p < 0.05$) during the middle and end compared to the beginning of the out-wintering period in heifers receiving G or F. In contrast, Hct increased ($p < 0.05$) in heifers receiving K between the beginning and middle of the out-wintering period. Heifers that received the trace mineral boluses had a lower ($p < 0.05$) mean MCV than those that did not, with an overall mean at the end of the out-wintering period of 44.8 and 43.3 μ$m^3$ in B- and B+ respectively. The provision of the trace mineral boluses resulted in a higher ($p < 0.05$) MCV in heifers receiving KB+ than KB-. In heifers receiving G, F or KB+, MCV did not change ($p > 0.05$) over the out-wintering period. In contrast, in heifers receiving G or F, MCH decreased ($p < 0.05$) between the start and the end of out-wintering. The provision of the trace mineral boluses had no effect ($p > 0.05$) on MCHC, with an overall mean of 36.8 g/dL. However, in heifers receiving F, MCHC was lower ($p < 0.05$) at the end of out-wintering compared to the beginning. In contrast, MCHC was higher ($p < 0.05$) at the middle compared to the beginning of out-wintering in heifers fed K, but was unchanged ($p > 0.05$) in heifers fed G. There was no effect of the mineral bolus on Plt count ($p > 0.05$), with an overall mean of 314 x103/mm$^3$.

*3.4. Animal performance*

Provision of the trace mineral boluses did not affect ($p > 0.05$) BW, with an overall mean of 415 kg over the out-wintering period (Table 6). In heifers receiving G, BW did not change between the start and middle of the out-wintering period, but increased ($p < 0.05$) between the middle and the end. In contrast, the BW of heifers receiving F increased ($p < 0.05$) between the start and middle, but was similar ($p > 0.05$) between the middle and the end of out-wintering. The BW of heifers receiving K increased ($p > 0.05$) at each measurement point during the out-wintering period. Provision of the trace mineral boluses did not affect ($p > 0.05$) ADG over the out-wintering period, with an overall mean of 245 g/d between the start and end of out-wintering.

Overall, the provision of the mineral boluses did not affect ($p > 0.05$) heifer

BCS, with a mean of 2.57. However, at the end of the out-wintering period, heifers that had received trace mineral boluses had a mean BCS that was 0.03 points higher ($p < 0.05$) than those that did not. Overall, heifers lost BCS over the out-wintering period ($p < 0.05$), with those receiving G or K losing BCS at each measurement point, whilst those receiving F lost BCS between the middle and end of out-wintering.

Coat length increased by 4.3 mm over the study period ($p < 0.001$), but bolus provision had no effect ($p > 0.05$), with a mean of 23.2 and 23.4 mm for B- and B+ respectively. There was no effect ($p > 0.05$) of forage source on the proportion of heifers that calved unassisted, with a mean value of 0.89, whilst the provision of trace mineral boluses also had no effect ($p = 0.877$) on the proportion of heifers that calved un-aided, with 0.11 and 0.10 (odds ratio = 0.95, 0.47 – 1.91 95% CI; *n* obs. = 166 B-, 165 B+) requiring some assistance to calve in B- and B+, respectively.

## 4. Discussion

### *4.1. Blood parameters*

In the current study the primary objective was to determine the effect of a combined trace mineral bolus on the mineral status and performance of replacement dairy heifers on commercial farms and out-wintered on pasture, fodder beet and kale. These forages were used as they are the main out-wintering forages used in temperate countries such as the UK, New Zealand and Ireland

(Atkins et al., 2014; Edwards et al., 2017; Keogh et al., 2009). A combined trace mineral bolus was used as some trace minerals (e.g. I) are not available in this form, and combined boluses represent commercial practice on many farms (Sinclair and Atkins, 2015). The provision of trace-mineral boluses in the current study had no effect on plasma Cu concentration in animals fed G or F, but concentrations were increased in heifers fed kale. Blood Cu typically ranges between 11 – 25.6 μmol/L (mean 18 μmol/L; Rushton, 1981), and plasma Cu of animals fed K without bolus was 10.3 μmol/L by the end of the out-wintering period indicating marginal status. The typical recommend dietary concentration of Cu is between 11-15 mg/kg DM (NRC, 2001), and therefore the dietary Cu concentration (predicted from the forage and supplementary feed) in the G, K and F treatments in the current study were considerably lower at 4.2, 2.1 and 4.6 mg Cu/kg DM, respectively. Additionally, the high S content in kale was likely to have had an antagonistic effect on Cu absorption due to the formation of thiomolybdates in the rumen (Suttle, 2010). Using the prediction equations of Suttle and McLauchlin (1976) and the mean forage S and Mo concentrations resulted in a predicted absorption co-efficient of Cu (%) of 4.5 and 4.4 in the grass and fodder beet fed animals respectively, but only 2.5% in the kale fed animals, further compounding the low dietary concentration in animals grazing this forage. However, Cu deficiency is not immediately reflected in plasma Cu, as hepatic Cu is metabolised to maintain homeostasis (Evans, 1973), and plasma Cu has been shown to be a poor indicator of hepatic reserves (Sinclair et al., 2013, 2017). The reduced SOD levels in kale fed heifers that did not receive a mineral bolus may also indicate that these animals were deficient in Cu, although all values were low compared to other studies that have fed additional S and Mo to dairy cattle (Sinclair et al., 2013, 2017). Animals fed kale have previously been reported to demonstrate symptoms of Cu deficiency and haemolytic anaemia due to ruminal production of SMCO (Barry et al., 1981). In the current study, heifers fed kale without a bolus had a lower Hb and RBC, but a greater MCV at the end of out-wintering than those fed kale with a bolus. These effects suggest the onset of anaemia in heifers fed kale, as elevated MCV is indicative of a bone marrow response and the presence of immature red blood cells (Otter, 2013). This negative effect of kale was, however, mitigated by the trace-mineral bolus, and Cu supplementation of animals fed kale has previously been reported to allow the recovery from haemolytic anaemia (Barry et al., 1981).

