

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

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19 ABSTRACT

20 The effects of a cobalt (Co), copper (Cu), selenium (Se), and iodine (I) trace-
21 mineral ruminal bolus on the mineral status and performance of out-wintered,
22 pregnant dairy heifers was investigated. Nine commercial farms grazing pasture
23 (G), kale (K), or fodder beet (F) were used (n=3 per forage), with forty heifers on
24 each farm randomly allocated to not receive (B-) or receive (B+) two combined
25 mineral boluses. Mean plasma Co concentrations were 0.021 and 0.041 $\mu\text{mol/L}$ in
26 B- and B+ respectively ($p < 0.001$), with serum vitamin B₁₂ also higher in heifers
27 receiving B+ than B- ($p < 0.001$). Mean plasma Se concentration was 0.50 and 0.82
28 $\mu\text{mol/L}$ in B- and B+ respectively, with heifers that received B+ also having a higher
29 ($p < 0.05$) mean blood GSH-Px concentration (30 and 76 U/mL haematocrit in B-
30 and B+ respectively). Providing a mineral bolus did not affect plasma Cu
31 concentration in heifers receiving G or F ($p < 0.05$), but was higher in KB+
32 compared to KB- ($p < 0.05$) at the middle and end of the out-wintering period.
33 Heifers receiving KB- also had a lower haemoglobin and red blood cell count, but
34 a higher mean corpuscular volume than KB+ at the end of the out-wintering period.
35 Animals receiving B- had a higher plasma thyroxine concentration ($p < 0.05$).
36 Neither the bolus nor forage type affected body weight ($p > 0.05$), however
37 condition score was higher ($p < 0.05$) in B+ at the end of the study. It is concluded
38 that the provision of a trace mineral bolus increased plasma concentrations of the
39 minerals supplied, with the greatest benefits in animals grazing kale, but these
40 increases were not translated into improved performance.

41

42 *Keywords:* brassica, dairy heifer, forages, wintering, minerals, vitamin B₁₂

43 **1. Introduction**

44

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

56 Out-wintering systems for replacement dairy heifers present particular
57 trace-mineral nutrition challenges which may impact animal health and
58 productivity. For instance, kale has been reported to be deficient in copper (Cu),
59 cobalt (Co) and iodine (I) (Grace et al., 2010). Brassicas' also contain
60 glucosinolates, which hydrolyse in the rumen to produce goitrogens, interfering
61 with I absorption and inhibiting thyroxine synthesis (Barry et al., 1981). Kale is
62 also high in sulphur (S), a Cu metabolism antagonist (Suttle, 2010), with much of
63 the S in kale contained in the anti-nutritional factor S-methylcysteine sulphoxide,
64 which causes damage to red blood cells and can lead to haemolytic anaemia (Barry,
65 2013). In addition, soil particles adhere to pasture during winter and may typically
66 contribute around 10% of dry matter intake (DMI), potentially inhibiting mineral

67 and element availability (Suttle et al., 1975), an effect that may be greater with
68 fodder beet grazed *in situ*. Fodder beet is also low in trace minerals such as Co and
69 selenium (Se; Atkins et al., 2018), although there is comparatively little published
70 data regarding the trace-mineral content of this forage. In addition, cold conditions
71 can increase thyroid activity (Tucker et al., 2007), and out-wintered animals may
72 benefit from additional I.

73 Achieving adequate live weight gain in heifers is generally considered an
74 important factor affecting both current and lifetime performance (Hoffman, 1997;
75 Le Cozler et al., 2011; Roche et al., 2015). Sub-optimal trace-mineral nutrition
76 during the heifer rearing period can impact on subsequent productivity with, for
77 example, low dietary concentration of Co or Cu restricting average daily gain (ADG)
78 in growing cattle (Mills et al., 1976; Schwarz et al., 2000), and Cu, Co, I and Se
79 supply are all important for immunity and fertility (Corah, 1996; Panousis et al.,
80 2001; Stabel et al., 1993). Recent surveys have indicated that in housed, winter-
81 fed dairy cows, minerals are generally supplied well in excess of requirements
82 (Sinclair and Atkins, 2015). In contrast, grazed animals present less control over
83 mineral nutrition (McDowell, 1996). Offering free-choice mineral licks can result
84 in in variable mineral intake between animals (Valk and Kogut, 1998), whilst
85 administering a reticulorumen trace-mineral bolus offers the opportunity to
86 deliver a consistent dose of selected trace-minerals throughout the grazing period
87 (Kendall et al., 2001). Despite mineral bolus use being common commercial
88 practice on many UK dairy farms (Sinclair and Atkins, 2015), the benefits of trace-
89 mineral supplementation on the mineral status, metabolism and performance in
90 lower-input, out-wintered heifer rearing systems are unclear. The aim of the

91 current study was to investigate the effects of a trace-mineral bolus on blood
92 mineral status and performance in replacement heifers out-wintered on pasture,
93 kale or fodder beet, in commercial, spring-calving pasture based dairy farms.

