**CSIRO** PUBLISHING

*Animal Production Science*, 2019, **59**, 921–932
https://doi.org/10.1071/AN17251

# Learned behaviours lead to bone ingestion by phosphorus-deficient cattle

*R. M. Dixon*[A,F], *M. T. Fletcher*[B], *K. L. Goodwin*[C], *D. J. Reid*[D], *D. M. McNeill*[E], *K. W. L. Yong*[B] *and J. C. Petherick*[A]

[A]The University of Queensland, Queensland Alliance for Agriculture and Food Innovation, Centre for Animal Science, PO Box 6014, Red Hill, Rockhampton, Qld 4701, Australia.
[B]The University of Queensland, Queensland Alliance for Agriculture and Food Innovation, Centre for Animal Science, Coopers Plains, Qld 4108, Australia.
[C]Department of Agriculture and Fisheries, Brian Pastures, PO Box 118, Gayndah, Qld 4625, Australia.
[D]Department of Agriculture and Fisheries, PO Box 6014, Red Hill, Rockhampton, Qld 4701, Australia.
[E]The University of Queensland, School of Veterinary Science, Gatton, Qld 4343, Australia.
[F]Corresponding author. Email: r.dixon77@uq.edu.au

**Abstract.** Grazing cattle deficient in phosphorus (P) often seek out and chew bones, apparently to obtain dietary P. To investigate this phenomenon heifers naïve to P deficiency were either fed a P-deficient diet (LowP) or grazed P-adequate pasture (AdeqP), and preference tests examined their attraction to weathered bones or a control of wood. During Phase 1 (Days 1–145), the LowP heifers developed severe P deficiency and pica, but demonstrated little attraction to weathered bones. During Phase 2 (Days 146–155), heifers were allowed to interact with and to chew a variety of weathered bones. After this experience LowP heifers were more attracted to bones during Phase 3 (Days 156–166) than during Phase 1 ($P < 0.05$), and more attracted than AdeqP heifers during either phase. Subsequently, in Phase 4 (Days 167–171), LowP heifers were more attracted than AdeqP heifers ($P < 0.01$) to weathered bones than to a control of wood, and in Phase 5 (Days 172–176) to bones with more extended weathering. During Phase 6 (Days 177–182), attraction was reduced when bones were placed inside a cloth bag. The olfactory constituents from weathered bones were dominated by aliphatic aldehydes and ketones, consistent with long-chain fatty acid breakdown. It was concluded that attraction of P-deficient cattle to seek and ingest bones is primarily a learned response. Smell, taste and visual appearance all appear to be important cues for attraction. Pica is likely important in causing P-deficient cattle to investigate unusual materials, including bones, resulting in cattle learning by making an association between bone chewing and P ingestion.

**Additional keywords:** bone chewing, learned behaviour, phosphorus deficiency, post-ingestive feedback.

Received 22 April 2017, accepted 22 February 2018, published online 28 May 2018

## Introduction

The importance of phosphorus (P) nutrition on the health, growth and reproduction, and the occurrence of nutritional deficiencies of P in grazing cattle in major rangeland regions of Australia, Africa and South America with low-P soils, are well known (Theiler and Green 1932; McCosker and Winks 1994; McDowell 1996; CSIRO 2007). The alleviation of nutritional deficiencies of P in cattle grazing rangelands dominated by P-deficient forages has often substantially increased growth, reproduction and herd productivity (Holmes 1990; Winks 1990; Dixon *et al*. 2011). In seasonally dry tropical rangeland environments the greatest responses to P supplementation by cattle occur during the rainy season since this is when dietary P requirements are highest for growth and lactation. However, effective P-supplementation strategies have often been poorly adopted by cattle managers in extensive rangelands (Niethe 2011). One important reason for such low adoption is that it is often difficult for cattle managers to achieve sufficient voluntary intake of commercial P supplements provided free choice during the rainy season when green and palatable forages are available. Characteristically, these supplements contain calcium phosphates as the source of P and are provided as low palatability loose mixes with salt, or as solidified feed blocks (McDowell 1996; Dixon *et al*. 2001, 2003).

Pica in cattle and other herbivores has long been recognised and is characterised by seeking out and ingestion of unusual objects such as sticks, soil and bones (Green 1925; Theiler and Green 1932; McCosker and Winks 1994). Pica, and in particular bone chewing, is considered indicative of a severe nutritional deficiency, most often of P. Bone-chewing behaviour has been reported in many species of ungulates, including cattle, antelope, sheep, deer and giraffes (Theiler and Green 1932;

 

Brothwell 1976; Langman 1978; Bowyer 1983; Bredin *et al*. 2008). A likely explanation for such bone chewing is that in rangeland landscapes with very P-deficient soils and forages bones comprise one of the few concentrated sources of P accessible to herbivores.

Blair-West *et al*. (1989, 1990, 1992) reported that some olfactory constituents of weathered bones are highly attractive to P-deficient cattle, but not to P-adequate cattle. Also, preference tests indicated that P-deficient cattle were strongly attracted to chew and ingest old weathered bones even when crushed and mixed with other materials, and also to some bone extracts (Blair-West *et al*. 1990). Such attraction occurred at a distance (e.g. >1 m) and also when the bones were covered with cloth. However, P-deficient cattle were not attracted to fresh bones, plaster models of bones, fresh or putrefying meat, or phosphate salts. This was in agreement with observations in earlier studies of attraction of P-deficient cattle to weathered bones, but not to calcium phosphate (Theiler and Green 1932; Gordon *et al*. 1954; Coppock *et al*. 1976). Blair-West *et al*. (1989, 1992) concluded that the attraction of P-deficient cattle to weathered bones depended primarily on olfactory constituents of weathered bones produced during decay, and that this attraction was innate.

In planning the present study the hypothesis of Blair-West *et al*. (1989, 1990, 1992) that P-deficient cattle were innately attracted to weathered bones, and that the olfactory components of weathered bones were implicated in this attraction, was accepted. The present study was intended to identify the olfactory components responsible for such innate attraction of P-deficient cattle to weathered bones. Phase 1 of the study was designed to validate a preference test bioassay similar to that used by Blair-West *et al*. (1989, 1992) but more appropriate for cattle grazing tropical rangelands. The preference test utilised young cattle adapted to a tropical environment and previously naïve to P deficiency, and initially examined the attraction of P-deficient and P-adequate heifers to weathered bones or to a control treatment (wood sticks). However, it became apparent that although P-deficient heifers developed pica they did not, in contrast with the previous studies, demonstrate a preferential attraction to weathered bones. Therefore, a series of phases of preference tests was conducted to examine the consequences of providing the heifers with the opportunity to interact with and ingest bones, their attraction to bones weathered for various intervals, and the roles of visual and olfactory cues in the attraction of heifers to bones.

## Experimental methods

### *Animals and feeding*

The study was conducted under Queensland Department of Agriculture and Fisheries Animal Ethics Committee Approval SA2012/05/384. Also, as required by *Queensland Stock Regulations 1988*, permission was obtained from the Chief Inspector of Stock for Queensland, Australia, to feed restricted animal material (bones) to cattle during the study as approved research. Heifers from a tropical composite genotype herd at the Belmont Research Station (Rockhampton, Qld, Australia) were weaned in April 2012 at 5–8 months of age and then grazed on an improved *Chloris gayana*-based pasture growing on fertile alluvial soils. In July 2012 20 heifers were selected on the basis of low flight speed as a measure of temperament (Burrow and Dillon 1997) and on liveweight (LW; mean 181 (s.d. 14) kg) to provide a homogeneous group expected to be docile and tractable and in replete P status. Following relocation to the Brian Pastures Research Facility (Gayndah, Qld, Australia), also located in a subtropical environment, the heifers grazed on senesced native pasture for 3 weeks and then the improved pasture described below for 4 weeks. Faeces were sampled on three occasions (21 July, 14 and 22 August 2012), and blood on one occasion (22 August 2012) to evaluate the P status of the heifers. The heifers were vaccinated for tick fever (Trivalent vaccine, Tick Fever Centre, Brisbane, Qld, Australia), botulism (Ultravac Botulinum, Pfizer Animal Health, Australia) and bovine ephemeral fever (Websters Pfizer Animal Health, Australia), and were treated for internal and external parasites (Paramectin, active ingredient abamectin, Jurox, Australia). The health of the heifers was monitored frequently.