Provision of trace-mineral boluses increased the concentration of Co in the plasma of heifers. The NRC (2001) stated the requirement for Co as 0.11 mg/kg DM, while others have estimated it as low as 0.06 mg/kg DM in grazing cattle (Clark et al., 1999). More recent studies in high yielding dairy cows has demonstrated that a dietary supply of 0.21 mg/kg DM is more than sufficient to meet the demands during the peri-parturient period and that there was no benefit to additional Co or vitamin $B_{12}$ (administered per os or injection) on performance or indicators of ketosis (Weerathilake et al., 2018). The Co content of grazed grass in this study was approximately 10 times above requirements and consequently additional Co would not have been expected to have any benefit. In contrast, the Co content of kale in this study was 0.05 mg/kg DM, approximately half the required dietary level (NRC, 2001). Within the rumen, elemental cobalt (Co) is used by bacteria to synthesise vitamin $B_{12}$ and animal status is generally assessed by measuring blood vitamin $B_{12}$ concentration. Vitamin $B_{12}$ has two principal metabolic functions in cattle; firstly, methlycobalamin acts as a co-factor in the transformation of methylmalonyl CoA to succinyl CoA which is then used within the liver for the synthesis of glucose from propionate (McDowell, 2000). Secondly, adenosylcobalamin is a co-factor in methionine synthase which is involved in the synthesis of methionine from homocysteine (McDowell, 2000). A threshold concentration of 150 pmol vitamin $B_{12}$/L has been suggested, above which there is little benefit to animal performance (Duplessis et al., 2017), although others have set the threshold at 90 pmol/L (Grace et al., 2014). In the current study, all serum $B_{12}$ concentrations were intermediate between these two threshold values. The low Co content of the kale may explain the decrease in vitamin $B_{12}$ status in un-supplemented heifers fed this forage over the out-wintering period compared to the increase in supplemented animals. Despite fodder beet fed animals receiving an adequate dietary concentration of Co, the vitamin $B_{12}$ status of heifers decreased over the out-wintering period, regardless of receiving a bolus. This reduction in vitamin $B_{12}$ status may have been as consequence of the low fibre and high water soluble content of fodder beet, as ruminal vitamin $B_{12}$ synthesis has been reported to be decreased in animals fed low roughage diets, most probably due to a low ruminal pH decreasing microbial synthesis (Smith et al., 1970; Walker and Elliot, 1972).

Plasma Se concentrations in the current study increased in heifers that received a trace mineral bolus across all the three forages, however, marginal limits for serum Se are 0.10 – 0.12 μmol/L (Suttle, 2010), and all treatments exceeded this threshold. Corresponding to plasma Se, plasma GSH-Px was increased in heifers receiving the bolus. A recommended Se content in the diet of cattle is 0.05 mg/kg DM in grazing situations where there is an adequate vitamin E supply (CSIRO, 2007). All the forages offered to the out-wintered heifers in this study were well in excess of this level of Se. However, even in the pasture, which had the highest concentration of Se at 0.23 mg/kg DM, levels were below the 0.3 mg/kg DM recommended by NRC (2001). Plasma GSH-Px concentration in heifers receiving pasture or fodder beet without a trace mineral bolus did not alter over the out-wintered period, further suggesting that Se concentration was adequate in the basal diet. However, there was a reduction from 46 to 18 U GSH-Px/mL Hct in heifers receiving kale without a bolus, despite kale containing a similar level of Se to the fodder beet (0.12 mg/kg DM). High dietary S may antagonise Se metabolism (Arthington, 2008), and plasma Se levels have been observed to decrease linearly with increasing dietary S concentration in dairy cows (Ivancic and Weiss, 2001). The S content in kale is known to be high and a mean concentration of 5.8 g/kg DM was recorded on the farms in the current study. However, increasing dietary S from 2.0 to 7.8 g/kg DM was previously observed to have little effect on GSH-Px concentration in cattle (Khan et al., 1987). Selenium deficiency has not previously been considered important with brassica diets, however the free amino acid s-methyl cysteine sulphoxide (SMCO) present in kale has been implicated in reduced GSH-Px activity of cattle and sheep (Barry et al., 1981). Barry et al. (1981) also suggested that Cu and Se status are related, as Cu containing SOD forms a coupling with Se containing GSH-Px in the erythrocytes, with SOD catalysing the reduction of superoxide anions to hydrogen peroxide and GSH-Px reducing hydrogen peroxide to water. As with GSH-Px, blood levels of SOD were also lower in the heifers fed kale without a bolus in this study.