94

95 **2. Material and methods**

96

97 *2.1. Animals, treatments and management*

98

99 Nine spring-calving, pasture-based dairy farms in the UK that were due to
100 out-winter heifers in 2012/2013 were used. The farms were selected to be
101 representative of out-wintering farms based on a survey of commercial practice
102 (Atkins et al., 2014). Three of the farms grazed kale (K), three fodder beet (F), and
103 three pastures which were composed predominately of perennial ryegrass (G).
104 The farms were located in the counties of Shropshire, Staffordshire and Hampshire
105 for K; Dumfries and Shropshire (x2) for F; and Derbyshire, Shropshire and
106 Somerset for G. The heifers were all Friesian/Jersey crosses, due to calve at 24
107 months of age from February 2013, and were destined for a grazed grass
108 production system. A sub-set of 40 primiparous heifers were randomly selected
109 on each study farm, resulting in a total of 360 heifers recruited onto the study.
110 Within each farm, the 40 heifers were paired according to body weight (BW) and
111 body condition score (BCS; Mulvany, 1977) and randomly allocated to one of two
112 treatments; un-supplemented (B-) or supplemented with trace-mineral boluses
113 (B+). The B+ heifers received two reticulorumen trace-mineral boluses (CoSeICure,
114 Telsol Ltd, Leeds, UK) according to the manufacturer's recommendations at the

115 start of the study. Each bolus contained Cu (13.4 g); Co (0.5 g); Se (0.15 g, as sodium
116 selenite) and I (1.0 g, as calcium iodate). No other mineral supplementation was
117 available during the study period. The heifers on each farm were kept together
118 throughout the winter and were managed within larger groups that included non-
119 trial heifers and received supplementary forage in the form of big bale perennial
120 ryegrass silage.

121

122 *2.2. Experimental routine and measurements*

123

124 The farms were visited on three occasions during the out-wintering period;
125 start (late October to early November 2012), middle (mid-December 2012), and
126 at the end of the out-wintering period (late January to early February 2013). The
127 initial visit coincided with the beginning of the out-wintering regime on each farm
128 (\pm one week), and the final visit with the end of the out-wintering feeding regime,
129 prior to the onset of calving. On each visit, samples of pasture or forage crop and
130 supplementary forage were collected and stored at -20°C prior to subsequent
131 analysis. Pasture samples were collected from 10 random positions in the
132 subsequent weeks grazing area at a height of approximately 4 cm. Kale samples
133 were collected by cutting 10 random plants from the subsequent weeks grazing
134 area at approximately 5-10 cm above ground level, just above the woody base. The
135 plants were then chopped into approximately 3 to 5 cm pieces, mixed, quartered,
136 and mixed again to obtain a 1 kg sub-sample. Fodder beet samples were collected
137 by pulling 10 random plants from the subsequent weeks grazing area. The leaves
138 of each plant were separated from the bulb and the weight of leaf and bulb

139 recorded. Leaves were then chopped and mixed, quartered and a sub sample
140 collected. Loose soil was washed from the bulb which was then cut into
141 approximately 2 cm cubes, mixed, quartered, and a 1 kg sub-sample obtained.
142 Samples of big bale silage were collected from the bales being fed on the day of the
143 visit. Crop and pasture yield, pre and post grazing, were assessed on each occasion
144 by quadrat cut (10 x 1 m² for K and F, and 10 x 0.1 m² for G), as described by Atkins
145 et al. (2018), and the area grazed, number of heifers, and silage fed to calculate the
146 proportion of crop and silage in the diet of each farm.

147 Beginning at approximately 1000 h on each visit body weight (BW) of the
148 heifers was recorded using electronic weigh-cells (Trutest, Auckland, New
149 Zealand), and body condition score (BCS) recorded. On visits 1 and 3, a hair length
150 sample was collected as described by Boyle et al. (2008). On each of the 3 visits
151 blood samples were collected from 12 pairs of study heifers via the coccygeal vein
152 into tubes (Becton Dickinson Vacutainer Systems, Plymouth, UK) containing
153 K₂EDTA, lithium heparin or without an anti-coagulant, and immediately stored on
154 ice until centrifuged at 1300 g and 4°C for 10 minutes. Plasma and serum were
155 decanted and stored at -20°C until subsequent analysis. In addition, a sub-sample
156 of whole blood was stored at -20°C prior to subsequent analysis. The farms
157 recorded calving date and calving-ease score as: 1. No assistance/ calved unaided,
158 2. Farmer assistance – normal presentation, 3. Farmer assistance – mal
159 presentation, 4. Vet assistance.

