CSIRO PUBLISHING

Animal Production Science, 2017, **57**, 2291–2303
https://doi.org/10.1071/AN17363

Review

New candidate markers of phosphorus status in beef breeder cows

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Abstract. Determining the phosphorus (P) status of cattle grazing P-deficient rangelands in northern Australia is important for improving animal production in these areas. Plasma inorganic P concentration is currently the best diagnostic marker of dietary P deficiency in growing cattle but is not suitable for assessing the P status of breeder cows, which often mobilise substantial bone and soft tissue reserves in late pregnancy and lactation. Markers of bone turnover offer potential as markers of P status in cattle, as they reflect bone mobilisation or bone formation. Recent experiments investigating the physiology of beef breeder cows during diet P deficiency have indicated that the ratio of plasma total calcium concentration to plasma inorganic P concentration might be suitable as a simple index of P deficiency. However, a more specific measure of increased bone mobilisation in P-deficient breeders is plasma concentration of C-terminal telopeptide of Type 1 collagen. Also, plasma concentration of bone alkaline phosphatase is a marker of defective bone mineralisation in dietary P deficiency. These candidate markers warrant further investigation to determine their predictive value for P deficiency in cattle.

Additional keywords: bone alkaline phosphatase, bone mobilisation, CTX-1, lactation.

Received 31 May 2017, accepted 7 August 2017, published online 19 September 2017

Introduction

The nutrient status of animals is key to determining their dietary requirements, but defining the status of a particular nutrient in animals is difficult. For the macromineral phosphorus (P), the term 'P status' is often used for soils, pastures and animals. In animal production, P status describes the P available for normal body functions and for a defined productive purpose, such as a slow growth, fast growth, or lactation. It includes readily available P in body fluids, as well as in P stores in tissues such as bone that may be mobilised under specific circumstances. P status broadly encompasses many different states, including P deficiency, P adequacy or P excess for immediate needs or over the longer term. For animal nutritionists, P status is often related to P balance, in terms of net gain or loss of P from the body. Whereas in clinical settings, P status is mainly defined by disease states and abnormalities related to, or a consequence of, biochemical and physiological changes that can be reversed by providing appropriate dietary P intake. In clinical P deficiency in cattle, overt signs of diminished performance include reduced feed intake, loss of liveweight and body condition, pica behaviour and poor reproductive performance. However, determining subclinical P deficiency, or optimal nutritional P intake, relies on the identification of biomarkers that reflect abnormalities in either critical tissue pools and/or nutrient stores. Further, to be useful as indicators of P status, such biomarkers need to be carefully evaluated for their specificity, sensitivity and predictive value relative to the desired animal-production process.

Phosphorus is a key limiting nutrient in beef production in northern Australia, South Africa and South America. Occasionally, P-deficient cattle have also been noted in northern America and Europe. Estimating the P requirements of grazing cattle for productive purposes such as growth and reproduction is problematic. Much work has concentrated on diagnosing P deficiency using serum or plasma P concentrations as a marker of P status (Wadsworth *et al*. 1990). In general, this

measure has been found to be a somewhat appropriate marker in growing animals, although it primarily reflects dietary P intake rather than bone P reserves. However, in breeder cows the validity of circulating P concentration as a marker of P status is questionable.

Many previous reviews have discussed the importance of P nutrition for grazing beef cattle in northern Australia (Cohen 1980; Littledike and Goff 1987; Ternouth 1990; Winks 1990; McCosker and Winks 1994) and also outside Australia (Karn 2001; Pfeffer *et al*. 2005). The present review will focus on the proposal that a more complete understanding of the physiology of P deficiency in breeder cows will elucidate new candidate markers of P status. Evidence from a large research project investigating the mobilisation of body P reserves to alleviate the adverse effects of P deficiency in breeder cows (Dixon *et al*. 2017) will be used to support this concept.

Calcium

Calcium (Ca) is essential for many physiological processes, including muscle contraction, blood coagulation, excitability of cells, cell signalling, enzyme activity and excocytosis of hormones and neurotransmitters. These critical functions in the body necessitate that the extracellular fluid concentration of Ca be strictly controlled by the main calciotropic hormones (parathyroid hormone (PTH), vitamin D3 and calcitonin), although many other hormones such as cortisol and oestrogens also contribute to Ca homeostasis.

Calcium is a key structural element of the skeleton and almost all (99%) body Ca stores occur in the bone matrix as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). Most of the remaining body Ca (0.9%) is located either in the plasma membrane or within organelles such as the endoplasmic reticulum. Consequently, extremely low Ca concentrations (100 nM) are normally found within the cytoplasm of cells in which the role of Ca is primarily as a signalling molecule.

Extracellular fluid (ECF) contains only a small fraction (0.1%) of body Ca; total Ca concentrations in ECF are ~2.5 mM. Approximately 50% (1.25–1.60 mM) of extracellular Ca is present as free ionised Ca^{2+}, the biologically active form of extracellular Ca. A small proportion of extracellular Ca (5%) is present as Ca^{2+} complexed with anions such as citrate, bicarbonate, phosphate or lactate, and the remainder (45% of ECF Ca) is bound to plasma proteins such as albumin and globulins. The size of the protein-bound fraction depends on blood pH, with acidic conditions increasing the fraction of total Ca in the ionised form (Rosol and Capen 1997). Concentrations of circulating albumin also influence the bioactive Ca^{2+} fraction; there is a linear relationship between albumin and free Ca^{2+} concentrations in cattle (Bienzle *et al*. 1993). This relationship may be important in beef cows with low intakes of P, protein and metabolisable energy grazing poor-quality senesced pastures, as albumin concentrations may be low under such circumstances. However, in lactating dairy cows, total Ca concentrations have been found to be a good indicator of the bioactive Ca^{2+} fraction (Lincoln and Lane 1990), the exception being cases of hypoalbuminemia (Seifi *et al*. 2005).