On Day 1 (13 September 2012) the heifers were stratified on LW and randomly allocated to two treatment groups (each $n = 10$). One group of heifers was held in a yard (20 m × 30 m) and fed a diet with a low P concentration (LowP). The second group continued to graze on a 16-ha paddock of improved pasture (predominantly *Bothriochloa insculpta* cv. Bisset (creeping bluegrass) and *B. pertusa* (Indian couch) grasses, with some *Stylosantheses* spp. and native forbs) on fertile soils to provide a diet expected to be adequate in P (AdeqP). The LowP diet contained (g/kg air-dry basis) 403 chopped wheat straw, 349 wheat flour, 166 sugar, 37 canola oil, 21 urea, 11 limestone, 5.5 ammonium sulfate, 5.4 salt, 0.90 Rumigro premix (BEC Feed Solutions, Brisbane, Qld, Australia) and 0.45 rumensin 100 (containing 100 g/kg monensin as sodium monensin; Elanco Animal Health, Sydney, NSW, Australia). The mineral premix did not include any source of P, and contained (g/kg) zinc 45, manganese 40, ferrous iron 20, copper 12, cobalt 0.5, iodine 0.5, selenium 0.1, antioxidant 20, vitamin E (DL-α-tocopheryl acetate) 15, and (IU/kg) vitamin A (retinyl acetate) 7 000 000 and vitamin $D_3$ (cholecalciferol) 1 400 000. This LowP diet was introduced to the group of heifers over 2 weeks with gradational increases in the proportions of flour and sugar, and was offered *ad libitum*; orts were on average 13% (range generally 5–15%) of intake. As the nutritive value of the pasture available to the grazing AdeqP heifers declined with the progression of dry season conditions, these heifers were supplemented with cottonseed meal (457 g air dry/heifer.day, fed twice weekly) from Day 36 until Day 134 when, with the commencement of the rainy season, new growth of pasture was initiated.

### *Phases of the experiment*

During Phase 1 (Days 1–145), LW was measured, blood and faeces sampled, and seven preference tests conducted at 2–3-week intervals. In the preference tests, individual heifers were offered a simultaneous choice between a control or a test feed bin of identical appearance in a circular yard and as described below. During Phase 1, the test bin contained four types of weathered bones, which comprised (i) three long and three flat bones from the skeleton of a heifer that had weathered for

3 months, (ii) fragmented bone of the skull of the same skeleton, (iii) three long and three flat bones from the skeleton of a steer that had weathered for 13 months, and (iv) three long and three flat bones from the skeletons of a group of mature cows that had weathered for 9 years. These stated ages of weathering of the bones were at Day 1, and thus increased as the experiment progressed. The long bones were major leg bones, while the flat bones were scapulae or ribs, or the fragmented skull described for (ii) above. The control bin contained three pieces of wood (0.4–0.5 m long, 30–50 mm in diameter and comprising branches from a dead tree). The bones or wood in the bins were covered with steel mesh grid with apertures 46 $mm^2$, so that the heifers could see and smell, but not chew, the test materials.

During Phase 2 (Days 146–155), each treatment group of heifers was given access as a group to a variety of test materials comprising four new groups of bones and to the same pieces of wood as used in Phase 1. The four groups of bones each consisted of three long and three flat bones from skeletons that, at the time of Phase 2, had weathered for (i) 1 month (B1), (ii) 4 months (B2), (iii) 8 months (B3), or (iv) 9 years (from the group of cows described above; B4). On Days 146, 147, 150, 151 and 155 the groups of test materials were offered simultaneously in five open troughs (0.25 m wide, 0.15 m deep and 2 m long) placed at least 1 m apart in a yard 20 m × 30 m. Each trough contained one of the test materials and remained in the same position throughout Phase 2, while the allocation of the test materials to troughs was changed at random each day. The heifers were able to access, and to sniff, lick and chew, the test materials. The LowP and AdeqP heifers were introduced into the yard in their respective groups, for 60 min on Day 146 and for 30 min on Days 147–155. The sniffing, licking and chewing behaviour (as described below) of the individual heifers in relation to the materials in the various troughs was recorded by an observer elevated 2 m from the ground and above the fence railing. The individual bones or pieces of wood were weighed before and after each test, after wiping with paper towelling to remove saliva or soil contamination, and the amounts of air-dry material ingested were calculated. LW was measured, and blood and faeces were sampled on Days 147 and 154.

During Phase 3 (Days 156–166), the individual preference tests conducted during Phase 1 were repeated on three occasions and by using the same test materials in the bins. The test materials in the bins were again covered with the steel mesh grid. LW was measured and blood and faeces were sampled on Days 161 and 166.

During Phase 4 (Days 167–171), three tests compared the preference of the heifers for a bin containing bones that had weathered for 9 years (B4), with a control bin containing wood. The mesh grids used in Phases 1 and 3 were removed so that the heifers could lick and chew the test materials. During Phase 5 (Days 172–176), four preference tests were conducted between bins containing bones that had been weathered for 0.5 years or for 9 years, and without the mesh grids. During Phase 6 (Days 177–182), four preference tests were conducted with each of the groups of bones tested in Phase 5 placed inside a cotton cloth bag (~40 cm × 60 cm), and again without the mesh grids. LW was measured and blood and faeces were sampled on Days 175 and 183.

### *Sampling, measurements and preference tests*

AdeqP heifers were mustered to the yards at ~07.30 h on days when sampling or preference tests were conducted. LW was measured without fasting. Body condition score was estimated on a scale of 1–5 (1 = thin, 5 = fat; CSIRO 2007). Faecal samples (~100 g wet faeces per animal) obtained from the rectum of each heifer were bulked for each group. Blood (10 mL per animal) was obtained by jugular puncture by using vacutainers, with lithium heparin as an anticoagulant. These samples were centrifuged (3000*g* for 10 min at ambient temperature) and the plasma was stored frozen (−20°C). The voluntary intake of the LowP group of heifers fed in yards was measured weekly from the feed offered and refused. Samples of each weekly batch of the LowP diet were retained for analyses of P, and samples representing intervals within each phase were bulked and retained for subsequent analyses. Feed and dried faecal samples were stored at ambient temperature.

Preference by heifers during Phases 1, 3 and 4 for bins containing either bones or wood, and during Phases 5 and 6 for bins containing bones with two intervals of weathering, was tested individually. The tests were conducted in a circular yard (10-m diameter) constructed from 2-m long × 1.8-m high portable cattle yard panels. Each group of heifers was moved from the feed yard or the paddock and held in a small yard near to the test yard. Each heifer was separated from its group without any planned sequence by the stockman, and was then held in a 2 m × 4 m yard for 30–60 s to allow the animal to settle and to turn in the direction opposite to the entry gate of the circular test yard. Each heifer entered the test yard through a bat-wing gate (i.e. a pair of spring-loaded gates opening at the centre when a lever was pulled) on the southern position of the test yard. Two cylindrical steel bins (570-mm diameter, 210 mm high) identical in appearance containing the test materials were placed 1 m inside the yard fence at the eastern and western positions. The bat-wing gates were then opened so that the heifer could enter the test yard. Each heifer remained in the yard for 120 s, during which time an observer recorded the activities. The heifer was then allowed to exit through a gate on the northern side of the test yard. The sequence in which each heifer within its group was tested was recorded.

The following behaviour of the heifers to each of the bins was recorded: M1, whether on entering the test yard the heifer showed interest by looking at a bin; M2, whether on entering the test yard the heifer walked directly to a bin; M3, whether at any time during the 120-s period, the heifer visited either of the bins; M4, the cumulative length of time (s/120 s) during which the heifer had its head over or in a bin; M5, the occurrence of any sniffing (inhalation without tongue contact with the bone or wood); M6, licking (tongue contact); M7, chewing (bone or wood in the mouth and with apparent mastication) of the material in a bin during Phases 4–6. M1 was a pen measurement and was not related to the specific bins. Measurement M4 (time at bins) was considered as a continuous variable, while the other measurements were binary variables scored for each bin as 0 for no and 1 for yes. On the first test occasion of Phase 1, the position of the two test bins (i.e. the control and the bones) was allocated at random for each heifer, while for each subsequent test the positions of the two bins were reversed relative to the previous test with the same heifer. Before the experiment commenced,

the heifers were accustomed to the preference test procedures by conducting preference tests on each heifer on five occasions, with both bins containing ~20 g chopped lucerne hay and without the grid in place.

### *Headspace sampling of olfactory constituents of bones*

Rib bones from the skeletons that provided the bones in B1–B4 of Phase 2 were transported to laboratories at the Health and Food Sciences Precinct (Coopers Plains, Brisbane, Australia) for chemical analysis of volatiles. These laboratory analyses were conducted after the commencement of the animal experimentation and, hence, the weathering time of these bones B1–B4 was ~2 months longer than the nominal times of weathering described above. The respective intact bone samples were enclosed in clamp-sealed commercial oven bags (35 cm × 48 cm, Glad®) or sealed into in-house manufactured Melinex bags (50 cm × 50 cm) with a PTFE/silicone septa seal entry for sampling of volatiles in the enclosed headspace around the bones. Pre-conditioned solid-phase microextraction fibres (75-μM carboxen–polydimethylsiloxane, Supelco, Bellefonte, PA, USA) were inserted through the septa and exposed to bone volatiles for both short (40 min) and long (18 h) intervals in preparation for gas chromatograph–mass spectrometer analysis for volatile organic compounds. In addition, each of the bone samples was mechanically fragmented into pieces ranging from 1 to 5 cm in size, and a 10-g sample of fragments was placed in a 100-mL serum vial with septum cap and crimp-seal. These were similarly sampled by solid-phase microextraction for both short (40 min) and long (18 h) intervals in preparation for gas chromatograph–mass spectrometer analysis of volatile organic compounds.