160

161 *2.3. Chemical analysis*

162

163 Forage samples were analysed for dry matter (DM) according to AOAC
164 (2012; 934.01) prior to milling through a 1 mm screen (Cyclone Mill, Retsch, Haan,
165 Germany). Dried, milled samples of fodder beet leaf and bulb were then bulked in
166 proportion to the measured amount to create a representative sample of the whole
167 crop. Crude protein (CP) concentration of the forages was determined by
168 combustion using a LECO FP 528 N analyser (Leco Corporation, St. Joseph, MI)
169 according to AOAC (2012; 990.03), neutral detergent fibre (NDF) content was
170 determined according to Van Soest et al. (1991), and water-soluble carbohydrate
171 (WSC) content as described by Thomas (1977). In addition, forage samples were
172 digested using the DigiPREP digestion system (Qmx Laboratories, Essex, UK), for
173 analysis by inductively coupled plasma mass spectrometry (ICP-MS; Thermo
174 Fisher Scientific Inc., Hemel Hempstead, UK), as described by Sinclair and Atkins
175 (2015). Briefly, 0.5 g duplicates of dried, milled sample were accurately weighed
176 into a DigiTUBE and 1 mL of concentrated HCl and 6 mL of concentrated HNO₃
177 added. The tubes were then heated over 30 min to 45 °C in a DigiPREP heating
178 block and held for 1 min before being increased over 25 min to 65 °C, held for
179 another 5 min, then increased over 15 min to 100 °C and refluxed for 40 min. The
180 digested samples were then diluted to 50 mL with purified water. On the same day
181 as analysis, a reagent blank of 2% HNO₃, 1% methanol and 0.1% Triton X (Sigma-
182 Aldrich Ltd., Gillingham, UK) was prepared using purified water, with Gallium (Ga)
183 as an internal standard. Calibration standards were prepared within an expected
184 range of the analytes for content of calcium (Ca), magnesium (Mg), phosphorous
185 (P), potassium, sodium (Na), S, Co, Se, Cu, iron (Fe), zinc (Zn), manganese (Mn),
186 and molybdenum (Mo). A 2.5 mL aliquot of digested sample was diluted with 2.5

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

195 Blood plasma samples were analysed for Co, Se, Cu, Zn, Fe and Mn by ICP-
196 MS as described by Cope et al. (2009). Briefly, 1 mL of lithium heparin plasma was
197 pipetted into auto-sampler tubes and diluted with 4 mL of reagent blank
198 consisting of 1% HNO₃, 1% methanol, 0.1% Triton X and Ga internal standard
199 before analysis. Plasma samples were analysed in duplicate, with a calibration
200 standard and blank routinely analysed amongst samples to assess instrument
201 performance. Fresh whole blood samples were analysed using a Vet Animal Blood
202 Counter (Woodley Equipment Company Ltd., Bolton, UK) to determine white blood
203 cells (WBC), red blood cells (RBC), haemoglobin (Hb), haematocrit (Hct), platelets
204 (Plt), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH)
205 and mean corpuscular haemoglobin concentration (MCHC). The performance of
206 the Vet Animal Blood Counter was assessed at each use, using a bovine whole
207 blood reference sample (Woodley Haematology Control, WD1154), with inter-
208 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,
209 MCH and MCHC respectively. Sub-samples of whole blood samples were analysed
210 for glutathione peroxidase (GSH-Px; intra-assay CV% 1.2) and superoxide

211 dismutase (SOD; intra-assay CV% 2.3) (Randox Laboratories, County Antrim, UK;
212 kit catalogue no. RS505 and SD125, respectively), using a Cobas Miras Plus auto
213 analyser (ABX Diagnostics, Bedfordshire, UK). Samples of blood serum were
214 analysed for β -hydroxybutyrate (3-OHB) and urea (Randox Laboratories, County
215 Antrim, UK; kit catalogue no. RB1008 and UR221; intra-assay CV% 3.5 and 2.8
216 respectively). Blood serum from the initial and final visit were also analysed for
217 vitamin B₁₂ (LOD = 33 pmol/L, and CV for low, med and high quality controls were
218 13.1, 9.3 and 7.9% respectively) and thyroxine (T₄; LOD = 3.9 nmol/L, and CV for
219 low, med and high quality controls were 8.7, 6.0 and 5.8% respectively) using the
220 ADIVA Centaur CP at the Animal and Plant Health Agency Scientific Laboratory,
221 Shrewsbury, Shropshire, UK.