Calcium can be stored as mineralised bone (hydroxyapatite) and can be resorbed or replenished as required to maintain Ca homeostasis. Such mechanisms are critical to cows undergoing annual reproductive cycles, with large demands for Ca and P during late pregnancy and early lactation. In addition to Ca in mineralised bone, a readily available of pool of Ca exists, predominantly as Ca salts, in extracellular bone fluid. Although this pool is relatively small compared to mineralised bone, it appears to be important for fine regulation of blood Ca concentrations (Parfitt 2003). However, osteoclastic bone resorption of mineralised bone appears to be the principal long-term process for substantial mobilisation of bone Ca reserves, and is often mediated by the key calciotropic hormones, namely PTH and vitamin D3. Nevertheless, there is evidence that PTH related protein (PTHrP) mediates bone resorption during lactation (Wysolmerski 2010; Kovacs 2016) and that osteocytic osteolysis contributes to bone turnover in lactation (Qing *et al*. 2012; Kovacs 2016).

Inorganic P

Phosphorus is found only in oxidised states and often in the form of phosphate (PO_4^{3-}). It is required by all living cells and plays critical roles in the structure of DNA and RNA, as a major component of cell membranes (phospholipids) and in energy transfer in cellular processes (phosphate–phosphate bonds). It is also involved in muscle contraction, maintenance of normal pH, oxygen transport, activation of enzymes and cell signalling. Most (80–85%) P in the body is present as hydroxyapatite. The remaining 15–20% occurs in soft tissues as a major intracellular anion and as a component of ATP/ADP, phosphoproteins, nucleic acids, phospholipids and other organic molecules. However, since only small amounts of phosphate are stored within cells, most of it being in the form of structural molecules, most cells depend on extracellular fluid P to meet their metabolic P requirements. In circumstances of chronic low extracellular P concentrations, cellular dysfunction, such as postpartum haemolysis, may occur (Goff 2000; Andrews *et al*. 2008). Therefore, although the extracellular fluid P pool constitutes only a very minor portion (<0.1%) of total body P, it is of critical importance.

In the blood circulation, P is present as an anionic phosphate, mainly in the dibasic form (HPO_4^{2-}) and a small amount is in the monobasic form ($H_2PO_4^-$); the ratio between these forms depends on pH. Phosphates in circulation are largely in the free anion form, but are also present as complexes with Na^+, Mg^{2+} or Ca^{2+}, and, to a lesser extent, proteins. Biochemical assays for P in blood usually determine only the inorganic P (Pi) fraction, but this is acceptable given that the P in extracellular fluid is almost entirely in the form of inorganic orthophosphate. The inorganic phosphate content of blood is often expressed as the elemental phosphorus concentration. In cattle, plasma inorganic P (PiP) concentrations often range from 1.1 to 2.3 mM, although concentrations are often higher (up to 2.5 mM) in younger growing animals fed high-P diets. High PiP concentrations in young animals have been reported to be partially due to high growth-hormone concentrations, which increase renal phosphate reabsorption (Blaine *et al*. 2015), but high PiP levels are also related to the high bone turnover that occurs in young animals (Braithwaite 1975). In severely P-deficient animals, PiP concentration is often <1 mM, and can be as low as 0.4 mM.

The key role of P is to support animal growth, both structurally (bone mineral and cell membranes) and metabolically. In animal nutrition, the focus has often been on the determining the dietary P intake required for various rates of growth and other production functions, and relating these to PiP concentrations. However, when considering the role of P in cellular metabolism, it is important to appreciate that although P plays a critical role in energetic processes involving the hydrolysis of phosphate–phosphate bonds contained in storage molecules such as ATP and phosphocreatine, elemental P itself is not consumed in these processes and can be recycled indefinitely. There are inevitable losses of P from the body, which in ruminants occurs mainly through faecal excretion (CSIRO 2007), but also through sweat and urinary excretion. Therefore, besides being required for growth, an essential role of P intake is to replace the inevitable P losses (Cashman and Flynn 1999). In addition, in cows, large amounts of P are required to support fetal growth and milk production. If dietary P is insufficient, the only alternative source is P present in body reserves. Furthermore, in young cows (e.g. first-calf cows), the need for dietary P is even greater because of a high demand for continuing bone growth as well as for reproduction. Understanding such complexities represents a major challenge to nutritionists wishing to define the dietary P requirements of reproducing cows. The concurrent needs for P to maintain body condition and bone mineral health are key to optimal reproductive output through the productive life of the animal. However, there are opportunities for breeders to mobilise and replenish P reserves during annual cycles (Dixon *et al*. 2017).

Hypophosphatemia

Inadequate P intake is often associated with hypophosphatemia, although dietary P deficiency does not necessarily cause hypophosphatemia, and similarly hypophosphatemia does not always indicate P deficiency (Knochel 1977). In humans, there are many identified causes of hypophosphatemia and various manifestations of P deficiency (Knochel 1977; Knochel 1981; Bansal 1990). In cattle, the primary cause of hypophosphatemia is dietary P deficiency (Andrews *et al*. 2008; Goff 2008); for an extensive review of P metabolism and hypophosphatemia in cattle, see Grünberg (2014).

In cows, acute hypophosphatemia can potentially occur during late pregnancy as fetal skeletal growth accelerates and removes Pi and Ca from maternal circulation (Goff 2000). In high-producing dairy cows close to parturition, the increase in Ca demand for colostrum production can result in parturient paresis (Goff 2000; DeGaris and Lean 2008). Sometimes a combination of hypocalcaemia and hypophosphatemia can develop, such as seen in 'downer' cows and further can be associated with hypoglycaemia as a result of decreased food intake around the time of parturition (Goff 2000). However, the hypophosphatemia is these situations is thought to be insignificant (Andrews *et al*. 2008), although subject to some debate (Grünberg (2014). Furthermore, in beef cows parturient paresis due to hypocalcaemia is uncommon, most likely being due to much lower colostrum and milk production and lower Ca demand than in dairy cows (Oetzel and Goff 2008). Therefore, although acute hypophosphatemia around the time of parturition is possible in beef cows, it is most likely to be associated with an ongoing dietary P deficiency (Goff 2000). Indeed, small decreases in serum Pi and Ca in healthy well fed beef cows around the time of parturition are typical (Dokovic *et al*. 2014).