### *Analytical procedures*

Samples of the LowP diet and of faeces were dried (60°C) before grinding. Feed samples were ground though a 1-mm screen using a laboratory mill (Christy and Norris, Chelmsford, United Kingdom). The feed samples were analysed for organic matter, total N, neutral detergent fibre, acid detergent fibre and lignin (AOAC 1984; Sweeney 1989). Samples of diet and faeces were digested in a nitric–perchloric acid mixture, and the concentrations of P and calcium (Ca) were analysed using an inductively coupled plasma spectrometer (Optima 7300 DV, Perkin Elmer, Waltham, MA, USA). Faecal samples from the AdeqP treatment heifers were ground through a 1-mm screen (Cyclotec mill, Model 1093, Foss Tecator AB, Hoganas, Sweden), redried at 60°C, cooled in a desiccator, and then scanned using a near-infrared spectrometer (NIRS, Foss 6500, NIRSystems Inc., Silver Spring, MD, USA) fitted with a spinning cup module. Chemometric analysis used ISI software (Intrasoft International, Port Matilda, PA, USA). Established calibration equations applicable to the pasture system were used to predict attributes of the diet selected by these grazing heifers from spectra of faeces; these attributes were the concentrations of non-grass (predominantly legume) and total nitrogen (N) in the diet, dry-matter (DM) digestibility and voluntary DM intake (Coates and Dixon 2008, 2011; Dixon and Coates 2009). The concentration of total P in the diet (g P/kg DM) selected by the heifers while grazing, both before the experiment and in the AdeqP treatment, was calculated from the concentration of total P in faeces (Dixon and Coates 2010), where Diet total P = 0.514 (faecal total P) – 0.12 (g/kg). Plasma inorganic P (PIP) concentration was measured colorimetrically (Wang *et al*. 1983).

Bone DM content was determined by heating to a constant weight at 105°C under an atmosphere of N (Thermogravimetric TGA 701 Analyser, LECO Corporation, St Joseph, Michigan, USA), while bone ash content was determined by further heating to 600°C. Bone ash was digested in concentrated hydrochloric acid and the P concentrations were measured colorimetrically (AOAC 1984). Calcium was measured in the same digest by atomic absorption flame spectroscopy using a nitrous oxide–acetylene flame, following with dilution in potassium chloride to prevent interference caused by ionisation of Ca (AOAC 1984).

For analysis of volatile organic compounds in the bone solid phase, microextraction fibres were manually injected into a gas chromatograph–mass spectrometer (Thermo GC Ultra gas chromatograph, coupled to a Thermo DSQ mass spectrometer) operating in positive electron-ionisation mode. Analyte separation was achieved using a Varian FactorFour™capillary column VF-5 ms (30 m × 0.25 mm i.d. × 0.25 μm) with helium as the carrier gas. The temperature of the splitless injector was held constant at 250°C. The oven temperature was programmed starting from 35°C (hold 2 min), followed by a temperature gradient to 80°C at 3°C/min, then to 120°C at 15°C/min and eventually to 300°C (hold 1 min) at 40°C/min. Full scan mode and SIM mode were used to monitor the volatile organic compounds in the bone samples. Individual volatile organic compounds were identified by comparison with library mass spectra in the 2011 version of the NIST/EPA/NIH Mass Spectal Library (NIST 11) and the search algorithm similarity index.

### *Statistical analyses*

Three heifers, one from the AdeqP group and two from the LowP group, were excluded from the data analyses for the reasons described below. Preference tests 5–7 in Phase 1, when the mean PIP of the LowP heifers was <1.0 mmol/L, were compared with Phase 3 tests to examine the effects of exposure to the variety of bones during Phase 2. LowP heifers during preference tests 5–7 (Days 98–124 of Phase 1) were considered, on the basis of their development of pica and the PIP concentration <1.0 mmol/L, to represent heifers in severe rather than moderate P deficiency.

For each phase, the binomial measurements M1–M3 and M5–M7 were examined as a generalised linear mixed model (GLMM) with a binomial distribution and a logit link function with fixed effects for P status (LowP and AdeqP) and bin (wood and bones) and random effects of heifer and test (Genstat, VSN International, Hemel Hempstead, UK). Due to numerous zero values in the time spent at the bins, the variable M4 was modelled using a two-part conditional GLMM for zero-inflated data; this comprised a binomial model with a logit link for the presence/absence and a conditional Poisson model with a log link for the time at the bin given a heifer visited the bin. The impact of experience in Phase 2 was tested by modelling the combined preference data from Phases 1 and 3 with a GLMM as above, but with the additional fixed effect of experience (pre- and

post-Phase 2) and a random effect of phase. During some phases there were insufficient data for analysis of the variables M1, M2 and M6 and, in these circumstances, these are not reported. A pairwise comparison of means was performed using a protected least significant difference test at a significance level of $P = 0.05$. In Phase 2, because the heifers were tested in their treatment groups, no statistical comparisons between P status, day or bone class could be made. The concentrations of DM, P and Ca in the various groups of bones examined in Phase 2 (B1–B4) were compared by one-way ANOVA.

## Results

### *Heifers, diets, LW gain and development of pica*

With one exception, the heifers were in good health throughout the experiment. On Day 128, during Phase 1, a heifer was observed to be in discomfort, demonstrated inappetence and had lost LW. Veterinary opinion was that this was likely due to pica-induced ingestion of stones, which may have obstructed the gastrointestinal tract; with continuing inappetence, the heifer was withdrawn from the experiment and it subsequently recovered. In addition, one heifer from each diet group consistently exhibited unsettled and nervous behaviour during both general handling of the group and when alone in the test pen; data from these heifers were excluded from the analyses.

On Day 1 of the experiment, heifer LW averaged 190 (s.d. 16) kg and a body condition score of 4.9 (s.d. 0.2). During the 183 days of the experiment the heifers fed the LowP diet gained 15 kg LW and lost 2.4 units of body condition score, while the AdeqP-diet heifers gained 90 kg LW and lost 2.0 units of body condition score. The LowP diet fed in the yards contained (g/kg DM) 934 (s.d. 7.1) organic matter, 25.1 (s.d. 0.85) total N, 292 (s.d. 23) neutral detergent fibre, 192 (s.d. 12) acid detergent fibre, and 20 (s.d. 2.1) lignin. The measured voluntary intake was, on average, 17 (s.d. 2) g DM/kg LW. The faecal NIRS measurements indicated that during the dry season (Days 1–134), the diet selected by the AdeqP-grazing heifers contained (g/kg DM), on average, 82 (s.d. 5) non-grass, 8.4 (s.d. 0.11) total N, with a DM digestibility of 559 (s.d. 16) and a voluntary intake of 23 (s.d. 1.8) g DM/kg LW. During the rainy season (Days 134–183), the diet selected contained (g/kg DM), on average, 63 (s.d. 32) non-grass, 17.0 (s.d. 1.1) total N, with a DM digestibility of 634 (s.d. 10) and a voluntary intake of 33 (s.d. 1.4) g DM/kg LW.

From Day 80, the heifers in the LowP group were observed to be demonstrating pica, with licking and attempted chewing of cables and rails of the yard fences, plastic trough legs, plastic pipe and weeds, and licking of soil. Also the presence of stones in the water trough indicated that the heifers were mouthing and possibly ingesting stones.