Thyroid regulation also involves Se, with the concentration of $T_4$ reported to increase in Se deficient animals (Arthur et al., 1993; Wichtel et al., 1996). Both kale and fodder beet fed heifers in the current study had similar dietary Se concentrations and also had higher concentrations of $T_4$ in the animals that did not receive a trace mineral bolus, despite a greater than required (0.33 mg/kg DM; NRC, 2001) I content in all three forages. This could indicate that thyroid function could have been restricted by the dietary Se content in kale and fodder beet fed heifers without a bolus and further work may be warranted to determine the effect of Se in animals fed these forages. It is also well established that brassica crops such as kale contain high levels of cyanogenic goitrogens which can be overcome by additional dietary I, which is reflected in serum $T_4$ concentrations, with values of 25-50 nmol/L indicating marginal status (Suttle, 2010). In the current study serum $T_4$ concentrations exceeded this threshold on all treatments, and were little affected by the provision of a bolus. In contrast, thiouracil-type goitrogens are not influenced by dietary I supply, but are generally not present in sufficiently high concentrations in brassicas such as kale, or in grass and fodder beet (Suttle, 2010).

*4.2. Animal performance*

The trace-mineral bolus appeared to have little effect on the physical performance of pregnant, growing heifers over the out-wintering period in the current study, although animals on all treatments were below the target weight at calving for similar systems (Roche et al., 2015). Heifers that received the trace-mineral boluses lost less body condition by the end of the study, however the effect was biologically small and may have little impact on production or fertility (Roche et al., 2015). Heifers fed F, and to a lesser extent G, tended to receive a lower dietary crude protein concentration, which may have been a limiting factor, but was not reflected in their performance or plasma urea concentrations. Inadequate energy intake may also have been limiting animal performance as the ADG of heifers in this study was low, and below that reported for other commercial herds (Atkins et al., 2013). Out-wintering systems have previously been reported to be able to support high levels of performance with an ADG of 1.10 kg/d in pregnant Holstein-Friesian heifers fed a diet consisting of 70% kale and 30% grass silage (Kennedy et al., 2012), and 1.24 or 0.95 kg/d in pregnant Holstein heifers receiving fodder beet or grazed grass with grass silage respectively (Atkins et al., 2018). Judson and Edwards (2008) reported that many farmers feeding kale to pregnant dairy cows in New Zealand and using a similar system to that described here, underestimated the crops herbage mass or overestimated the cows' intake. The difference in performance reported by previous studies involving out-wintered heifers and those in this study could therefore be in part due to low or inaccurate feed allocation.

## 5. Conclusion

There was no effect of forage source or provision of a Co, Se, I and Cu containing trace-mineral boluses on animal performance, except body condition prior to calving which was slightly higher in heifers receiving boluses. Provision of trace-mineral boluses increased plasma concentrations of the minerals supplied except for Cu in heifers fed grass or fodder beet. Despite the increase in plasma Co with heifers fed fodder beet, serum vitamin $B_{12}$ decreased in heifers fed this forage. The blood metabolite and haematology results suggest that the trace-mineral bolus was effective at counteracting many of the anti-nutritional factors associated with kale. The use of a trace-mineral bolus when out-wintering pregnant heifers is therefore recommended, particularly for heifers grazing kale, but further research is required to more accurately define mineral requirements amongst the different forages.

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**Conflict of interest statement**

The authors declare that there is no conflict of interest regarding the publication of this article.