PiP concentration as a marker of P status

A simple marker of P status would be helpful in defining P deficiency in animals. Extending the ideas of Cashman and Flynn (1999), a marker of nutrient status should be a physiological or biochemical marker that reflects the cellular effect of the nutrient, the dietary intake of the nutrient or available body reserves of the nutrient. To fully assess nutrient status, possibly a combination of such indicators is required. For P deficiency in cattle, early workers (Theiler *et al*. 1937) proposed the use of blood P concentration as a simple measure, but several disadvantages with the use of blood P have been identified (Cohen 1973), the most important being that a substantial proportion of P in whole blood occurs as phospholipids in erythrocytes. Thus, serum or PiP concentration was regarded as more appropriate (Palmer *et al*. 1930; Cohen 1974). In cattle, other indicators of P status, including the concentration of P in faeces (Belonje and Berg 1980; Wadsworth *et al*. 1990), and attributes of rib bone obtained at biopsy (Cohen 1973, 1974; Little 1984; Beighle *et al*. 1993; Beighle 1999) have all been examined with mixed success (reviewed by Karn 2001).

Generally, PiP concentration is accepted as the best diagnostic marker of P status in growing cattle, reflecting the current P intake. Some limitations of this measure include that it may not be a suitable indicator in reproducing cows due to substantial mobilisation of body P reserves in late pregnancy and lactation, and also that it does not provide information about body P reserves, especially those present in bone. In addition, PiP can be affected by physiological events around the time of blood sampling. In humans, variability in PiP concentrations has been associated with recent consumption of food, especially glucose, insulin release, muscular activity and hyperventilation. These lower PiP concentration by causing a shift of P from the extracellular fluid into cells as part of normal physiological responses (Bansal 1990). In cattle, unexplained daily variability in PiP was noted in early studies (Palmer *et al*. 1930). Furthermore, rapid movement of Pi into cells has also been observed as a stress response to blood sampling (Gartner *et al*. 1965). A low metabolisable energy intake and dehydration (Rollinson and Bredon 1960) can also affect PiP. Although some early studies reported differences between blood samples obtained from the jugular versus tail veins, more recent thorough studies have found no differences or small differences (Fletcher *et al*. 2016; Quigley *et al*. 2016). Despite the above issues, PiP concentrations remain a simple and easy-to-measure marker of the P status in cattle that has biological validity, at least for some circumstances.

Since PiP is a measure of the extracellular fluid P pool, it is reasonable that it will reflect the immediate availability of inorganic P for metabolic processes. Moreover, PiP concentrations are related to dietary P intake and this appears to be established across many animal species (Cashman and Flynn 1999; Dias *et al*. 2011). It has been demonstrated (Cohen 1974) that PiP concentrations exhibit acute and hourly

responses to P intake in cattle. Studies in lactating goats have demonstrated that changes in dietary P content rapidly affect PiP concentrations, regardless of whether the animals are P-adequate or P-deficient (Rodehutscord *et al.* 1994). However, it should be noted that not all studies in cattle have found a close relationship between P intake and PiP concentrations (Gartner *et al.* 1982). If animals can draw on P held in storage in bone, the amount of P in the blood is, at best, poorly related to P intake. Serum Pi concentrations can take many months to decrease to below normal concentrations following introduction of P-deficient diets in growing heifers (Gartner *et al.* 1982). Presumably, this is because P is mobilised from body reserves to maintain homeostasis. Similarly, in lactating dairy cows, PiP concentrations declined markedly only during the second lactation, some 15 months after the introduction of low-P diets (Valk *et al.* 2002). Others have specifically concluded that PiP is quite limited as the sole determinate of inadequate P intakes of lactating dairy cows (Forar *et al.* 1982). Taken together, the evidence supports the hypothesis that PiP concentrations reflect dietary P intake, but not necessarily in cattle that are mobilising substantial bone stores, such as breeders.

The data for defining diagnostic thresholds for PiP as an indicator were mainly derived from studies conducted some 25–30 years ago, principally from large research programs with growing cattle (Wadsworth *et al.* 1990; Coates 1994). From comprehensive studies on growing steers and heifers across four geographical zones in northern Australia, it was concluded that 'plasma Pi successfully diagnosed P deficiency in young, growing cattle in 90–100% of cases' (Wadsworth *et al.* 1990). However, this conclusion was conditional on obtaining samples late in the growing (wet) season and using local reference values (Wadsworth *et al.* 1990). When these conditions were omitted from the analysis, the predictive value was poor (only 55%). PiP concentrations were highly correlated with liveweight gain during the growing (wet) season, but the relationship varied markedly among properties (Wadsworth *et al.* 1990).

From a physiological perspective, the normal range for any plasma metabolite (such as PiP) reflects homeostatic mechanisms and represents a state of equilibrium essential for normal biological function. However, it is a dynamic equilibrium regulated by physiological processes and, therefore, different physiological states (growth, pregnancy, lactation) will influence set points within the normal range. For blood minerals, this concept is best exemplified by higher Ca and PiP concentrations in young, growing heifers than in mature cows. Another perspective is to accept that not all levels of PiP within the normal range are equally beneficial to cell functioning. However, evidence for such an idea in the case of PiP is not available. The normal PiP concentration range is broad (1.3–2.6 mM) in comparison to that for Ca (2.1–2.5 mM) in mature dairy cows (Goff 2006). This highlights that the physiological control of Ca, rather than P, is the primary issue in normal body function.

The emphasis in research on cattle grazing P-deficient rangelands has been to define P deficiency associated with PiP concentrations at the lower end of the normal range (e.g. Wadsworth *et al.* 1990). However, defining P deficiency in breeders is difficult, and current recommendations rely on PiP concentrations of sentinel growing animals grazing the same paddocks as breeders. Essentially, the PiP concentrations of sentinels provide information about the P status of the paddock, and, thus, indirectly inform about the P status of breeders. As an alternative, we examined whether circulating markers of bone metabolism would better define P status in breeders than do PiP concentrations.