222

223 2.4. Statistical analysis

224

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

235

236 **3. Results**

237

238 *3.1. Feed analysis and blood mineral concentration*

239

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

251 Calcium concentration was highest in kale, and lowest in fodder beet, with
252 an intermediate concentration in grass ($p < 0.05$), whereas P and potassium
253 concentration were similar ($p > 0.05$) across all forages (Table 2). The
254 concentration of Na and Mg were higher ($p < 0.05$) in fodder beet compared with
255 grass or kale. In contrast, kale had a higher ($p < 0.05$) concentration of S than either
256 grass or fodder beet. Grass had a higher concentration ($p < 0.05$) of both Mn and
257 Co compared to kale or fodder beet, while Fe and Cu concentrations were lower (p
258 < 0.05) in kale than either grazed grass or fodder beet. The concentration of Zn

259 also tended ($p < 0.1$) to be lowest in kale, while Se tended ($p < 0.1$) to be highest in
260 grass compared to the other 2 forages. Fodder beet had a lower concentration (p
261 < 0.05) of Mo compared to grass or kale. The mineral content of the supplementary
262 forage was similar across farms ($p > 0.05$). Total diet mineral intake differed
263 between forage source ($p < 0.05$) for Ca, being highest in K and lowest in F; Co,
264 where G was highest and K lowest; Cu, where K was lower than G or F; Fe, where
265 G was highest and K lowest; and Mo, where G and K were higher than F.

266 Supplementation with mineral boluses increased plasma concentrations of
267 Co, Se and Cu ($p < 0.05$; Table 3). Mean plasma Co was 0.021 and 0.041 $\mu\text{mol/L}$ in
268 B- and B+ respectively, with the concentration being higher at the middle and end
269 of the out-wintering period in B+ compared to the beginning ($p < 0.05$). Mean
270 plasma Se concentration was 0.50 and 0.82 $\mu\text{mol/L}$ in B- and B+ respectively, with
271 heifers that received a trace mineral bolus having higher concentrations at the
272 middle and end of out-wintering compared to the beginning ($p < 0.05$). In heifers
273 receiving GB-, plasma Se concentration was higher at the end of the out-wintering
274 period compared to animals receiving FB- ($p < 0.05$), but similar to those receiving
275 KB-. Mean plasma Cu concentration was 11.3 and 14.5 $\mu\text{mol/L}$ in B- and B+
276 respectively, and the provision of a mineral bolus did not affect plasma
277 concentrations in heifers receiving G or F ($p > 0.05$), but in heifers receiving K,
278 plasma Cu was higher ($p < 0.05$) in animals receiving a bolus (KB+) at the middle
279 and end of the out-wintering period compared to those that did not receive a bolus
280 (KB-). There was no effect of providing a mineral bolus on plasma Mn, Fe, Zn or Mo
281 concentration ($p < 0.05$), which averaged 0.06, 62.3 12.6 and 0.48 $\mu\text{mol/L}$
282 respectively. Plasma Fe concentration was lower ($p < 0.05$) at the end of the out-

283 wintering compared to the beginning in heifers receiving G or F, while in heifers
284 receiving K, plasma Zn was lower ($p < 0.05$) at the middle compared to the
285 beginning. Plasma Zn concentration was also lower ($p < 0.05$) in heifers receiving
286 G at the middle of the out-wintering period, while in heifers fed F, Zn was lower (p
287 < 0.05) at the end. In heifers receiving G, plasma Mo concentration was higher (p
288 < 0.05) at the middle and end of the out-wintering period compared to the
289 beginning. However, in heifers receiving K, plasma Mo was lower ($p < 0.05$) at the
290 end of out-wintering compared to the beginning, while plasma Mo was similar ($p >$
291 0.05) over the out-wintering period in heifers receiving F.

292

293 *3.2. Blood vitamin, enzyme and metabolite concentrations*

294

295 Heifers that received the trace-mineral boluses had a higher ($p < 0.05$)
296 mean serum vitamin B₁₂ concentration than those that did not receive the bolus,
297 with mean concentrations of 116 and 128 pmol/L for B- and B+ respectively (Table
298 4). However, in heifers receiving G, serum vitamin B₁₂ concentration did not differ
299 between the start and end of the out-wintering period ($p > 0.05$), while in heifers
300 receiving F, concentrations were lower ($p < 0.05$) at the end of out-wintering
301 compared to the beginning. In contrast, heifers receiving kale along with the
302 mineral boluses (KB+), had a higher ($p < 0.05$) serum vitamin B₁₂ concentration at
303 the end of out-wintering compared to KB-. Heifers that received the trace-mineral
304 boluses had a higher ($p < 0.001$) mean blood SOD concentration than un-
305 supplemented heifers at the end of the out-wintering period, with mean
306 concentrations of 2067 and 2338 U/g Hb for B- and B+ respectively. However;