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**Table 1**
Limit of detection (LOD), limit of quantification (LOQ), intra-assay CV%, and certified reference material results of ICP-MS analysis

| | | | | | Certified reference material | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | intra-assay CV% | | EU BCR-129, hay | | | EU BCR-708, dairy concentrate | | |
| Element | LOD, µl/l | LOQ, µl/l | Feed | Plasma | Certified level | Analysed level | % recovered | Certified level | Analysed level | % recovered |
| | | | | | g/kg DM | | | | | |
| Ca | 55.9 | 186 | 1.0 | | 6.40 ± 0.10 | 6.10 ± 0.41 | 95 | 4.8 ± 0.5 | 4.5 ± 0.3 | 93 |
| Na | 15.7 | 52.4 | 0.6 | | 3.49 | 3.77 ± 0.04 | 108 | | | |
| Mg | 0.46 | 1.52 | 0.4 | | 1.45 ± 0.04 | 1.50 ± 0.01 | 104 | 1.47 ± 0.22 | 1.57 ± 0.018 | 107 |
| P | 4.75 | 15.8 | 0.7 | | 2.36 ± 0.07 | 2.37 ± 0.03 | 100 | 4.7 ± 0.4 | 4.7 ± 0.1 | 100 |
| K | 1.53 | 5.10 | 0.4 | | 33.8 ± 0.8 | 36.1 ± 0.5 | 107 | | | |
| S | 99.5 | 332 | 4.0 | | 3.16 ± 0.04 | 2.74 ± 0.37 | 87 | | | |
| | | | | | mg/kg DM | | | | | |
| Co | 0.01 | 0.02 | 1.2 | 6.4 | 0.121 | 0.099 ± 0.047 | 81 | | | |
| Cu | 0.04 | 0.12 | 1.2 | 2.3 | 10 | 9.7 ± 0.1 | 97 | 37 ± 4 | 42 ± 1.4 | 114 |
| Se | 0.04 | 0.13 | 5.9 | 1.7 | 0.025 | 0.024 ± 0.010 | 97 | | | |
| Zn | 0.09 | 0.32 | 1.1 | 1.7 | 32.1 ± 1.7 | 28.0 ± 6.1 | 87 | | | |
| Fe | 1.90 | 6.34 | 0.6 | 1.2 | 114 | 102.9 ± 5.9 | 90 | | | |
| Mn | 0.01 | 0.03 | 0.6 | 5.3 | 72 | 76.3 ± 2.6 | 106 | | | |
| Mo | 0.01 | 0.02 | 0.7 | 2.5 | 1 | 1.0 ± 0.1 | 96 | | | |

**Table 2**
Chemical composition of the grazed and supplementary forage and total diet fed to pregnant dairy heifers out-wintered between November 2012 and February 2013 on nine commercial spring-calving farms. Heifers grazed either pasture (G), kale (K) or fodder beet (F), supplemented with baled grass silage (n=3)

| | Grazed forage | | | | | Supplementary forage | | | | | Total diet | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | G | K | F | SED | *P*-value | G | K | F | SED | *P*-value | G | K | F | SED | *P*-value |
| DM, g/kg | 161 | 134 | 158 | 12.9 | 0.151 | 388 | 368 | 541 | 150.7 | 0.495 | 343 | 192 | 324 | 68.2 | 0.131 |
| CP, g/kg DM | 128 | 164 | 87 | 24.7 | 0.053 | 113 | 121 | 101 | 17.1 | 0.555 | 115 | 153 | 92 | 19.6 | 0.054 |
| NDF, g/kg DM | 521 | 302 | 179 | 24.5 | <0.001 | 601 | 554 | 628 | 38.5 | 0.230 | 583 | 365 | 402 | 45.9 | 0.007 |
| WSC, g/kg DM | 100 | 256 | 494 | 37.7 | <0.001 | 19 | 29 | 78 | 33.4 | 0.244 | 35 | 201 | 288 | 64.5 | 0.021 |
| Macro-mineral, g/kg DM | | | | | | | | | | | | | | | |
| Ca | 7.49 | 15.2 | 3.23 | 0.707 | <0.001 | 5.91 | 5.64 | 3.34 | 1.376 | 0.202 | 6.39 | 12.8 | 3.45 | 0.844 | <0.001 |
| Na | 0.65 | 1.20 | 4.75 | 0.908 | 0.008 | 2.45 | 1.32 | 2.93 | 1.564 | 0.600 | 2.21 | 1.22 | 3.96 | 1.244 | 0.163 |
| Mg | 1.55 | 1.64 | 2.55 | 0.295 | 0.027 | 1.80 | 1.95 | 1.99 | 0.481 | 0.923 | 1.79 | 1.72 | 2.31 | 0.384 | 0.316 |
| P | 2.77 | 3.26 | 2.06 | 0.492 | 0.123 | 2.79 | 2.91 | 2.94 | 0.529 | 0.954 | 2.77 | 3.18 | 2.52 | 0.489 | 0.439 |
| K | 16.8 | 31.7 | 24.9 | 5.71 | 0.101 | 24.0 | 28.0 | 20.6 | 4.99 | 0.396 | 22.5 | 30.8 | 21.3 | 3.79 | 0.089 |
| S | 1.90 | 5.84 | 0.49 | 1.182 | 0.010 | 2.23 | 2.25 | 3.24 | 1.823 | 0.823 | 2.19 | 4.93 | 1.97 | 1.487 | 0.166 |
| Trace-mineral, mg/kg DM | | | | | | | | | | | | | | | |
| Co | 1.05 | 0.05 | 0.28 | 0.20 | 0.007 | 0.29 | 0.14 | 0.11 | 0.088 | 0.166 | 0.43 | 0.08 | 0.19 | 0.068 | 0.006 |
| Cu | 7.74 | 1.64 | 4.86 | 0.95 | 0.002 | 3.52 | 3.64 | 3.97 | 1.076 | 0.911 | 4.20 | 2.14 | 4.61 | 0.743 | 0.033 |
| Se | 0.23 | 0.12 | 0.12 | 0.048 | 0.084 | 0.24 | 0.14 | 0.12 | 0.131 | 0.656 | 0.21 | 0.12 | 0.12 | 0.099 | 0.577 |
| I | 1.92 | 0.62 | 2.53 | 1.863 | 0.604 | 0.44 | 0.40 | 0.88 | 0.488 | 0.577 | 0.80 | 0.52 | 1.39 | 0.903 | 0.639 |
| Zn | 38.3 | 15.4 | 37.0 | 9.13 | 0.080 | 28.2 | 20.2 | 22.6 | 7.07 | 0.549 | 29.6 | 16.6 | 30.2 | 7.10 | 0.176 |
| Fe | 1709 | 127 | 1168 | 310.4 | 0.010 | 805 | 351 | 359 | 333.5 | 0.361 | 1276 | 183 | 770 | 198.4 | 0.004 |
| Mn | 213 | 15 | 57 | 20.3 | <0.001 | 209 | 87 | 260 | 98.1 | 0.270 | 217 | 33 | 160 | 75.3 | 0.117 |
| Mo | 1.29 | 0.97 | 0.13 | 0.201 | 0.003 | 1.06 | 1.31 | 0.74 | 0.307 | 0.256 | 1.11 | 1.06 | 0.41 | 0.240 | 0.049 |