Biochemical markers of bone metabolism

The bone health of animals can be assessed directly from bone-biopsy samples and histomorphometric analyses. Such approaches have recently been used in cattle to assess rib cortical bone and hip trabecular bone by using new biopsy techniques (Kidd *et al.* 2014, 2016; Dixon *et al.* 2017). Whereas these approaches are useful methods for assessing bone remodelling, they have drawbacks in that the biopsies require surgery, animals cannot be sampled repeatedly, measurements are labour-intensive and they are unsuitable for routine evaluation in industry.

Measurement of circulating biochemical markers of bone turnover provides a less invasive approach to assess bone processes in cattle than does bone biopsy (Dixon *et al.* 2017), and such markers are now used routinely in human clinical medicine for diagnosis of bone diseases and response to treatment (Seibel 2005, 2006). Furthermore, bone turnover markers have an advantage in that they provide a measure of whole-body bone metabolism, rather than metabolism at a specific bone site used for biopsy. They also provide near real-time information on bone activity. Their use in veterinary medicine and animal research has been increasing over the past 15 years and commercial assay kits are readily available for many biomarkers (Allen 2003; Camassa *et al.* 2017).

Biochemical markers of bone metabolism provide indirect measures of the process of bone turnover and the underlying and opposing processes of bone formation by osteoblasts and bone resorption by osteoclasts. In growing animals, bone turnover involves bone modelling, and although the processes of bone formation and bone resorption are spatially distinct, most bone surfaces are constantly being modelled. Growing young animals generally have increased bone turnover with consequent higher concentrations of the biochemical markers of bone metabolism. In growing animals, bone formation exceeds bone resorption so that there is net bone gain. In mature animals, bone turnover is characterised by the process of remodelling whereby bone is usually removed and replaced at the same site and there is, overall, no change, net gain or net loss of bone. Thus, healthy mature animals generally exhibit low and stable concentrations of bone markers, with the exception peri-parturient breeder cows that mobilise substantial bone reserves.

Several biochemical markers have been identified and can be categorised as indicators of either bone formation or bone resorption, reflecting the activity of osteoblasts and osteoclasts respectively (Allen 2003). Such markers have proved useful in the diagnosis of specific bone diseases or in monitoring the response of treatments for bone disorders (Hlaing and Compston 2014). Overall, the sensitivity and specificity of such markers critically determine their clinical usefulness. Many of the markers described in early research lacked

specificity, as they were present in non-skeletal as well as skeletal tissues, and gave mixed outcomes (Barton *et al*. 1987; Moore *et al*. 2000; Peterson *et al*. 2005). However, a variety of markers have now been developed that may be useful in ruminant research (Allen 2003; Herrmann 2011; Camassa *et al*. 2017). Wide variability among animals and diurnal variation in concentration have been described for some markers and some are likely to be of limited individual diagnostic value, but possibly will be more useful to monitor changes over time in individuals, or for diagnosis of groups of animals (Allen 2003). Furthermore, bone metabolism markers provide insight into bone physiology and may be useful as functional indicators of the adequacy of Ca and P intake (Cashman and Flynn 1999). Consequently, some biochemical markers are ideal candidates as markers of bone metabolism and P status in cattle, including cattle grazing P-deficient rangelands. In the present review, two biochemical markers of bone metabolism, C-terminal telopeptide of Type 1 collagen (CTX-1) and bone alkaline phosphatase (BALP), will be discussed in detail as these markers are widely used to estimate bone resorption and bone formation, but these are certainly not the only or necessarily the best markers for each purpose across all circumstances.

C-terminal telopeptide of Type 1 collagen: a marker of bone resorption

Bone-resorption markers are either enzymes produced by osteoclasts or the degradation products of Type I collagen in bone. C-terminal telopeptides of Type I collagen comprise the latter, being fragments of Type I collagen composed of short peptide sequences from the non-helical domain of this molecule (Chubb 2012). Such cross-linked peptides of degraded bone collagen are readily detectable in circulation before being cleared by urinary excretion.

Despite being a widely used marker of bone resorption, the specificity of CTX-1 is not entirely resolved. Some reviewers propose that CTX-1 is not bone-specific because Type I collagen is also expressed in skin, dentine, tendons, uterus and fetal adnexa, and metabolism of those tissues might contribute to circulating CTX-1 concentrations. However, the antibodies used in CTX-1 assays (serum Crosslaps ELISA, Immunodiagnostic Systems, Boldon Business Park, UK) have been designed to have high specificity for Type I collagen derived from mature bone. In addition, circumstantial evidence from many bone metabolism studies clearly suggests that this marker is bone specific and there appears to be no published data to the contrary (Chubb 2012). Diurnal variability in CTX-1 concentration in humans has been well characterised, with a peak in early morning and nadir in the afternoon (Chubb 2012). Such variability has been examined in cattle, but only in calves (Matsuo *et al*. 2014).

Bone alkaline phosphatase: a bone-formation marker

All current bone-formation markers are products in blood derived from osteoblasts and are secreted proteins, either an enzyme or precursor peptides of collagen synthesis. BALP is an enzyme (EC 3.1.3.1) solely produced by osteoblasts (Hoffmann and Solter 2008). It is expressed on the external surface of the cell membrane (i.e. an ecto-enzyme) of either osteoblasts or matrix vesicles derived from osteoblasts (Golub 2009). It is anchored by a phospho-glycan link to phosphatidylinositol, which is embedded in the cell membrane and is released into circulation by the action of phosphatidylinositol glycan hydrolase (Orimo 2010). BALP belongs to a family of widely expressed alkaline phosphatases that generally act as hydrolytic enzymes capable of dephosphorylating various molecules, including nucleotides and proteins. However, the specific role of BALP in bone has been the subject of much debate. There is evidence to support the hypothesis that BALP is involved in the hydrolysis of phosphate esters on the osteoblast cell surface, resulting in higher extracellular (interstitial fluid) Pi concentrations (Orimo 2010). Other evidence supports a more specific role for BALP in hydrolysing inorganic pyrophosphate (PPi) to Pi (Anderson *et al*. 2004; Orimo 2010). It is thought that PPi acts as an inhibitor of bone mineralisation, and that BALP, through hydrolysing PPi, not only produces Pi ($PP_i + H_2O \rightarrow 2\ P_i + 2\ H^+$), but also prevents accumulation of PPi as an inhibitor of bone mineralisation. Nevertheless, BALP is widely reported as a specific marker of bone formation (Hoffmann and Solter 2008), but perhaps should be more appropriately considered a marker of bone mineralisation.