307 there was no main effect ($p > 0.05$) of forage source, although heifers fed KB- had
308 the lowest ($p < 0.05$) SOD at the end of the out-wintering period compared to any
309 of the other treatments. Heifers that received the boluses also had a higher ($p <$
310 0.05) mean blood GSH-Px concentration than un-supplemented animals, with a
311 mean concentration at the end of out-wintering of 30 and 76 U/mL Hct in B- and
312 B+ respectively. In heifers that received the trace mineral boluses, blood GSH-Px
313 concentration increased ($p < 0.05$) by the end of the out-wintering period
314 irrespective of forage source, but in animals that did not receive a mineral bolus
315 and grazed kale (KB-), blood GSH-Px concentration decreased ($p < 0.05$). The
316 serum concentration of T₄ did not differ between treatments at the beginning of
317 the study ($p > 0.05$), with heifers that received B+ having a similar ($p < 0.05$)
318 concentration at the end of the out-wintering period compared to the beginning,
319 while those that received B- had a higher ($p < 0.05$) concentration. Provision of the
320 mineral boluses did not affect serum 3-OHB concentration ($p > 0.05$), with an
321 overall mean of 0.38 mmol/L in B- and B+. However, heifers receiving K had a
322 higher concentration of 3-OHB at the end of out-wintering compared to the
323 beginning ($p < 0.05$), whereas in animals receiving F, serum 3-OHB concentration
324 decreased with time ($p < 0.05$). Serum urea concentration was not affected by
325 providing mineral boluses ($p > 0.05$), with an overall mean of 4.1 mmol/L for B-
326 and B+. In contrast, for heifers receiving any of the forages, serum urea
327 concentration was lower at the end of out-wintering period than the beginning (p
328 < 0.05).

329

330 *3.3. Haematology*

331

332 Provision of the mineral boluses had no effect ($p > 0.05$) on WBC, with an
333 overall mean count of 8.27 and 8.57 $\times 10^3$ cells/mm³ in B- and B+ respectively,
334 although there was a trend ($p < 0.1$) for fewer WBC at the end compared to the
335 beginning of the out-wintering period in heifers receiving F along with a bolus
336 (FB+; Table 5). Similarly, the provision of the trace mineral boluses had no effect
337 ($p > 0.05$) on Hb, with an overall mean concentration of 12.2 g/dL blood. However,
338 there was an interaction between time and forage, with Hb being lower ($p < 0.05$)
339 during the middle and end compared to the beginning of the out-wintering period
340 in heifers receiving G or F. In contrast, in heifers fed K, Hb was higher ($p < 0.05$) in
341 the middle compared to the beginning of the out-wintering period. The mineral
342 boluses had no effect ($p > 0.05$) on RBC, with a mean count of 7.73 and 7.79 $\times 10^3$
343 cells/mm³ in B- and B+, respectively. However, in heifers receiving G or F, RBC was
344 lower ($p < 0.05$) during the middle and end compared to the beginning of the out-
345 wintering period. In contrast, at the end of out-wintering, heifers receiving KB+
346 had a higher ($p < 0.05$) RBC concentration than those receiving KB-. The mineral
347 boluses had no effect ($p > 0.05$) on blood Hct volume, with an overall mean of 33.4
348 and 33.3 % for B- and B+ respectively, but there was an effect of time, with Hct
349 being lower ($p < 0.05$) during the middle and end compared to the beginning of
350 the out-wintering period in heifers receiving G or F. In contrast, Hct increased ($p <$
351 0.05) in heifers receiving K between the beginning and middle of the out-wintering
352 period. Heifers that received the trace mineral boluses had a lower ($p < 0.05$) mean
353 MCV than those that did not, with an overall mean at the end of the out-wintering
354 period of 44.8 and 43.3 μm^3 in B- and B+ respectively. The provision of the trace

355 mineral boluses resulted in a higher ($p < 0.05$) MCV in heifers receiving KB+ than
356 KB-. In heifers receiving G, F or KB+, MCV did not change ($p > 0.05$) over the out-
357 wintering period. In contrast, in heifers receiving G or F, MCH decreased ($p < 0.05$)
358 between the start and the end of out-wintering. The provision of the trace mineral
359 boluses had no effect ($p > 0.05$) on MCHC, with an overall mean of 36.8 g/dL.
360 However, in heifers receiving F, MCHC was lower ($p < 0.05$) at the end of out-
361 wintering compared to the beginning. In contrast, MCHC was higher ($p < 0.05$) at
362 the middle compared to the beginning of out-wintering in heifers fed K, but was
363 unchanged ($p > 0.05$) in heifers fed G. There was no effect of the mineral bolus on
364 Plt count ($p > 0.05$), with an overall mean of $314 \times 10^3/\text{mm}^3$.