**Table 3** Plasma trace-mineral concentration at the beginning, middle and end of a three month out-wintering period, in pregnant crossbred dairy heifers out-wintered on either grazed pasture (G), kale (K) or fodder beet (F), without (B-) or with (B+) a trace-mineral bolus

| | Treatment | | | | | | | *P*-value† | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| item | GB- | KB- | FB- | GB+ | KB+ | FB+ | SED | B | Fo | B x Fo | T | B x T | Fo x T | B x Fo x T |
| Cobalt, µmol/L | | | | | | | | | | | | | | |
| start | 0.020 | 0.023 | 0.017 | 0.020 | 0.021 | 0.021 | 0.0092 | 0.626 | 0.965 | 0.213 | | | | |
| mid | 0.022 | 0.023 | 0.022 | 0.069 | 0.064 | 0.061 | 0.0076 | <0.001 | 0.884 | 0.318 | <0.001 | <0.001 | 0.283 | 0.327 |
| end | 0.023 | 0.021 | 0.021 | 0.042 | 0.034 | 0.041 | 0.0083 | <0.001 | 0.883 | 0.472 | | | | |
| Selenium, µmol/L | | | | | | | | | | | | | | |
| start | 0.49 | 0.51 | 0.26 | 0.51 | 0.53 | 0.33 | 0.146 | 0.032 | 0.374 | 0.234 | | | | |
| mid | 0.70 | 0.48 | 0.48 | 1.17 | 1.24 | 1.05 | 0.203 | <0.001 | 0.793 | <0.001 | <0.001 | <0.001 | 0.002 | 0.009 |
| end | 0.56 | 0.50 | 0.45 | 0.88 | 0.88 | 0.81 | 0.153 | <0.001 | 0.869 | 0.536 | | | | |
| Copper, µmol/L | | | | | | | | | | | | | | |
| start | 13.7 | 11.2 | 12.6 | 15.1 | 9.9 | 15.1 | 3.46 | 0.187 | 0.583 | 0.051 | | | | |
| mid‡ | 12.9 | 10.8 | 13.2 | 14.5 | 17.6 | 16.3 | 2.54 | <0.001 | 0.937 | <0.001 | 0.002 | <0.001 | <0.001 | <0.001 |
| end | 11.6 | 10.3 | 12.0 | 12.4 | 16.2 | 15.0 | 1.93 | <0.001 | 0.702 | <0.001 | | | | |
| Zinc, µmol/L | | | | | | | | | | | | | | |
| start | 12.4 | 13.8 | 12.4 | 12.3 | 15.4 | 12.9 | 4.27 | 0.147 | 0.894 | 0.391 | | | | |
| mid‡ | 11.2 | 11.2 | 11.9 | 10.7 | 11.2 | 12.1 | 3.95 | 0.856 | 0.974 | 0.755 | <0.001 | 0.399 | <0.001 | 0.831 |
| end‡ | 13.6 | 10.3 | 11.5 | 13.7 | 10.3 | 10.9 | 3.08 | 0.610 | 0.669 | 0.765 | | | | |
| Iron, µmol/L | | | | | | | | | | | | | | |
| start | 82.2 | 78.3 | 65.6 | 78.4 | 77.6 | 63.3 | 10.20 | 0.591 | 0.203 | 0.957 | | | | |
| mid | 65.0 | 58.9 | 52.5 | 68.0 | 59.1 | 61.0 | 8.92 | 0.290 | 0.486 | 0.641 | <0.001 | 0.532 | 0.085 | 0.961 |
| end | 51.4 | 58.9 | 45.8 | 49.0 | 58.5 | 47.9 | 8.35 | 0.945 | 0.322 | 0.860 | | | | |
| Manganese, µmol/L | | | | | | | | | | | | | | |
| start | 0.05 | 0.07 | 0.05 | 0.07 | 0.07 | 0.06 | 0.025 | 0.451 | 0.834 | 0.540 | | | | |
| mid | 0.06 | 0.07 | 0.06 | 0.05 | 0.07 | 0.06 | 0.014 | 0.698 | 0.495 | 0.855 | 0.320 | 0.994 | 0.984 | 0.244 |
| end | 0.05 | 0.08 | 0.07 | 0.08 | 0.08 | 0.06 | 0.020 | 0.824 | 0.484 | 0.213 | | | | |
| Molybdenum, µmol/L | | | | | | | | | | | | | | |
| start | 0.44 | 0.38 | 0.44 | 0.44 | 0.35 | 0.44 | 0.223 | 0.669 | 0.952 | 0.885 | | | | |
| mid‡ | 0.68 | 0.35 | 0.48 | 0.75 | 0.34 | 0.42 | 0.207 | 0.658 | 0.280 | 0.114 | 0.001 | 0.922 | <0.001 | 0.869 |
| end‡ | 0.70 | 0.27 | 0.48 | 0.81 | 0.27 | 0.43 | 0.344 | 0.762 | 0.475 | 0.117 | | | | |