### *Phosphorus intake, faecal P and blood PIP*

At Belmont Research Station, when the heifers were grazing improved pasture, the faecal P concentration was 6.2 g P/kg DM, indicating that diet P concentration was ~3.1 g P/kg DM. At Brian Pastures Research Facility, during the 8 weeks before the experiment commenced, the faecal P concentration of all the heifers averaged 4.1 ($n = 3$, s.d. 1.6) g P/kg DM. During the experiment, the faecal P concentration of the grazing AdeqP heifers during the dry season (Days 1–134) averaged 3.2 (s.d. 0.40), and during the rainy season (Days 135–183) 5.2 (s.d. 1.58) g P/kg DM (Fig. 1*a*). The concentration of P in the diet of the AdeqP heifers, estimated from P in the faeces, averaged 1.5 (s.d. 0.20) and 2.5 (s.d. 0.81) g P/kg DM during the dry season and the rainy season respectively. The concentration of P in the diet fed to the LowP heifers averaged 0.72 (s.d. 0.135) g P/kg DM from Day 43 through to the end of the experiment. A higher P concentration for this diet from Days 12–35 (1.4–1.8 g P/kg DM) was a consequence of an abnormally high P concentration in the batch of wheat flour used to prepare the diet during this interval. The PIP concentration of all the heifers 3 weeks before the treatments commenced was 2.3 (s.d. 0.29) mmol/L. On Day 27, the PIP concentrations were similar for the LowP and the AdeqP heifers (1.7 (s.d. 0.16) and 1.9 (s.d. 0.31) mmol/L respectively; Fig. 1*b*). The PIP of the LowP heifers decreased progressively from Day 27 to Day 98; by Day 77, PIP averaged 1.0 (s.d. 0.28) mmol/L and during the remainder of the experiment it generally

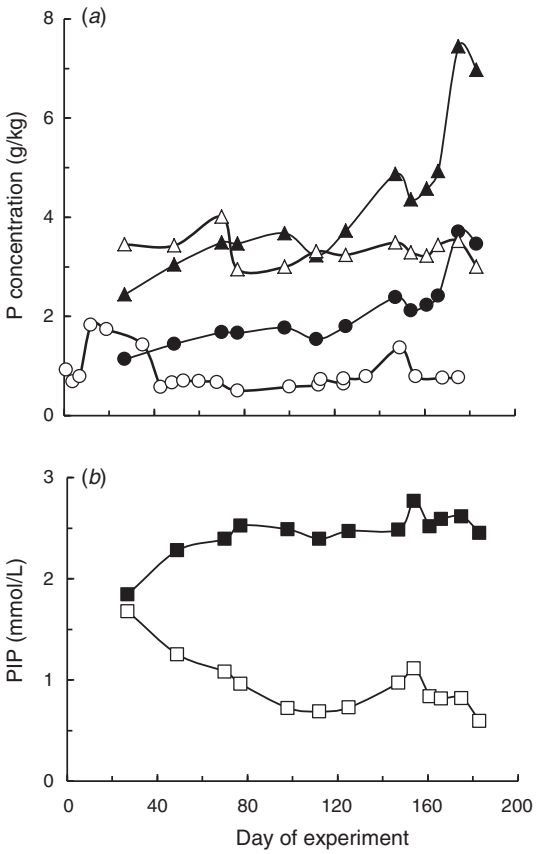


**Fig. 1.** The concentrations of (*a*) total phosphorus (P) in the diet and faeces and (*b*) plasma inorganic P (PIP) of heifers fed a diet of low P concentration in yards (LowP), and of heifers that grazed pasture expected to supply adequate amounts of dietary P (AdeqP). The concentrations of P in the diet (○) and faeces (●) of LowP heifers, and in the diet (Δ) and faeces (▲) of AdeqP heifers, are shown in Fig. 1*a*. The concentrations of PIP in LowP (□) and AdeqP (■) heifers are shown in Fig. 1*b*. The concentration of P in the diet of AdeqP heifers was calculated from the concentration in faeces as described in the Materials and Methods.

ranged from 0.7 to 0.8 mmol/L. The coefficient of variation within a sampling day in the LowP heifers ranged from 13% to 29%. In the AdeqP heifers PIP concentrations tended to increase until Day 70, and then to remain in the range of 2.4–2.6 mmol/L.

### *Behaviour of the heifers during Phases 1–3*

During the preference tests from Day 24 to Day 65 (Tests 1–4), when the PIP of the LowP heifers was >1.0 mmol/L, the heifers in both treatment groups exhibited limited attraction to the test materials in the bins. On average, only 29% of heifers displayed interest by looking at a bin (measurement M1), 31% visited one of the bins (M3), and those heifers that did visit bins spent, on average, only 14 s/120 s (i.e 14 s of the 120 s during each test) at the bins (M4). During the preference tests from Day 86 to Day 114 (Tests 5–7 of Phase 1), when the average PIP of LowP heifers was <1.0 mmol/L, similar proportions of heifers from the two treatment groups displayed interest by looking at a bin (M1) (32%) and visited bins (M3) (26%), and these proportions were not influenced by the P status of the heifers ($P > 0.10$). Among the heifers that did visit the bins, the LowP heifers spent more time at the bins (regardless of content) than did the AdeqP heifers (M4; 9 and 4 s/120 s respectively; $P = 0.05$). Irrespective of P status, heifers that visited bins tended ($P = 0.07$) to spend more time at the bones bin than the control bin (M4; 9 and 4 s/120 s respectively), and a greater proportion of heifers sniffed at the bone than the control bin (M5; 27% and 10% respectively; $P < 0.05$). Nevertheless, only a minority of heifers visited the bins and these visits were only brief, indicating limited attraction by heifers in either treatment group to the bones during Phase 1.

On the first occasion of Phase 2 (Day 146) when heifers were allowed access to the variety of bones, all of the heifers in each P status sniffed, licked or chewed bones at least in one of the groups of B1–B4. In addition, ~half of the heifers in each treatment group (4 of 8 of the LowP heifers, and 5 of 9 of the AdeqP heifers), sniffed or licked the control wood. Over the 5 days of Phase 2, the LowP heifers appeared to exhibit more sniffing and licking of the bones weathered for 1 or 4 months (B1 and B2 respectively), than of the bones weathered for 8 months or 9 years (B3 and B4 respectively; Table 1). On only 3% of the 40 opportunities when the LowP heifers were given access to the bones did any of these heifers chew the B1 or B2 bones, but on 30% and 40% of opportunities some of these heifers chewed the B3 and B4 bones respectively. Despite comparable chewing of the B3 and B4 bones, the LowP heifers ingested 42.6 g of B4 bone per heifer, but only 6.2 g of B3 bone per heifer, over the 5 days. The amounts of B1 and B2 bone ingested were comparable to the amount of B3 bone ingested (Table 1). Most of the B4 bone and total bone ingested was consumed on the first occasion that bones were offered on Day 146 (95% and 72% respectively). Also, almost all (96%) of the B4 bone ingested was flat bone rather than long bone (Table 1). The AdeqP heifers also extensively sniffed and licked each of the groups of bones, but the frequency of sniffing appeared to be higher, and the frequency of licking lower, than for the LowP heifers (Table 1). There was little chewing of any of the bones by the AdeqP heifers. The AdeqP heifers ingested 6.0 g air-dry bone/heifer on Day 146, and 17.0 g air-dry bone/heifer in total. Although there was appreciable sniffing and licking of the wood control by both the LowP and the AdeqP heifers, there was negligible ingestion by either group (Table 1).

When the results of the preference tests for Phases 1 and 3 were compared, the main effects of heifer P status (i.e. LowP versus AdeqP), experience during Phase 2 (Phase 1 versus Phase 3), and test materials in the bins (i.e. bones versus control) on the behaviour of the heifers were generally observed ($P < 0.05$ to $P < 0.01$; Table 2). There tended ($P = 0.051$) to be an interaction

**Table 1. Phase 2 (Days 146–155): the mean ingestion over five occasions on 5 days of flat and long air-dry bones that had been weathered for 1 month (B1), 4 months (B2), 8 months (B3) or 9 years (B4) at the commencement of the experiment, or of pieces of wood as a control by the heifers of low phosphorus (LowP) and adequate P (AdeqP) status**
In addition, the percentage of occasions when heifers in LowP or AdeqP status demonstrated sniffing, licking and chewing behaviours during the 5 days when the bones were offered is given

| Origin of bones | Bone ingested (g/heifer) | | Heifer behaviour (%) | | |
|---|---|---|---|---|---|
| | Flat | Long | Sniffing | Licking | Chewing |
| | | *LowP* | | | |
| B1 | 0.8 | 6.8 | 23 | 55 | 3 |
| B2 | 6.4 | 4.9 | 28 | 58 | 3 |
| B3 | 5.0 | 1.2 | 8 | 43 | 30 |
| B4 | 41.1 | 1.5 | 5 | 43 | 40 |
| Total bone | 67.6 | | | | |
| Wood | 3.3 | | 18 | 15 | 0 |
| | | *AdeqP* | | | |
| B1 | 0.6 | 4.4 | 58 | 38 | 4 |
| B2 | 3.1 | 3.4 | 42 | 22 | 0 |
| B3 | 0.3 | 0.7 | 34 | 20 | 0 |
| B4 | 1.3 | 3.4 | 46 | 26 | 0 |
| Total bone | 17.0 | | | | |
| Wood | 1.9 | | 32 | 8 | 0 |