The activity of all circulating alkaline phosphatases is readily detectable as 'total' alkaline phosphatase (ALP) activity and is a widely performed test in clinical biochemistry (Hoffmann and Solter 2008). However, in adult humans, only about half of the circulating ALP is the bone isoform. The other major isoform is derived from the liver. Both isoforms are encoded by the same ALPL gene and differ in post-translational modifications in their carbohydrate moiety. The relative proportions of the bone to liver isoforms in other species are lower, with the bone isoform constituting only ~30% and 20% of total ALP in dogs and horses respectively (Hoffmann and Solter 2008). Cattle appear to be similar (Anderson, unpubl. obs.). However, despite ALP being detectable in circulation, it is noteworthy that none of the circulating isoforms has a discernable physiological function. They appear metabolically inert as their activity occurs only under alkaline conditions (Kaplan 1972). Therefore, alkaline phosphatase enzymes, including BALP, act only locally at sites at which they are formed and the concentration in blood of an isoform is indicative of local production.

ALP is an early marker of increased bone turnover. Increased ALP concentrations in blood in the absence of liver disease are indicative of either normal growth in young animals or of bone disease in mature animals. The latter includes osteoblastic tumours, rickets, osteomalacia and osteoporosis. However, the absolute change in the bone isoform BALP is often small in these bone diseases and a change in concentration may be obscured by the liver-derived isoform when total ALP is measured. ALP has proved most useful in the diagnosis of Paget's disease, which is characterised by excessive bone turnover and remodelling (Seibel 2006). Although total ALP is economical and easy to measure for clinical biochemistry, there is a trend for increasing use of immunoreactive assay methodologies (ELISA and IRMA) in human clinical practice to measure BALP, due to their high specificity for the bone isoform.

New investigations

A recent large research program investigated P nutrition of the breeder beef cows in northern Australia. The capacity of breeders to use body reserves to alleviate the adverse effects of extended intervals of dietary P deficiency were examined and animal-production and bone-biopsy responses are discussed in an associated paper (Dixon *et al*. 2017). Here, the physiology of dietary P deficiency is discussed, with reference to results from two experiments in mature breeders during lactation (Benvenutti *et al*. 2015; Dixon *et al*. 2016; Anderson *et al*. 2016*b*, 2017). Potential new candidate markers of P status are presented.

Plasma mineral concentrations

At calving, mature cows exhibited normal plasma mineral concentrations (Table 1). Cows were expected to be in adequate P status at calving (liveweight 474 ± 57 kg, body condition score 3.5 ± 0.6) after being given adequate dietary P (estimated intake of 14–19 g P/day) throughout the last trimester of pregnancy (Dixon *et al*. 2016). Mean PiP concentrations of cows fed adequate P diets decreased in early lactation, before recovering in later lactation (Table 1). Such a small decrease in PiP in early lactation is typical of dairy cows and reflects the P demand for milk production (Forar *et al*. 1982). In contrast, the PIP concentrations of cows fed very low P diets (20–40% of their calculated P requirements) rapidly and markedly decreased in early lactation (<0.7 mmol/L), indicating that these cows were severely P deficient.

In these experiments, plasma total Ca concentrations increased during lactation in cows fed both adequate-P and low-P diets (Table 1), although the changes observed were within the normal range for plasma total Ca (2.1–2.8 mmol/L). However, a highly significant ($P < 0.0001$) diet effect was observed, with much higher Ca plasma concentrations in cows fed the low-P diet (Table 1). Such an increase most likely indicates mobilisation of bone mineral, since almost all of the body Ca reserves occur in bone. In the low-P diet, the increased demand of P for milk production, together with the need to maintain P homeostasis, would activate physiological mechanisms to mobilise bone P reserves, with a concomitant release of Ca from bone. Indeed, increased bone mobilisation is a mandatory characteristic of early lactation in all mammals (Horst *et al*. 2005) to meet the mineral (mainly Ca) demands of milk production and occurs despite high Ca and P intakes (Ramberg *et al*. 1970; Braithwaite 1983; Horst *et al*. 2005). In the current experiments, other results supported the hypothesis that substantial bone mobilisation was observed in cows fed low-P diets. The P concentration (P per unit surface area of cortical bone; PSACB) of rib bone obtained by biopsy was reduced by 16% during early lactation (Dixon *et al*. 2017). Therefore, an increase in plasma Ca observed within the normal range in P-deficient cows is indicative of mobilisation of bone P reserves. Plasma Ca concentrations in lactation represent the net flux between various body compartments (Ramberg *et al*. 1970). It should also be noted that cows fed the low-P diet described above mobilised substantial soft-tissue reserves in early lactation, losing ~50–60 kg liveweight (Dixon *et al*. 2017). Whereas such degradation of body tissues would contribute to P homeostasis as soft tissue contains a substantial amount of P, the corresponding Ca yield from soft-tissue

Table 1. Mean (±s.e.m.) of plasma minerals, hormones and markers of bone mobilisation and formation in mature Droughtmaster (*Bos indicus*-cross) breeder cows at calving, after 4 weeks of lactation and after 12–14 weeks of lactation for phosphorus (P)-adequate or P-deficient diets

All cows were fed P-adequate diets during the third trimester of pregnancy and had a good body condition score at calving. Results have been pooled from two experiments (Benvenutti *et al*. 2015; Dixon *et al*. 2016; Anderson *et al*. 2016*b*, 2017). $n = 30$ at calving and n = 14–16 per group for lactation. BALP, bone alkaline phosphatase; PTH, parathyroid hormone. Values within a row followed by the same letter are not significantly different from the calving value (at $P = 0.05$). *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ (for difference between low- and adequate-P diets during lactation)