365

366 *3.4. Animal performance*

367

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

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

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

385 Coat length increased by 4.3 mm over the study period ($p < 0.001$), but
386 bolus provision had no effect ($p > 0.05$), with a mean of 23.2 and 23.4 mm for B-
387 and B+ respectively. There was no effect ($p > 0.05$) of forage source on the
388 proportion of heifers that calved unassisted, with a mean value of 0.89, whilst the
389 provision of trace mineral boluses also had no effect ($p = 0.877$) on the proportion
390 of heifers that calved un-aided, with 0.11 and 0.10 (odds ratio = 0.95, 0.47 – 1.91
391 95% CI; n obs. = 166 B-, 165 B+) requiring some assistance to calve in B- and B+,
392 respectively.

393

394 **4. Discussion**

395

396 *4.1. Blood parameters*

397

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

403 (Atkins et al., 2014; Edwards et al., 2017; Keogh et al., 2009). A combined trace
404 mineral bolus was used as some trace minerals (e.g. I) are not available in this form,
405 and combined boluses represent commercial practice on many farms (Sinclair and
406 Atkins, 2015). The provision of trace-mineral boluses in the current study had no
407 effect on plasma Cu concentration in animals fed G or F, but concentrations were
408 increased in heifers fed kale. Blood Cu typically ranges between 11 – 25.6 $\mu\text{mol/L}$
409 (mean 18 $\mu\text{mol/L}$; Rushton, 1981), and plasma Cu of animals fed K without bolus
410 was 10.3 $\mu\text{mol/L}$ by the end of the out-wintering period indicating marginal status.
411 The typical recommend dietary concentration of Cu is between 11-15 mg/kg DM
412 (NRC, 2001), and therefore the dietary Cu concentration (predicted from the
413 forage and supplementary feed) in the G, K and F treatments in the current study
414 were considerably lower at 4.2, 2.1 and 4.6 mg Cu/kg DM, respectively.
415 Additionally, the high S content in kale was likely to have had an antagonistic effect
416 on Cu absorption due to the formation of thiomolybdates in the rumen (Suttle,
417 2010). Using the prediction equations of Suttle and McLauchlin (1976) and the
418 mean forage S and Mo concentrations resulted in a predicted absorption co-
419 efficient of Cu (%) of 4.5 and 4.4 in the grass and fodder beet fed animals
420 respectively, but only 2.5% in the kale fed animals, further compounding the low
421 dietary concentration in animals grazing this forage. However, Cu deficiency is not
422 immediately reflected in plasma Cu, as hepatic Cu is metabolised to maintain
423 homeostasis (Evans, 1973), and plasma Cu has been shown to be a poor indicator
424 of hepatic reserves (Sinclair et al., 2013, 2017). The reduced SOD levels in kale fed
425 heifers that did not receive a mineral bolus may also indicate that these animals
426 were deficient in Cu, although all values were low compared to other studies that

427 have fed additional S and Mo to dairy cattle (Sinclair et al., 2013, 2017). Animals
428 fed kale have previously been reported to demonstrate symptoms of Cu deficiency
429 and haemolytic anaemia due to ruminal production of SMC₂O (Barry et al., 1981).
430 In the current study, heifers fed kale without a bolus had a lower Hb and RBC, but
431 a greater MCV at the end of out-wintering than those fed kale with a bolus. These
432 effects suggest the onset of anaemia in heifers fed kale, as elevated MCV is
433 indicative of a bone marrow response and the presence of immature red blood
434 cells (Otter, 2013). This negative effect of kale was, however, mitigated by the
435 trace-mineral bolus, and Cu supplementation of animals fed kale has previously
436 been reported to allow the recovery from haemolytic anaemia (Barry et al., 1981).

437 Provision of trace-mineral boluses increased the concentration of Co in the
438 plasma of heifers. The NRC (2001) stated the requirement for Co as 0.11 mg/kg
439 DM, while others have estimated it as low as 0.06 mg/kg DM in grazing cattle
440 (Clark et al., 1999). More recent studies in high yielding dairy cows has
441 demonstrated that a dietary supply of 0.21 mg/kg DM is more than sufficient to
442 meet the demands during the peri-parturient period and that there was no benefit
443 to additional Co or vitamin B₁₂ (administered per os or injection) on performance
444 or indicators of ketosis (Weerathilake et al., 2018). The Co content of grazed grass
445 in this study was approximately 10 times above requirements and consequently
446 additional Co would not have been expected to have any benefit. In contrast, the
447 Co content of kale in this study was 0.05 mg/kg DM, approximately half the
448 required dietary level (NRC, 2001). Within the rumen, elemental cobalt (Co) is
449 used by bacteria to synthesise vitamin B₁₂ and animal status is generally assessed
450 by measuring blood vitamin B₁₂ concentration. Vitamin B₁₂ has two principal