**Table 2. Phases 1 and 3: the effects of phosphorus status of the heifers (P status: low P (LowP) and adequate P (AdeqP)), of access to a variety of bones during Phase 2 (experience), the material presented in the feed bins (bin), and the interaction between phosphorus (P) status and experience, on measurements of behaviour of the heifers during the preference tests**

Measurements are the logit values, with back-transformed means in parenthesis, of the percentage of heifers exhibiting 'interest by looking at a bin' on entry to the test yard (M1), the percentage of heifers visiting bins (M3), the percentage exhibiting sniffing activity (M5), and log value of the time spent at bins by those heifers that visited bins (M4). The test offered heifers a choice between a control (wood) or a mixture of bones weathered for between 3 months and 9 years. Values are the means of three preference tests conducted on eight or nine heifers per diet treatment. Values for M1, M3 and M5 are logit values of percentage of heifers; and those for M4 are log values of time at the bins (s/120 s). The values for the measurement of the percentage of heifers 'walking direct to a bin' on entering the test yard (M2) are not given because insufficient numbers of heifers exhibited this behaviour for this measurement to be analysed statistically. The measurement of interest (M1) was a pen measurement and was not related to the specific bins. n.s., not significant ($P > 0.10$). n.d., not determined

| Treatment | Interest in bins (M1) | Visit to bins (M3) | Time at bins (M4) | Sniffing activity (M5) |
|---|---|---|---|---|
| | | *P status* | | |
| LowP | 0.39 (60) | –0.05 (49) | 2.42 (11) | –1.06 (26) |
| AdeqP | –0.50 (38) | –0.99 (27) | 1.85 (6) | –1.34 (21) |
| s.e.d. | 0.46 | 0.30 | 0.26 | 0.33 |
| Probability | $P = 0.060$ | $P < 0.01$ | $P < 0.05$ | n.s. |
| | | *Experience* | | |
| Pre-experience | –0.71 (33) | –1.11 (25) | 1.64 (5) | –1.42 (19) |
| Post-experience | 0.60 (64) | –0.07 (52) | 2.62 (14) | –0.98 (27) |
| s.e.d. | 0.45 | 0.30 | 0.30 | 0.33 |
| Probability | $P < 0.01$ | $P < 0.001$ | $P < 0.01$ | n.s. |
| | | *Bin* | | |
| Control (wood) | n.d. | –0.91 (29) | 1.38 (4) | –1.70 (16) |
| Mixed bones | n.d. | –0.13 (47) | 2.89 (18) | –0.70 (33) |
| s.e.d. | n.d. | 0.30 | 0.28 | 0.32 |
| Probability | n.d. | $P < 0.01$ | $P < 0.001$ | $P < 0.01$ |
| | | *P status × experience* | | |
| Probability | $P = 0.051$[A] | n.s. | n.s. | n.s. |

[A]Although the *P*-value for the interaction between P status and experience was only $P = 0.051$, a pairwise comparison of means was applied at $P = 0.05$. The interaction means were 33% and 83% for LowP heifers pre- and post-experience respectively, and 33% and 44% for AdeqP heifers pre- and post-experience, respectively.

between P status and experience for the interest of heifers in the bins (M1); a greater percentage (83%) of LowP heifers post-experience showed interest in the bins (regardless of materials in the bin) than heifers from either diet group pre-experience (each 33% of the heifers) or the AdeqP heifers post-experience (44%). A significantly ($P < 0.01$) greater proportion of LowP than AdeqP heifers visited one of the bins, and for those heifers that did visit bins, more time was spent at the bins by LowP than AdeqP heifers (M4; $P < 0.05$; Table 2). Experience during Phase 2 increased both the proportion of heifers (regardless of P status) visiting bins (M3; 52% versus 25%; $P < 0.001$) and the time spent at bins by those heifers that visited bins (M4; $P < 0.01$). A greater proportion of heifers visited the bones bin than the control bin (M3; 47% versus 29%; $P < 0.001$), and the heifers that did visit bins spent more time at the bones bin ($P < 0.001$). Also a greater percentage of heifers exhibited sniffing of the bones bin than the control bin (M5; 33 versus 16%; $P < 0.01$; Table 2).

### *Behaviour of the heifers during Phases 4–6*

In Phase 4, when heifers were offered either bones weathered for 9 years or a control of wood and with the mesh grids removed, the percentage of heifers exhibiting interest in the bins on entering the test yard (M1) was greater for the LowP than the AdeqP heifers (88% versus 19%; $P < 0.001$; Table 3). In addition, a greater proportion of the LowP than AdeqP heifers visited the bins (M3; 78 versus 24%; $P < 0.001$), but there was no effect of P status ($P > 0.10$) on the time spent at the bins by those that visited the bins (M4). Among heifers that did visit the bins, more time was spent at the bones bin than the control bin ($P < 0.05$). Licking activity (M6) was greater for LowP than AdeqP heifers, and greater for the bones than the control (both $P < 0.01$; Table 3).

In Phase 5, when heifers were offered bones weathered for 0.5 or 9 years and with the mesh grids removed, a greater proportion of the LowP heifers than AdeqP heifers went directly to a bin on entry (M2; 35% versus 4%; $P < 0.001$), and a greater proportion visited the bins (M3; 82% versus 36%, $P < 0.001$; Table 4). Heifers that visited bins spent more time at the bin containing the bones weathered for 9 years (M4; 56 versus 39 s/120 s), a greater percentage tended to go directly to this bin (M2; $P = 0.058$), and a greater percentage tended to lick the bones weathered for 9 years than bones weathered for 0.5 years (M6; $P = 0.061$). In addition, there was an interaction between the P status of the heifers and the age of weathering ($P < 0.001$) on sniffing behaviour (M5); a greater percentage of LowP heifers sniffed bones weathered for 0.5 years than 9 years (63% and 12% respectively), while the sniffing activity of AdeqP heifers (on average, 31%) was not affected by the length of time of weathering of the bones.

**Table 3. Phase 4: the effects of phosphorus status of the heifers (P status: low P (LowP) and adequate P (AdeqP)) and the material presented in the feed bins (bin) on measurements of behaviour of the heifers during the preference tests**

Measurements are the logit values, with back-transformed means in parenthesis, of the percentage of heifers exhibiting 'interest by looking at a bin' on entry to the test yard (M1), percentage of heifers visiting bins (M3), sniffing (M5) and licking (M6) of material in bins, or the log value of the time spent at bins by those heifers that visited bins (M4). The test offered heifers a choice between a control (wood) or bones weathered for 9 years with the mesh grids removed so that the heifers could lick and chew the test materials. Values for M1, M3, M5 and M6 are logit values of percentage of heifers; and those for M4 are log values of time at the bins (s/120 s). The interactions were not significant ($P > 0.05$). The values for the measurement of the percentage of heifers 'walking direct to a bin' on entering the test yard (M2) are not given because insufficient number of heifers exhibited this behaviour for this measurement to be analysed statistically. Since the measurement of interest (M1) was a pen measurement, it was not related to the specific bins; n.d., not determined. n.s., not significant ($P > 0.10$)

| Treatment | Interest in bins (M1) | Visit to bins (M3) | Time at bins (M4) | Sniffing activity (M5) | Licking activity (M6) |
|---|---|---|---|---|---|
| | | | *P status* | | |
| LowP | 1.95 (88) | 1.29 (78) | 3.83 (46) | –0.98 (27) | –0.29 (43) |
| AdeqP | –1.48 (19) | –1.18 (24) | 3.76 (43) | –1.48 (19) | –2.40 (8) |
| s.e.d. | 0.81 | 0.63 | 0.32 | 0.62 | 0.64 |
| Probability | $P < 0.001$ | $P < 0.001$ | n.s. | n.s. | $P < 0.01$ |
| | | | *Bin* | | |
| Control | n.d. | –0.00 (50) | 3.53 (34) | –1.06 (26) | –1.92 (13) |
| Bones | n.d. | 0.11 (53) | 4.07 (58) | –1.41 (20) | –0.76 (32) |
| s.e.d. | n.d. | 0.42 | 0.23 | 0.42 | 0.44 |
| Probability | n.d. | n.s. | $P < 0.05$ | n.s. | $P < 0.01$ |

**Table 4. Phases 5 and 6: the effects of phosphorus status of the heifers (P status: low P (LowP) and adequate P (AdeqP)) and the time of weathering (0.5 or 9 years) of the bones presented in the feed bins (bin) on behaviour of the heifers during the preference tests when bones were exposed or placed within cloth bags**