Blood marker	Diet P	Calving	Early lactation (4 weeks)	Later lactation (12–14 weeks)
Inorganic P (mM)	Adequate	1.94 ± 0.08a	1.40 ± 0.13b	1.76 ± 0.02c
	Low		0.59 ± 0.07****	0.42 ± 0.02****
Total calcium (Ca; mM)	Adequate	2.12 ± 0.03a	2.21 ± 0.02b	2.27 ± 0.03c
	Low		2.37 ± 0.02****	2.54 ± 0.04****
Ca to P ratio	Adequate	1.16 ± 0.07a	1.84 ± 0.21b	1.35 ± 0.08a
	Low		5.00 ± 0.70****	6.78 ± 0.59****
PTH (pg/mL)	Adequate	256 ± 39a	289 ± 111a	199 ± 48a
	Low		271 ± 193 NS	38 ± 14**
1,25-diOH vit D (pM)	Adequate	231 ± 32a	172 ± 21a	119 ± 9b
	Low		209 ± 31NS	296 ± 34****
CTX-1 (ng/mL)	Adequate	0.45 ± 0.05a	1.68 ± 0.17b	1.11 ± 0.12c
	Low		4.29 ± 0.41****	5.58 ± 0.68****
BALP (U/L)	Adequate	15.5 ± 1.7a	11.1 ± 0.8b	13.8 ± 1.0c
	Low		15.9 ± 2.0*	29.3 ± 5.7**

mobilisation is insignificant. An alternative explanation is that the increased plasma Ca concentration was the result of greater absorption of Ca from the gastrointestinal tract and a likely reduction in Ca loss through urinary excretion. Such processes are under physiological control by the major calciotropic hormones, parathyroid hormone (PTH) and 1,25-dihydroxy vitamin D (1,25-diOH vit D). In the above experiment, concentrations of PTH and 1,25-diOH vit D were not changed in early lactation, but in later lactation, cows fed low-P diets had markedly ($P < 0.01$) reduced plasma PTH concentrations and plasma 1,25-diOH vit D concentrations remained high ($P < 0.0001$; Table 1; Anderson *et al*. 2017).

Reduced PTH concentrations in the cows fed low-P diets are most likely a consequence of the increase in plasma Ca concentration. We have observed similar increases in blood Ca concentration within the normal homeostatic range, together with marked reductions in plasma PTH concentration, frequently to undetectable levels, in both growing animals (McNeill *et al*. 2016) and pregnant cows (Anderson *et al*. 2016*a*) with severe dietary P deficiency. Furthermore, since PTH is suppressed, increased plasma 1,25-diOH vit D concentrations most likely reflect a direct action of low plasma Pi on expression of the enzyme 1 α-hydroxylase, which is responsible for conversion of the precursor 25-hydroxy vitamin D to active 1,25-diOH vit D in the kidneys. This finding is contrary to those of previous studies, mainly with growing sheep, which did not find evidence for a direct effect of low Pi on activation of vitamin D (Breves *et al*. 1985). Such results led to a dogma that low PiP concentrations do not stimulate active 1,25-diOH vit D expression in ruminants (Scott *et al*. 1997), quite unlike monogastrics (Fraser 1980). However, the changes in calciotropic hormones and plasma minerals we observed in lactating beef cows fed low-P diets are consistent with those reported for young non-pregnant beef cows fed a long-term P-deficient diet (Blair-West *et al*. 1992). Furthermore, the increased 1,25-diOH vit D concentrations in beef cows fed low-P diets are consistent with results for lactating dairy cows (Horst and Reinhardt 1983; Goff 2000, 2006).

Intriguingly, the different PTH and 1,25-diOH vit D responses in lactating cows as a consequence of low-P diets would have opposing physiological effects. Increased 1,25-diOH vit D concentrations would promote greater duodenal absorption of both Ca and P together with increased bone resorption, resulting in much higher circulating pools of Ca and Pi. In contrast, reduced PTH concentration would decrease bone resorption, increase Ca urinary excretion and decrease urinary and salivary P loss, reducing Ca, while retaining P in the circulating pool. The end result is that plasma Ca concentrations increase in cows as a consequence of attempting to maintain P homeostasis. Moreover, the increase in Ca concentrations occurred within the normal homeostatic range. With higher plasma Ca concentrations, there would be increased urinary loss simply due to filtration of a more concentrated solute, and this would normally limit any increase in plasma Ca concentration as a result of bone mobilisation. Such physiological mechanisms could explain why circulating Ca concentrations in P-deficient cattle are often reported as being normal or elevated (Craig *et al*. 2016). We propose that an elevated Ca concentration within the normal homeostatic range is an indication of bone mobilisation.

Calcium-to-phosphorus ratio in plasma

A simple indicator of P deficiency in lactating cows would be a measure that reflects net mobilisation of bone mineral reserves when dietary P intake becomes insufficient to maintain P homeostasis. The divergent results above for PiP (large decrease) versus plasma total Ca (small increase) of cows fed low-P diets suggest that a ratio of these blood minerals might be a potential sensitive index of P deficiency. In early lactation, the plasma Ca : P ratio increased slightly in cows on P-adequate diets, before returning to calving levels in later lactation (Table 1). Not surprisingly, cows fed low-P diets differed ($P < 0.0001$) in the Ca : P ratio in plasma from P-adequate cows (Table 1).