451 metabolic functions in cattle; firstly, methylcobalamin acts as a co-factor in the
452 transformation of methylmalonyl CoA to succinyl CoA which is then used within
453 the liver for the synthesis of glucose from propionate (McDowell, 2000). Secondly,
454 adenosylcobalamin is a co-factor in methionine synthase which is involved in the
455 synthesis of methionine from homocysteine (McDowell, 2000). A threshold
456 concentration of 150 pmol vitamin B₁₂/L has been suggested, above which there
457 is little benefit to animal performance (Duplessis et al., 2017), although others
458 have set the threshold at 90 pmol/L (Grace et al., 2014). In the current study, all
459 serum B₁₂ concentrations were intermediate between these two threshold values.
460 The low Co content of the kale may explain the decrease in vitamin B₁₂ status in
461 un-supplemented heifers fed this forage over the out-wintering period compared
462 to the increase in supplemented animals. Despite fodder beet fed animals
463 receiving an adequate dietary concentration of Co, the vitamin B₁₂ status of heifers
464 decreased over the out-wintering period, regardless of receiving a bolus. This
465 reduction in vitamin B₁₂ status may have been as consequence of the low fibre and
466 high water soluble content of fodder beet, as ruminal vitamin B₁₂ synthesis has
467 been reported to be decreased in animals fed low roughage diets, most probably
468 due to a low ruminal pH decreasing microbial synthesis (Smith et al., 1970; Walker
469 and Elliot, 1972).

470 Plasma Se concentrations in the current study increased in heifers that
471 received a trace mineral bolus across all the three forages, however, marginal
472 limits for serum Se are 0.10 – 0.12 µmol/L (Suttle, 2010), and all treatments
473 exceeded this threshold. Corresponding to plasma Se, plasma GSH-Px was
474 increased in heifers receiving the bolus. A recommended Se content in the diet of

475 cattle is 0.05 mg/kg DM in grazing situations where there is an adequate vitamin
476 E supply (CSIRO, 2007). All the forages offered to the out-wintered heifers in this
477 study were well in excess of this level of Se. However, even in the pasture, which
478 had the highest concentration of Se at 0.23 mg/kg DM, levels were below the 0.3
479 mg/kg DM recommended by NRC (2001). Plasma GSH-Px concentration in heifers
480 receiving pasture or fodder beet without a trace mineral bolus did not alter over
481 the out-wintered period, further suggesting that Se concentration was adequate in
482 the basal diet. However, there was a reduction from 46 to 18 U GSH-Px/mL Hct in
483 heifers receiving kale without a bolus, despite kale containing a similar level of Se
484 to the fodder beet (0.12 mg/kg DM). High dietary S may antagonise Se metabolism
485 (Arthington, 2008), and plasma Se levels have been observed to decrease linearly
486 with increasing dietary S concentration in dairy cows (Ivancic and Weiss, 2001).
487 The S content in kale is known to be high and a mean concentration of 5.8 g/kg DM
488 was recorded on the farms in the current study. However, increasing dietary S from
489 2.0 to 7.8 g/kg DM was previously observed to have little effect on GSH-Px
490 concentration in cattle (Khan et al., 1987). Selenium deficiency has not previously
491 been considered important with brassica diets, however the free amino acid s-
492 methyl cysteine sulphoxide (SMCO) present in kale has been implicated in reduced
493 GSH-Px activity of cattle and sheep (Barry et al., 1981). Barry et al. (1981) also
494 suggested that Cu and Se status are related, as Cu containing SOD forms a coupling
495 with Se containing GSH-Px in the erythrocytes, with SOD catalysing the reduction
496 of superoxide anions to hydrogen peroxide and GSH-Px reducing hydrogen
497 peroxide to water. As with GSH-Px, blood levels of SOD were also lower in the
498 heifers fed kale without a bolus in this study.

499 Thyroid regulation also involves Se, with the concentration of T₄ reported
500 to increase in Se deficient animals (Arthur et al., 1993; Wichtel et al., 1996). Both
501 kale and fodder beet fed heifers in the current study had similar dietary Se
502 concentrations and also had higher concentrations of T₄ in the animals that did not
503 receive a trace mineral bolus, despite a greater than required (0.33 mg/kg DM;
504 NRC, 2001) I content in all three forages. This could indicate that thyroid function
505 could have been restricted by the dietary Se content in kale and fodder beet fed
506 heifers without a bolus and further work may be warranted to determine the effect
507 of Se in animals fed these forages. It is also well established that brassica crops
508 such as kale contain high levels of cyanogenic goitrogens which can be overcome
509 by additional dietary I, which is reflected in serum T₄ concentrations, with values
510 of 25-50 nmol/L indicating marginal status (Suttle, 2010). In the current study
511 serum T₄ concentrations exceeded this threshold on all treatments, and were little
512 affected by the provision of a bolus. In contrast, thiouracil-type goitrogens are not
513 influenced by dietary I supply, but are generally not present in sufficiently high
514 concentrations in brassicas such as kale, or in grass and fodder beet (Suttle, 2010).