Measurements are the logit values, with back-transformed means in parenthesis, of heifers 'walking direct to a bin' (M2) and that visited a bin (M3), and the percentage sniffing (M5) or licking (M6) of the bones in the bins, and the log values of the time the heifers spent at the bins that they visited (M4). The test offered heifers a choice between bones with the mesh grids removed, so that the heifers could see, lick and chew the test materials (exposed, Phase 5) or with the bones placed within cloth bags (covered, Phase 6). Values for M2, M3, M5 and M6 are logit values of percentage of heifers; and those for M4 are log values of time at the bins (s/120 s). The statistical model for the measurement of 'interest in a bin' by the heifers when they entered the test yard (M1) did not converge and results are thus not presented. n.s., not significant ($P > 0.10$)

| Treatment | Direct to bin (M2) | Visit to bins (M3) | Time at bins (M4) | Sniffing activity (M5) | Licking activity (M6) |
|---|---|---|---|---|---|
| | | | *Phase 5, exposed bones, P status* | | |
| LowP | –0.61 (35) | 1.49 (82) | 3.80 (45) | –0.54 (37) | –0.21 (45) |
| AdeqP | –3.23 (4) | –0.56 (36) | 3.90 (49) | –0.87 (30) | –1.04 (26) |
| s.e.d. | 0.62 | 0.53 | 0.23 | 0.39 | 0.54 |
| Probability | $P < 0.001$ | $P < 0.001$ | n.s. | n.s. | n.s. |
| | | | *Phase 5, exposed bones, weathering time of bones* | | |
| 0.5 years | –2.39 (8) | 0.15 (54) | 3.67 (39) | –0.23 (44) | –0.95 (28) |
| 9 years | –1.45 (19) | 0.78 (68) | 4.02 (56) | –1.18 (24) | –0.30 (43) |
| s.e.d. | 0.49 | 0.35 | 0.16 | 0.36 | 0.35 |
| Probability | $P = 0.058$ | n.s. | $P < 0.05$ | $P < 0.01$ | $P = 0.061$ |
| P status × bones | n.s. | n.s. | n.s. | $P < 0.001$[A] | n.s. |
| | | | *Phase 6, covered bones, P status* | | |
| LowP | –1.77 (15) | 1.64 (84) | 3.03 (21) | 0.65 (66) | –0.89 (29) |
| AdeqP | –2.85 (5) | –0.14 (47) | 3.68 (40) | –0.28 (43) | –1.44 (19) |
| s.e.d. | 0.88 | 0.63 | 0.27 | 0.70 | 0.93 |
| Probability | n.s. | $P < 0.05$ | $P < 0.05$ | n.s. | n.s. |
| | | | *Phase 6, covered bones, weathering time of bones* | | |
| 0.5 years | –2.17 (10) | 0.83 (70) | 3.65 (39) | 0.18 (55) | –0.83 (30) |
| 9 years | –2.45 (8) | 0.67 (66) | 3.05 (21) | 0.18 (55) | –1.50 (18) |
| s.e.d. | 0.39 | 0.35 | 0.19 | 0.34 | 0.35 |
| Probability | n.s. | n.s. | $P < 0.01$ | n.s. | $P = 0.057$ |
| | | | *P status × weathering time of bones* | | |
| Probability | n.s. | n.s. | n.s. | $P = 0.077$ | n.s. |

[A]Interaction means of 63% and 28% for bones weathered for 0.5 years and 12% and 33% for bones weathered for 9 years for LowP and AdeqP heifers respectively.

In Phase 6, when each of the groups of bones were enclosed in a cloth bag, a higher percentage of LowP than AdeqP heifers visited the bins (M3; 84% versus 47%; $P < 0.05$). However, among these heifers that visited the bins, AdeqP heifers spent more time than LowP heifers at the bins (M4; 40 versus 21 s/120 s; $P < 0.05$). The heifers spent more time at bins containing bones weathered for only 0.5 years than at those containing bones weathered for 9 years (M4; 39 versus 21 s/120 s; $P < 0.01$), and a greater percentage tended ($P = 0.057$) to exhibit licking activity (M6) of these less-weathered bones (Table 4).

### *Identified volatile organic components in bone samples*

Analysis of volatile components detected in the enclosed headspace around the bone specimens showed that the samples contained a variety of volatile organic compounds (Table 5). Due to the low intensity of peaks, particularly for the B3 and B4 samples, the identification of some components obtained from the 40-min collections was difficult, and the overnight collections (18 h) provided more readily identifiable results. The volatiles identified included a range of short-chain aldehydes ($C_6$–$C_9$), alcohols ($C_5$–$C_9$), acid esters, ketones, hydrocarbons, aromatics and dimethyl disulfide. A comparison of volatile components detected from bone samples that had been weathered for 3 months (B1), 6 months (B2), 10 months (B3) or 9 years (B4) at the time of laboratory analysis is shown in Table 5. The B1 and B2 bones showed the greatest range of volatiles, and similar profiles were obtained for both whole bones and bone fragments. Collections for the longer time of 18 h showed a composition similar to that observed for the 40-min collections, although there was some distortion with the longer collection interval occurring towards the less volatile components. Predominant volatile organic compounds in these less weathered samples (B1 and B2) were $C_6$–$C_9$ aldehydes, particularly hexanal ($C_6$). These aldehydes (particularly hexanal) were present at high concentrations in both B1 and B2 samples and, although present in older bones, they were present in these at markedly lower concentrations. The volatiles observed in the headspace of the more weathered B3 and B4 bones were present at much lower concentrations than they were in the B1 and B2 bone samples. The B3 and B4 samples had similar concentrations of volatiles, and the major volatile organic compounds were identified as linear aliphatic ketones and aldehydes (Table 5).

**Table 5. Major volatile organic compounds measured in the enclosed headspace of rib bones that had been weathered for 3 months (B1), 6 months (B2), 10 months (B3) or 9 years (B4)**

Identified volatile organic compounds are ordered by gas chromatograph retention, with relative abundance scored from low or barely detectable (+) to major components (+++++) on an ordinal scale. Similar volatile profiles were obtained from both intact and fragmented bones. For older B3 and B4 bones, discernible volatiles were detected only with longer (18 h) collections, whereas for B1 and B2 bones, observed volatile organic compounds were determined in 40-min collections

| Compound | B1 | B2 | B3 | B4 |
|---|---|---|---|---|
| Acetone | ++ | ++ | + | + |
| Butanal | ++ | ++ | – | – |
| Pentanal | +++ | +++ | – | – |
| Pentanol | + | + | – | – |
| Hexanal | +++++ | ++++ | + | – |
| Hexanol | + | + | – | – |
| 2-Heptanone | + | + | + | – |
| Heptanal | +++ | ++++ | + | + |
| Heptanol | + | + | + | – |
| 2-Octanone | ++ | ++ | – | – |
| Octanal | +++ | ++++ | + | + |
| Undecene[A] | + | + | – | – |
| 2-Nonanone | + | ++ | + | – |
| Nonanal | ++ | +++ | ++ | ++ |

[A]Tentative identification.

### *Bone analysis*

The rib bones with various degrees of weathering contained similar concentrations of P (102–115 g/kg DM) and Ca (172–218 g/kg DM; Table 6). The ash content of the bones increased ($P < 0.001$) with weathering, from 628 and 585 g/kg DM in the B1 and B2 bones respectively, to 709 g/kg DM in the B4 bones weathered for 9 years.

## Discussion

The limited attraction of the LowP treatment heifers to weathered bones in the preference tests during Phase 1 provided clear evidence that the attraction of P-deficient cattle to weathered bones is not an innate response, as concluded from the studies of Blair-West *et al*. (1989, 1990, 1992). During this phase, these heifers had developed pica and had PIP of <1.0 mmol/L, but their attraction to weathered bones was not different from that observed in the AdeqP treatment heifers. Rather, the present study provided strong evidence that this attraction is associated primarily with the development of a positive post-ingestive feedback response following the opportunity and experience for P-deficient animals to ingest bones. In the present study, the increased attraction of P-deficient animals to weathered bones following experience was demonstrated by the increase in the proportion of LowP treatment heifers showing interest in bins containing bones (M1), from 33% to 83% and then 88% in Phases 1, 3 and 4 respectively. Similarly, it can be calculated that the proportion of LowP heifers visiting the bin containing bones (M3) increased from 44% to 72%, 78% and 82% in Phases 1, 3, 4 and 5 respectively. Attraction by P-deficient cattle to weathered bones by the development of a post-

**Table 6. Measured dry matter (DM), ash, phosphorus (P) and calcium (Ca) concentrations (g/kg DM) of rib bones that had been weathered for 3 months (B1), 6 months (B2), 10 months (B3) or 9 years (B4)**

The values are the average of analyses of two samples. n.s., not significant; $P > 0.10$. Means not followed by a common letter are significantly different (at $P = 0.05$)

| Bone sample | DM | Ash | P | Ca |
|---|---|---|---|---|
| B1 | 943a | 628c | 104 | 197 |
| B2 | 941a | 585d | 102 | 179 |
| B3 | 899c | 665b | 115 | 172 |
| B4 | 918b | 709a | 109 | 218 |
| s.e.d. | 1.6 | 6.3 | 12.5 | 15.3 |
| Probability | $P < 0.001$ | $P < 0.001$ | n.s. | n.s. |

ingestive feedback response is comparable with the development of responses to a variety of diet constituents reported in several studies with sheep (Provenza 1995, 1996; Arsenos and Kyriazakis 1999; Hills *et al*. 1999). Although the AdeqP heifers in the present study investigated the bones by sniffing and licking, and ingested a small amount of bone when given the opportunity during Phase 2, they consistently exhibited a lower attraction than did LowP heifers to the weathered bones in the subsequent phases. The development of pica in response to a severe P deficiency is likely to have an important role in the establishment of a positive post-ingestive feedback, since, in a natural rangeland environment, pica would be expected to cause P-deficient cattle to ingest carcasses and bones and thus would lead to a post-ingestive feedback from bone-chewing. It is clear that once attraction to weathered bones is established cattle can demonstrate very strong attractions including an extraordinary ability to find bones and bone fragments in pastures (Green 1925; Blair-West *et al*. 1989, 1992).