†B = main effect of bolus, Fo = main effect of forage, B x Fo = interaction of bolus and forage, T = main effect of time, B x T = interaction of bolus and time, Fo x T = interaction of forage and time, B x Fo x T = interaction of bolus, forage and time

‡means adjusted for initial value covariate

**Table 4**
Blood vitamin, enzyme and metabolite concentrations at the beginning and end of a three month out-wintering period, in pregnant crossbred dairy heifers out-wintered on either grazed pasture (G), kale (K) or fodder beet (F), without (B-) or with (B+) a trace-mineral bolus

| | Treatment | | | | | | | *P*-value† | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| item | GB- | KB- | FB- | GB+ | KB+ | FB+ | SED | B | Fo | B x Fo | T | B x T | Fo x T | B x Fo x T |
| Vitamin $B_{12}$, pmol/L | | | | | | | | | | | | | | |
| start | 128 | 126 | 126 | 138 | 119 | 133 | 9.0 | 0.412 | 0.468 | 0.105 | <0.001 | 0.020 | <0.001 | 0.010 |
| end‡ | 126 | 116 | 107 | 144 | 132 | 109 | 11.7 | <0.001 | 0.106 | 0.039 | | | | |
| §SOD, U/g Hb | | | | | | | | | | | | | | |
| start | 2126 | 2368 | 2002 | 2021 | 2174 | 1995 | 307.3 | 0.195 | 0.707 | 0.630 | <0.079 | <0.001 | <0.001 | <0.054 |
| end‡ | 2117 | 1731 | 2353 | 2385 | 2190 | 2438 | 200.0 | <0.001 | <0.088 | 0.128 | | | | |
| GSH-Px, U/mL Hct | | | | | | | | | | | | | | |
| start | 41 | 46 | 19 | 39 | 41 | 20 | 15.8 | 0.215 | 0.447 | 0.393 | | | | |
| mid‡ | 42 | 22 | 37 | 57 | 61 | 55 | 9.8 | <0.001 | 0.731 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| end‡ | 40 | 18 | 33 | 70 | 79 | 75 | 8.2 | <0.001 | 0.752 | <0.001 | | | | |
| $T_4$, nmol/L | | | | | | | | | | | | | | |
| start | 76 | 67 | 85 | 74 | 68 | 90 | 11.6 | 0.482 | 0.322 | 0.374 | <0.001 | 0.034 | <0.001 | 0.228 |
| end‡ | 95 | 81 | 80 | 95 | 75 | 72 | 17.7 | 0.009 | 0.610 | 0.234 | | | | |
| 3-OHB, mmol/L | | | | | | | | | | | | | | |
| start | 0.47 | 0.35 | 0.54 | 0.42 | 0.36 | 0.42 | 0.191 | 0.054 | 0.840 | 0.128 | | | | |
| mid | 0.28 | 0.36 | 0.35 | 0.28 | 0.39 | 0.31 | 0.078 | 0.853 | 0.604 | 0.124 | <0.001 | 0.266 | <0.001 | 0.469 |
| end‡ | 0.42 | 0.47 | 0.29 | 0.42 | 0.46 | 0.32 | 0.070 | 0.792 | 0.079 | 0.679 | | | | |
| Urea, mmol/L | | | | | | | | | | | | | | |
| start | 5.1 | 5.6 | 4.3 | 5.0 | 5.2 | 4.3 | 0.91 | 0.137 | 0.581 | 0.353 | | | | |
| mid | 3.7 | 4.7 | 2.8 | 3.5 | 4.8 | 2.9 | 0.66 | 0.788 | 0.051 | 0.305 | <0.001 | 0.616 | <0.001 | 0.564 |
| end | 4.3 | 3.7 | 3.2 | 4.0 | 3.8 | 3.0 | 0.65 | 0.312 | 0.363 | 0.309 | | | | |