515

516 *4.2. Animal performance*

517

518 The trace-mineral bolus appeared to have little effect on the physical
519 performance of pregnant, growing heifers over the out-wintering period in the
520 current study, although animals on all treatments were below the target weight at
521 calving for similar systems (Roche et al., 2015). Heifers that received the trace-
522 mineral boluses lost less body condition by the end of the study, however the effect

523 was biologically small and may have little impact on production or fertility (Roche
524 et al., 2015). Heifers fed F, and to a lesser extent G, tended to receive a lower dietary
525 crude protein concentration, which may have been a limiting factor, but was not
526 reflected in their performance or plasma urea concentrations. Inadequate energy
527 intake may also have been limiting animal performance as the ADG of heifers in
528 this study was low, and below that reported for other commercial herds (Atkins et
529 al., 2013). Out-wintering systems have previously been reported to be able to
530 support high levels of performance with an ADG of 1.10 kg/d in pregnant Holstein-
531 Friesian heifers fed a diet consisting of 70% kale and 30% grass silage (Kennedy
532 et al., 2012), and 1.24 or 0.95 kg/d in pregnant Holstein heifers receiving fodder
533 beet or grazed grass with grass silage respectively (Atkins et al., 2018). Judson and
534 Edwards (2008) reported that many farmers feeding kale to pregnant dairy cows
535 in New Zealand and using a similar system to that described here, underestimated
536 the crops herbage mass or overestimated the cows' intake. The difference in
537 performance reported by previous studies involving out-wintered heifers and
538 those in this study could therefore be in part due to low or inaccurate feed
539 allocation.

540

541 **5. Conclusion**

542

543 There was no effect of forage source or provision of a Co, Se, I and Cu
544 containing trace-mineral boluses on animal performance, except body condition
545 prior to calving which was slightly higher in heifers receiving boluses. Provision of

546 trace-mineral boluses increased plasma concentrations of the minerals supplied
547 except for Cu in heifers fed grass or fodder beet. Despite the increase in plasma Co
548 with heifers fed fodder beet, serum vitamin B₁₂ decreased in heifers fed this forage.
549 The blood metabolite and haematology results suggest that the trace-mineral
550 bolus was effective at counteracting many of the anti-nutritional factors associated
551 with kale. The use of a trace-mineral bolus when out-wintering pregnant heifers is
552 therefore recommended, particularly for heifers grazing kale, but further research
553 is required to more accurately define mineral requirements amongst the different
554 forages.

555

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557

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561

562 **Conflict of interest statement**

563

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

566

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726

727

728

Table 1

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

Element	LOD, µl/l	LOQ, µl/l	Certified reference material							
			intra-assay CV%		EU BCR-129, hay			EU BCR-708, dairy concentrate		
			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	<i>P</i> -value	G	K	F	SED	<i>P</i> -value	G	K	F	SED	<i>P</i> -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

item	Treatment						SED	B	Fo	B x Fo	P-value†			
	GB-	KB-	FB-	GB+	KB+	FB+					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

item	Treatment						SED	B	Fo	B x Fo	P-value [†]			
	GB-	KB-	FB-	GB+	KB+	FB+					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	<0.001	<0.001	<0.001	<0.001
mid [‡]	42	22	37	57	61	55	9.8	<0.001	0.731	<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	<0.001	0.266	<0.001	0.469
mid	0.28	0.36	0.35	0.28	0.39	0.31	0.078	0.853	0.604	0.124				
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	<0.001	0.616	<0.001	0.564
mid	3.7	4.7	2.8	3.5	4.8	2.9	0.66	0.788	0.051	0.305				
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; T₄ = 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

item	Treatment						SED	Bo	Fo	B x Fo	P-value†			
	GB-	KB-	FB-	GB+	KB+	FB+					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 ^s	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 ^s	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 ^s	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 ^s	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 ^s	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 ^s	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 ^s	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 ^s	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 ^s	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 ^s	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 ^s	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 ^s	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 ^s	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 ^s	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

item	Treatment						SED	B	Fo	P-value [†]			
	GB-	KB-	FB-	GB+	KB+	FB+				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			
end	26.2	24.9	24.3	26.2	24.6	26.1	1.41	0.282	0.617	0.121	<0.001	0.715	0.125

[†]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. Interaction of B, Fo and T was not significant ($p > .05$)

[‡]means adjusted for initial value covariate