The heifers used in the study were considered a particularly appropriate model to examine behaviour in relation to bones. The heifers were obtained as weaners from a research station herd where evidence of P deficiency, including bone-chewing, had never been observed over decades (R. A. Hunter, pers. comm.). Following weaning, and before their entry into the experiment, the heifers grazed pastures on fertile alluvial soils and were thus expected to be in replete P status as well as being naïve to behaviour associated with P deficiency. The initial replete P status of the heifers was confirmed by their high PIP and faecal P concentrations before the experiment commenced. It was in response to consuming a P-deficient diet in the experiment that the LowP treatment heifers developed severe P deficiency, with a progressive decrease in PIP to less than 1.0 mmol/L and with concurrent development of pica. These observations are in accord with numerous reports of the gradual development of P deficiency and pica in cattle ingesting diets low in P concentration (Green 1925; Theiler and Green 1932; McCosker and Winks 1994) and with the reports of Blair-West *et al*. (1989, 1992) that cattle exhibit strong attraction to weathered bones when the PIP concentration decreases to less than 1.0 mmol/L. This latter threshold is appreciably lower than the PIP concentration (1.3–1.8 mmol/L) below which young growing cattle substantially increase voluntary intake and LW gain in response to P supplements (Wadsworth *et al*. 1990; Coates 1994; CSIRO 2007).

It is clear from the studies of Blair-West *et al*. (1989, 1992) that in at least some circumstances the attraction of P-deficient cattle to weathered bones is associated with olfactory compounds from the bones. The variety of volatile organic compounds measured on the various groups of bones in the present study, which included a range of short-chain aldehydes ($C_6$–$C_9$), alcohols ($C_5$–$C_9$), acid esters, ketones, hydrocarbons, aromatics and dimethyl disulfide, were consistent with those observed during decay of other animal remains (Hoffman *et al*. 2009; DeGreeff and Furton 2011). The major identified volatiles comprising $C_6$–$C_9$ aldehydes, and the linear aldehydes of this type, are derived from the breakdown of fats and fatty acids (Lee *et al*. 2007). Hexanal is a common breakdown product from the omega-6 family of fatty acids such as linoleic acid (C18:2; Shahidi 2001). Cattle bones, and particularly bone marrow, are rich in palmitic (C16:0), stearic (C18:0) and oleic (C18:1) acids; oleic acid or cis-9-octadecenoic acid are the most predominant and there is only a low percentage of linoleic (C18:2) acid (Abd-El-Aal and Mohamed 1989). By comparison, bovine muscle and adipose fat contain higher concentrations of linoleic acid (C18:2; Wood *et al*. 2008). In the present study, it was noted that decomposing muscle and fat were still evident on the less weathered B1 and B2 bones, and this may be the source of the higher concentrations of volatile aldehydes including hexanal (from linoleic oxidation) in these bones. The more weathered B3 and B4 bones in the present study were typically clean bones, with no adhering muscle or fat, and aldehyde volatiles would likely have been derived from bone marrow oxidation, consistent with the observed lower hexanal concentrations. Although the P-deficient heifers in the present study displayed attraction to weathered bones, it was not possible to identify the specific behavioural cues that attracted the heifers. The observations of sniffing and licking of the bones during each of the phases by the P-deficient heifers after they had gained experience in Phase 2 implicated olfactory components and taste as cues. This is in accord with the conclusions of Blair-West *et al*. (1989, 1990, 1992) from comparable preference tests, and with field observations (Green 1925; Theiler and Green 1932) that the olfactory constituents of weathered bones have an important role. However, visual cues may also have been involved with the different responses to bones and the control wood in Phases 3 and 4, and to bones with different degrees of weathering and either visible or enclosed in cloth bags in Phases 5 and 6.

Irrespective of the cues involved in attraction of the P-deficient heifers in the present study, it was clear that the amount of bone ingested during Phase 2 was also affected by other factors. Although both groups of heifers investigated each of the types of bone in Phase 2, there was extensive chewing only of the more weathered B3 and B4 bones. Furthermore, despite comparable frequencies of chewing of these groups of bones, the amount of extensively weathered B4 flat bone ingested by the LowP heifers was much greater than of long bone weathered for the same interval, or of B3 bone weathered for 8 months (Table 1). There may be several possible explanations. First, the heifers may have ingested more of the B4 flat bones because they were easier to chew and ingest; the texture of the outside surfaces of these bones, particularly at the extremities, was soft and chalky to the extent that the bone could be easily scratched or probed to a depth of several millimetres with a metal point. In contrast, the bones that had been weathered for only a few months had hard surfaces, and were presumably more difficult for the heifers to chew and ingest. Second, even though the measured profiles of olfactory compounds were similar for the bones weathered for various intervals, the heifers may have been able to identify constituents, or combinations of constituents, that were not identified with the procedures used; such differences may have been important aspects of the olfactory cue(s). Third, the olfactory cues associated with putrification of carcasses may reduce attraction due to negative post-ingestive feedback caused, for example, through subclinical illness or as an innate response. There may be benefits for herbivores to ingest old weathered bones with reduced concentrations of potentially debilitating organisms (Theiler 1920). In addition, there is evidence that herbivores generally avoid areas with odour of carrion or

excreta (Minson 1990; Lev-Yadun and Gutman 2013). The present study was consistent with the greater attraction to and chewing of bones weathered for longer intervals (e.g. 1.5 years) (Blair-West *et al*. 1989, 1992), and to 'old sweet bones' than to 'stinking rotten bones' observed by Green (1925) and Theiler and Green (1932). A further consideration is that the cues associated with development of a post-ingestive feedback response will presumably depend on the circumstances in which it was developed and reinforced, and thus there is not likely to be any unique profile of olfactory, taste or visual cues. For example, post-ingestive feedback responses, including subclinical P deficiency, can be associated with a variety of synthetic flavours (Villalba *et al*. 2006, 2008).

In conclusion, the attraction of the P-deficient heifers to investigate and chew weathered bones appears to primarily depend on the development of a positive post-ingestive feedback response following from opportunities for experience and interactions with bones. Olfactory and taste compounds and visual cues appear to be involved. Pica appears to have an important role by causing P-deficient cattle to explore their environment and ingest bones, which would establish a post-ingestive feedback response for P-deficient cattle to ingest bones.

## Conflicts of interest

The authors declare no conflicts of interest.

## Acknowledgements

We thank Tracy Longhurst, Tim Grant, the staff team at the Brian Pastures Research Facility, Michael Gravel, Brian Burren and Peter Isherwood for skilled technical assistance with the animal experimentation and laboratory analyses. The financial support of Meat and Livestock Australia, and the support of the Department of Agriculture and Fisheries (Queensland) and AgForce Queensland for access to animal research facilities and cattle is gratefully acknowledged.

## References

Abd-El-Aal MH, Mohamed MS (1989) A comparative study on bone fats from different species of animals. *Food Chemistry* **31**, 93–103. doi:10.1016/0308-8146(89)90020-4

AOAC (1984) 'Official methods of analysis of the Association of Official Analytical Chemists. Section 7.099 (a).' 14th edn (AOAC: Washington, DC)

Arsenos G, Kyriazakis I (1999) The continuum between preferences and aversions for flavoured foods in sheep conditioned by administration of casein doses. *Animal Science* **68**, 605–616. doi:10.1017/S1357729800050633

Blair-West JR, Denton DA, Nelson JF, McKinley MJ, Radden BG, Ramshaw EH (1989) Recent studies of bone appetite in cattle. *Acta Physiologica Scandinavica* **136**(Suppl. 583), 53–58.