†B = main effect of bolus, Fo = main effect of forage, B x Fo = interaction of bolus and forage, T = main effect of time, B x T = interaction of bolus and time, Fo x T = interaction of forage and time, B x Fo x T = interaction of bolus, forage and time
‡means adjusted for start value covariate
§SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; $T_4$ = thyroxine; 3-OHB = β-hydroxybutyrate

**Table 5**
Haematology at the beginning, middle and end of a three month out-wintering period, in pregnant crossbred dairy heifers out-wintered on either grazed pasture (G), kale (K) or fodder beet (F), without (B-) or with (B+) a trace-mineral bolus

| | Treatment | | | | | | | *P*-value[†] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| item | GB- | KB- | FB- | GB+ | KB+ | FB+ | SED | Bo | Fo | B x Fo | T | B x T | Fo x T | B x Fo x T |
| [‡]WBC, $10^3$/mm$^3$ | | | | | | | | | | | | | | |
| start | 9.63 | 7.72 | 8.36 | 7.73 | 8.17 | 9.84 | 1.187 | 0.985 | 0.653 | 0.002 | | | | |
| mid[§] | 7.99 | 8.27 | 8.74 | 8.42 | 9.26 | 9.28 | 0.834 | 0.050 | 0.563 | 0.773 | 0.002 | 0.442 | 0.103 | 0.022 |
| end[§] | 7.82 | 7.98 | 7.81 | 7.83 | 8.58 | 7.92 | 0.567 | 0.488 | 0.581 | 0.711 | | | | |
| Hb, g/dL | | | | | | | | | | | | | | |
| start | 15.5 | 10.5 | 13.9 | 14.8 | 10.8 | 13.8 | 1.11 | 0.559 | 0.001 | 0.356 | | | | |
| mid[§] | 11.6 | 12.5 | 11.3 | 11.3 | 12.5 | 11.9 | 1.02 | 0.742 | 0.605 | 0.381 | <0.001 | 0.719 | <0.001 | 0.205 |
| end[§] | 11.8 | 11.3 | 12.0 | 11.2 | 12.2 | 11.3 | 1.02 | 0.622 | 0.977 | 0.030 | | | | |
| RBC, $10^3$/mm$^3$ | | | | | | | | | | | | | | |
| start | 9.51 | 7.32 | 8.67 | 8.84 | 7.38 | 8.50 | 0.649 | 0.143 | 0.035 | 0.316 | | | | |
| mid[§] | 7.24 | 7.69 | 7.26 | 6.96 | 8.22 | 7.48 | 0.323 | 0.431 | 0.009 | 0.124 | <0.001 | 0.241 | <0.001 | 0.359 |
| end[§] | 7.44 | 6.67 | 7.94 | 7.06 | 7.59 | 7.54 | 0.701 | 0.774 | 0.691 | 0.002 | | | | |
| Hct, % | | | | | | | | | | | | | | |
| start | 42.8 | 29.4 | 36.9 | 39.9 | 30.0 | 36.7 | 3.03 | 0.314 | 0.002 | 0.189 | | | | |
| mid[§] | 31.7 | 33.9 | 30.4 | 30.6 | 34.4 | 31.4 | 2.30 | 0.847 | 0.315 | 0.418 | <0.001 | 0.597 | <0.001 | 0.506 |
| end[§] | 32.4 | 31.5 | 33.2 | 30.5 | 33.5 | 31.5 | 3.00 | 0.447 | 0.938 | 0.044 | | | | |
| MCV, µm$^3$ | | | | | | | | | | | | | | |
| start | 45.2 | 44.2 | 43.0 | 45.4 | 44.0 | 43.3 | 1.16 | 0.695 | 0.167 | 0.823 | | | | |
| mid[§] | 43.1 | 46.1 | 42.9 | 43.3 | 44.8 | 42.9 | 1.24 | 0.202 | 0.183 | 0.013 | <0.001 | <0.001 | <0.001 | <0.001 |
| end[§] | 42.9 | 48.6 | 42.9 | 42.8 | 44.7 | 42.5 | 0.77 | <0.001 | <0.001 | <0.001 | | | | |
| MCH, pg | | | | | | | | | | | | | | |
| start | 16.5 | 15.6 | 16.1 | 17.0 | 15.4 | 16.6 | 0.50 | 0.183 | 0.016 | 0.509 | | | | |
| mid[§] | 16.0 | 17.9 | 15.7 | 16.2 | 16.9 | 15.9 | 0.54 | 0.583 | 0.011 | 0.008 | <0.001 | 0.018 | <0.001 | 0.561 |
| end[§] | 16.0 | 17.7 | 15.2 | 15.9 | 16.4 | 15.0 | 0.37 | 0.005 | <0.001 | 0.003 | | | | |
| MCHC, g/dL | | | | | | | | | | | | | | |
| start | 36.7 | 35.4 | 37.6 | 37.7 | 35.2 | 38.5 | 1.27 | 0.184 | 0.091 | 0.629 | | | | |
| mid[§] | 36.6 | 38.7 | 37.4 | 37.2 | 37.5 | 38.2 | 0.70 | 0.528 | 0.041 | 0.105 | <0.001 | 0.575 | <0.001 | 0.501 |
| end[§] | 36.9 | 36.4 | 36.3 | 36.9 | 36.5 | 36.2 | 1.08 | 0.948 | 0.820 | 0.933 | | | | |
| Plt, $10^3$/mm$^3$ | | | | | | | | | | | | | | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| start | 337 | 217 | 254 | 316 | 248 | 288 | 91.5 | 0.599 | 0.641 | 0.593 | | | | |
| mid§ | 310 | 298 | 320 | 368 | 323 | 257 | 79.0 | 0.900 | 0.789 | 0.185 | 0.091 | 0.968 | 0.061 | 0.389 |
| end§ | 330 | 239 | 366 | 349 | 258 | 348 | 89.4 | 0.854 | 0.508 | 0.796 | | | | |