As noted above, if PiP concentration predominantly reflects P intake and total Ca concentration reflects the amount of bone mobilisation during P deficiency, then the Ca : P ratio is a candidate index of P status in breeders. Most studies have regarded the increase in blood Ca concentration within the normal homeostatic range as unimportant. For example, in early work on P deficiency in cattle, Theiler *et al*. (1927, pp. 312–313) stated in respect of Ca that ‘the differences lie in the normal range of variation in normal cattle and it would, therefore, be unwise to attach any significance to them’. However, similar divergent plasma Ca (increase) versus P (decrease) responses within the normal homeostatic range have been noted in studies on young pigs (Liesegang *et al*. 2002) and sheep fed low-P diets (Fraser 1932; Ewer 1951; Scott *et al*. 1997), often associated with rickets (Ewer 1951; Field *et al*. 1975; Breves *et al*. 1985), in humans with hypophostemia (Knochel 1977; Parfitt and Villanueva 1982) and in dairy cows fed low-P diets during early lactation (Knowlton and Herbein 2002; Puggaard *et al*. 2014). Older biomedical and human studies (Howland and Kramer 1922; Shipley *et al*. 1926; Albright *et al*. 1931, 1932) have often highlighted the importance of the so-called calcium–phosphorus product (often written as Ca × P ion product) as being important in the physical-chemical process of bone mineralisation, although these hypotheses have been criticised as being invalid, particularly with regard to calcification of soft tissue (O’Neill 2007). In dairy cows, most studies have, as might be expected, focussed on hypocalcaemia during the peri-parturient period. In this scenario, the plasma Ca : P ratio would be much lower than normal. Indeed, high dietary P (and Ca) intake in late pregnancy in dairy cows increases PiP concentration while decreasing plasma Ca concentration (Kichura *et al*. 1982), and high-P diets during the weeks before calving are not recommended as they increase the incidence of parturient paresis (Hemingway 1967; Jorgensen 1974; Goff 2000; DeGaris and Lean 2008). Furthermore, young sheep fed low-Ca diets have a decreased Ca and increased P response in circulation (Fraser 1932; Field *et al*. 1975). In rabbits fed oats (high P content), there was an inverse relationship between serum Ca and Pi concentrations (Bourne and Campbell 1932). However, more research is required to

establish whether plasma Ca : P ratio is a simple and reliable indicator of P nutrient status, either dietary P deficiency or P excess in cows, across a wide range of circumstances.

CTX-1 as a marker of bone resorption

Plasma CTX-1 concentrations were low at calving (Table 1), indicating a low rate of bone resorption. Other studies in dairy cows (Ekelund *et al*. 2006; Puggaard *et al*. 2014), ewes and goat does (Liesegang *et al*. 2006; Liesegang 2008) have indicated that circulating CTX-1 concentrations are usually low in late pregnancy up to parturition, provided there is adequate Ca and P nutrition. Sometimes a small increase in CTX-1 concentration can be observed in very late pregnancy (Ekelund *et al*. 2006), presumably due to increasing demand for skeletal development of the fetus. In lactating cows fed P-adequate diets, there was an increase in plasma CTX-1 concentration in the first month of lactation, followed by a decline thereafter, but CTX-1 concentrations remained higher during lactation than at calving (Table 1). This response is similar to that reported for dairy cows (Puggaard *et al*. 2014). In contrast, in cows fed low-P diets, CTX-1 concentrations increased markedly ($P < 0.0001$) during the first weeks of lactation and remained substantially elevated throughout lactation (Table 1). Such high levels of circulating CTX-1 are usually observed only in very early lactation in high-producing dairy cows fed adequate diets (Liesegang *et al*. 1998; Ekelund *et al*. 2006; Puggaard *et al*. 2014), reflecting the large Ca demand for milk production. However, serum CTX-1 concentrations in dairy cows can increase substantially in early lactation when they are fed diets with low P content (Puggaard *et al*. 2014). Overall, the sustained high plasma concentrations of CTX-1 directly support the paradigm that there is an extensive bone mobilisation in P-deficient lactating cows. This is in agreement with bone-biopsy evidence in these animals (Dixon *et al*. 2017).

To further validate the plasma Ca : P ratio as a potential indicator of bone mobilisation, the relationship between plasma Ca : P ratio and plasma CTX-1 concentrations was examined across all the results obtained from pregnant and lactating mature cows in two experiments, regardless of dietary P intake. These two measures were highly correlated (Fig. 1), with a positive linear relationship (CTX-1 = 0.71 × Ca : P – 0.01; $r^2 = 0.78$, slope $P < 0.0001$). Given that CTX-1 is a well characterised marker of bone resorption, this correlation analysis supports the hypothesis that plasma Ca : P ratio is a good measure of bone mobilisation in P-deficient breeders. Therefore, using the existing measure of PiP concentrations together with plasma Ca concentrations (as a ratio) is a simple and possibly a better diagnostic test of dietary P deficiency in mature breeders than is PiP alone. However, further testing, especially across a wide range of dietary Ca and P intakes, is necessary to define the specificity and predictive value of this measure and its usefulness for diagnosis. Nonetheless, CTX-1 is widely accepted as a valid, specific and sensitive marker of bone mobilisation. In early lactating dairy cows, plasma CTX-1 concentrations were found to be a good indicator of bone turnover (Liesegang *et al*. 1998; Puggaard *et al*. 2014). Furthermore, other markers of bone resorption (Camassa *et al*. 2017) should be investigated as potential markers of P status.

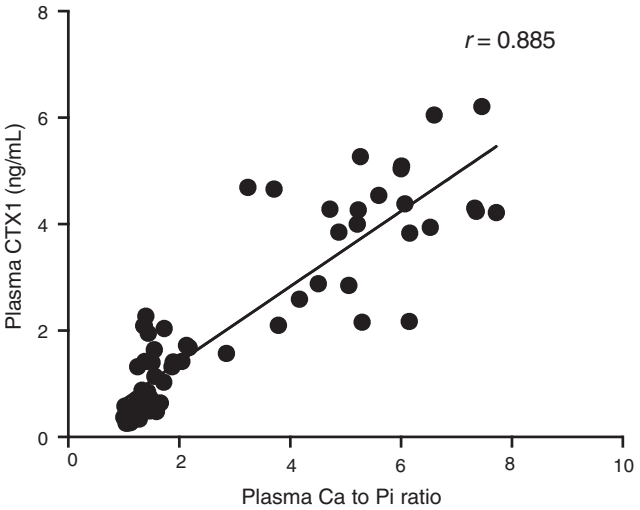

Fig. 1. Relationship between the ratio of plasma total calcium (Ca) to inorganic phosphorus (P) concentrations and plasma C-terminal telopeptides of Type I collagen (CTX-1). Linear regression analysis: CTX-1 = 0.71 × Ca/P – 0.01; $R^2 = 0.78$, slope $P < 0.0001$). Data were obtained from mature breeder cows ($n = 66$) during late pregnancy and lactation, irrespective of dietary P intake (low or adequate). Results were pooled from two experiments (Benvenutti *et al*. 2015; Dixon *et al*. 2016; Anderson *et al*. 2016*b*, 2017).