Blair-West JR, Denton DA, Ramshaw EH (1990) Olfactory cueing in phosphorus deficient cattle. *Proceedings of the Australian Physiological and Pharmacological Society* **21**, 114P.

Blair-West JR, Denton DA, McKinley MJ, Radden BG, Ramshaw EH, Wark JD (1992) Behavioural and tissue responses to severe phosphorus depletion in cattle. *The American Journal of Physiology* **263**, R656–R663.

Bowyer RT (1983) Osteophagia and antler breakage among Roosevelt elk. *California Fish and Game* **69**, 84–88.

Bredin IP, Skinner JD, Mitchell G (2008) Can osteophagia provide giraffes with phosphorus and calcium? *The Onderstepoort Journal of Veterinary Research* **75**, 1–9. doi:10.4102/ojvr.v75i1.82

Brothwell D (1976) Further evidence of bone chewing by ungulates: the sheep of North Ronaldsay, Orkney. *Journal of Archaeological Science* **3**, 179–182. doi:10.1016/0305-4403(76)90085-6

Burrow HM, Dillon RD (1997) Relationships between temperament and growth in a feedlot and commercial carcass traits of *Bos indicus* crossbreds. *Australian Journal of Experimental Agriculture* **37**, 407–411. doi:10.1071/EA96148

Coates DB (1994) The effect of phosphorus as fertilizer or supplement on pasture and cattle productivity in the semi-arid tropics of north Australia. *Tropical Grasslands* **28**, 90–108.

Coates DB, Dixon RM (2008) Development of NIRS analysis of faeces to estimate non-grass proportions in the diets selected by cattle grazing tropical pastures. *Journal of Near Infrared Spectroscopy* **16**, 471–480. doi:10.1255/jnirs.815

Coates DB, Dixon RM (2011) Developing robust faecal near infrared spectroscopy calibrations to predict diet dry matter digestibility in cattle consuming tropical forages. *Journal of Near Infrared Spectroscopy* **19**, 507–519. doi:10.1255/jnirs.967

Coppock CE, Everett RW, Belyea RL (1976) Effect of low calcium or low phosphorus diets on free choice of calcium phosphate by lactating dairy cows. *Journal of Dairy Science* **59**, 571–580. doi:10.3168/jds.S0022-0302(76)84241-5

CSIRO (2007) 'Nutrient requirements of domesticated ruminants.' (CSIRO Publishing: Melbourne)

DeGreeff LE, Furton KG (2011) Collection and identification of human remains volatiles by non-contact, dynamic airflow sampling and SPME–GC/MS using various sorbent materials. *Analytical and Bioanalytical Chemistry* **401**, 1295–1307. doi:10.1007/s00216-011-5167-0

Dixon RM, Coates DB (2009) NIR spectroscopy of faeces to evaluate the nutrition and physiology of herbivores. A review. *Journal of Near Infrared Spectroscopy* **17**, 1–31. doi:10.1255/jnirs.822

Dixon RM, Coates DB (2010) The relationship between the concentration of phosphorus in the diet and in faeces of cattle grazing tropical grass and grass–legume pastures. *Advances in Animal Biosciences* **2**, 337.

Dixon RM, Smith DM, Porch I, Petherick JC (2001) Effects of experience of young cattle on their voluntary intake of supplements. *Australian Journal of Experimental Agriculture* **41**, 581–592. doi:10.1071/EA00172

Dixon RM, White A, Fry P, Petherick JC (2003) Effects of supplement type and previous experience on variability in intake of supplements by heifers. *Australian Journal of Agricultural Research* **54**, 529–540. doi:10.1071/AR02091

Dixon RM, Coates DB, Holmes W, English B, Rolfe J (2011) Phosphorus nutrition and management: overcoming constraints to wider adoption. In 'Proceedings of the northern beef research update conference', 3–4 August 2011, Darwin. pp. 102–109. (North Australia Beef Research Council: Qld)

Gordon JG, Tribe DE, Graham TC (1954) The feeding behaviour of phosphorus-deficient cattle and sheep. *The British Journal of Animal Behaviour* **2**, 72–74. doi:10.1016/S0950-5601(54)80035-9

Green HH (1925) Perverted appetites. *Physiological Reviews* **5**, 336–348. doi:10.1152/physrev.1925.5.3.336

Hills J, Kyriazakis I, Nolan JV, Hinch GN, Lynch JJ (1999) Conditioned feeding responses in sheep to flavoured foods associated with sulphur doses. *Animal Science* **69**, 313–325. doi:10.1017/S1357729800050888

Hoffman EM, Curran AM, Dulgerian N, Stockham RA, Eckenrode BA (2009) Characterization of the volatile organic compounds present in the headspace of decomposing human remains. *Forensic Science International* **186**, 6–13. doi:10.1016/j.forsciint.2008.12.022

Holmes WE (1990) Phosphorus and beef production in northern Australia. 11. A case study approach to the economic assessment of phosphorus supplementation. *Tropical Grasslands* **24**, 250–255.

Langman VA (1978) Giraffe pica behaviour and pathology as indicators of nutritional stress. *The Journal of Wildlife Management* **42**, 141–147. doi:10.2307/3800701

Lee J, Kim D-H, Chang P, Lee J (2007) Headspace–solid phase microextraction (HS–SPME) analysis of oxidized volatiles from free fatty acids (FFA) and application for measuring hydrogen donating antioxidant activity. *Food Chemistry* **105**, 414–420. doi:10.1016/j.foodchem.2006.12.059

Lev-Yadun S, Gutman M (2013) Carrion odor and cattle grazing: evidence for plant defense by carrion odor. *Communicative & Integrative Biology* **6**, e26111. doi:10.4161/cib.26111

McCosker T, Winks L (1994) 'Phosphorus nutrition of beef cattle in northern Australia.' (Meat Research Corporation: Sydney)

McDowell LR (1996) Feeding minerals to cattle at pasture. *Animal Feed Science and Technology* **60**, 247–271. doi:10.1016/0377-8401(96)00983-2

Minson DJ (1990) 'Forage in ruminant nutrition.' (Academic Press: London)

Niethe GE (2011) New R and D for better herd health and productivity. In 'Proceedings of the northern beef research update conference', 3–4 August 2011, Darwin. pp. 34–40. (North Australia Beef Research Council: Qld)

Provenza FD (1995) The role of learning in food preferences of ruminants: Greenhalgh and Reid revisited. In 'Ruminant physiology: digestion, growth and reproduction'. (Eds WV Engelhardt, S Leonhard-Marek, G Breves, D Giesecke) pp. 233–247. (Ferdinand Enke Verlag: Stuttgart, Germany)

Provenza FD (1996) Acquired aversions as the basis for varied diets of ruminants foraging on rangelands. *Journal of Animal Science* **74**, 2010–2020. doi:10.2527/1996.7482010x

Shahidi F (2001) Headspace volatile aldehydes as indicators of lipid oxidation in foods. *Advances in Experimental Medicine and Biology* **488**, 113–123. doi:10.1007/978-1-4615-1247-9_9

Sweeney RA (1989) Generic combustion method for determination of crude protein in feeds. *Journal – Association of Official Analytical Chemists* **72**, 770–774.

Theiler A (1920) The cause and prevention of lamziekte. *Journal of the Department of Agriculture Union of South Africa* **1**, 221–244.

Theiler A, Green HH (1932) Aphosphorosis in ruminants. *Nutrition Abstracts and Reviews* **1**, 359–385.

Villalba JJ, Provenza FD, Hall JO, Peterson C (2006) Phosphorus appetite in sheep: dissociating taste from postingestive effects. *Journal of Animal Science* **84**, 2213–2223. doi:10.2527/jas.2005-634

Villalba JJ, Provenza FD, Hall JO (2008) Learned appetites for calcium, phosphorus, and sodium in sheep. *Journal of Animal Science* **86**, 738–747. doi:10.2527/jas.2007-0189

Wadsworth JC, McLean RW, Coates DB, Winter WH (1990) Phosphorus and beef production in northern Australia. 5. Animal phosphorus status and diagnosis. *Tropical Grasslands* **24**, 185–196.

Wang J, Chen CC, Osaki S (1983) Optimization of the phosphorus–UV reagent. *Clinical Chemistry* **29**, 1255.

Winks L (1990) Phosphorus and beef production in northern Australia. 2. Responses to phosphorus by ruminants. *Tropical Grasslands* **24**, 140–158.

Wood JD, Enser M, Fisher AV, Nute GR, Sheard PR, Richardson RI, Hughes SI, Whittington FM (2008) Fat deposition, fatty acid composition and meat quality: a review. *Meat Science* **78**, 343–358. doi:10.1016/j.meatsci.2007.07.019