†B = main effect of bolus, Fo = main effect of forage, B x Fo = interaction of bolus and forage, T = main effect of time, B x T = interaction of bolus and time, Fo x T = interaction of forage and time, B x Fo x T = interaction of bolus, forage and time

‡WBC = white blood cell; Hb = haemoglobin; RBC = red blood cells; Hct = haematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; Plt = platelets.

§means adjusted for initial value covariate

**Table 6**
Average daily gain (ADG), body weight (BW), body condition score (BCS) and coat length at the beginning, middle and end of a three month out-wintering period, in pregnant crossbred dairy heifers out-wintered on either grazed pasture (G), kale (K) or fodder beet (F), without (B-) or with (B+) a trace-mineral bolus

| | Treatment | | | | | | | *P*-value† | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| item | GB- | KB- | FB- | GB+ | KB+ | FB+ | SED | B | Fo | B x Fo | T | B x T | Fo x T |
| ADG,g/d | 205 | 393 | 136 | 145 | 438 | 152 | 198.0 | 0.988 | 0.452 | 0.169 | | | |
| BW, kg | | | | | | | | | | | | | |
| start | 428 | 399 | 385 | 430 | 400 | 383 | 16.8 | 0.816 | 0.086 | 0.291 | | | |
| mid‡ | 411 | 429 | 412 | 408 | 432 | 413 | 13.3 | 0.674 | 0.387 | 0.117 | <0.001 | 0.968 | <0.001 |
| end‡ | 423 | 435 | 413 | 418 | 439 | 414 | 15.3 | 0.968 | 0.415 | 0.135 | | | |
| BCS, 1 – 5 point scale | | | | | | | | | | | | | |
| start | 2.73 | 2.71 | 2.56 | 2.73 | 2.73 | 2.54 | 0.130 | 1.000 | 0.421 | 0.769 | | | |
| mid | 2.60 | 2.58 | 2.61 | 2.63 | 2.55 | 2.60 | 0.057 | 0.752 | 0.709 | 0.330 | <0.001 | 0.146 | <0.001 |
| end | 2.44 | 2.45 | 2.41 | 2.48 | 2.46 | 2.47 | 0.068 | 0.024 | 0.952 | 0.376 | | | |
| Coat length, mm | | | | | | | | | | | | | |
| start | 20.8 | 20.5 | 21.4 | 21.8 | 20.0 | 22.1 | 0.87 | 0.423 | 0.057 | 0.345 | <0.001 | 0.715 | 0.125 |
| end | 26.2 | 24.9 | 24.3 | 26.2 | 24.6 | 26.1 | 1.41 | 0.282 | 0.617 | 0.121 | | | |

†B = main effect of bolus, Fo = main effect of forage, B x Fo = interaction of bolus and forage, T = main effect of time, Bo x T = interaction of bolus and time, Fo x T = interaction of forage and time. Interaction of B, Fo and T was not significant (*p* > .05)
‡means adjusted for initial value covariate