An interesting physiological issue is the identity of the signal for increased bone mobilisation in cows fed P-deficient diets. Bone resorption appears to be independent of PTH, as PTH is markedly suppressed by increased plasma Ca concentration. As mentioned above, a likely cause of increased bone resorption is increased 1,25-diOH vit D concentration. This hypothesis is supported by a study that showed that 1,25-diOH vit D can mobilise mineralised bone when given to thyroparathyroidectomised rats fed low-P diets (Castillo *et al*. 1975). However, additional hormones may be involved in driving bone resorption in P deficiency. In lactation, it has been proposed that mammary-derived PTHrP, not PTH, is the critical hormone responsible for induction of bone Ca resorption during lactation (Wysolmerski *et al*. 1995; Kovacs 2016). This evidence is derived from rodents and humans (Wysolmerski 2010), whereas, in cattle, PTHrP remains difficult to detect in circulation (Onda *et al*. 2006; Filipović *et al*. 2008). However, because increased blood Ca concentration in P deficiency would inhibit PTHrP secretion from the mammary gland (Wysolmerski 2010), similar to its effect on PTH, this related protein hormone is unlikely to be driving bone resorption in P-deficient breeders. It should be noted that increased bone mobilisation in P-deficient cows also occurs in pregnancy (Anderson *et al*. 2016*a*), during which PTHrP is thought to be unimportant (Kovacs 2016). Therefore, the hormonal signal mediating such an effect remains to be determined. Alternatively, it is possible that local low extracellular-fluid Pi concentrations in bone directly affect osteoclast activity (Raisz and Niemann 1969; Baylink *et al*. 1971; Binswanger *et al*. 1971).

BALP as a marker of bone mineralisation

In early lactation, plasma BALP concentrations were reduced in cows fed high-P diets, while, in contrast, BALP concentrations in P-deficient cows increased substantially (Table 1). These results may seem contradictory for a marker of bone formation. However, a plausible explanation relates to the biological function of this enzyme. BALP is an enzyme that is expressed on the external surface of the osteoblast cell membrane, where it is thought to remove (hydrolyse) phosphate from various molecules and make the P available locally for hydroxyapatite formation. It is considered a marker of bone formation because circulating concentrations of BALP reflect the activity of osteoblasts, but, more specifically, BALP reflects the process of bone mineralisation. In cows fed low-P diets, there is low availability of Pi for bone mineralisation. A consequence is that BALP as an enzyme is upregulated in bone and acts locally to counteract whole-body P deficiency. In essence, BALP appears to be an indicator of defective mineralisation in P-deficient cows.

Studies in cattle have shown total ALP activity to be consistently higher in heifers than cows over the peri-parturient period (Jonsson *et al*. 2013), although some earlier reports found no differences among dry cows, normal postparturient cows and cows with parturient paresis (Lappeteläinen *et al*. 1993). However, measurement of total ALP activity in cattle is of limited diagnostic value, even for hepatic disease, due to the broad range of reference values (0–500 U/L; Jackson and Cockcroft 2002). Some variability in cattle can be attributed to breed differences, with *Bos indicus* breeds having higher concentrations than *Bos taurus* breeds within age groups (Kunkel *et al*. 1953; Gahne 1967; McComb *et al*. 1979). It is possible to increase the specificity of total ALP tests for the bone-specific isoform by using lectin precipitation and heat inactivation before testing, and, in general, this has yielded more definitive results. Higher and more variable bone ALP activity was observed in young growing cattle than in mature animals (Yamagishi *et al*. 2009), with a decline in bone ALP activity noted in cows with increasing parity/age (Mohebbi *et al*. 2010). Furthermore, in early lactation in dairy cows, serum ALP activity increased (Sato *et al*. 2005) and this was attributable to increases in both bone and liver isoforms. In subsequent studies, measurement of BALP concentrations using an ELISA, which generally has a higher specificity for the bone isoform, confirmed that BALP concentration is increased markedly in young, growing cattle, whereas in aged cows, BALP concentrations are less variable, with a mean value of <20 U/L (Kim *et al*. 2010; Sato *et al*. 2013). Such concentrations in mature dairy cows are similar to our observations in beef cows fed P-adequate diets (Table 1). Furthermore, and as we also observed, plasma BALP concentrations peaked at calving and then declined during the first few weeks of lactation (Sato *et al*. 2013). Although the mechanisms that control BALP expression are unknown, it is appropriate that bone formation, including mineralisation, is suppressed in very early lactation.

It is well known that total ALP activity is increased in most forms of rickets and osteomalacia due to an increase in the concentration of the bone isoform (BALP), reflecting increased osteoblastic activity in these bone diseases. However, ALP activity is also increased in other metabolic diseases such as primary and secondary hyperparathyroidism (Craig *et al*. 2016), and together with issues of specificity (as discussed earlier), ALP does not provide definitive diagnosis in metabolic bone diseases. No previous studies have measured BALP specifically in P-deficient cattle. Lambs fed low-P diets had higher BALP concentrations, associated with less well mineralised bone reflecting the extent of the bone surface covered by active bone-forming cells (Scott *et al*. 1997). Similarly, in the P-deficient cows in our studies, histomorphological analysis showed significant increases in osteoid volume in trabecular (hip) bone biopsies from P-deficient cows (Kidd *et al*. 2016). Such histological results clearly indicate osteomalacia in P-deficient cows. We, therefore, propose that BALP is a valuable indicator of defective bone mineralisation in P-deficient mature cows.

Concluding perspectives

Biochemical markers of bone metabolism in blood are candidates for the diagnosis of P deficiency in cattle. Recent evidence supports the idea that both CTX-1 (as a marker of bone resorption) and BALP (as a marker of defective bone mineralisation) both have potential for defining P status in breeder cows. Further, the total Ca to inorganic P concentration ratio in plasma also appears to be a useful and simple measure of bone mobilisation in P-deficient cows. More work is required to confirm the sensitivity and specificity of such markers of P status, and to determine their predictive value in diagnosis of P deficiency in cattle.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The research project at the focus of this review was supported by Meat and Livestock Australia (Project B.NBP.0689) and the Queensland Department of Agriculture and Fisheries. We gratefully acknowledge the technical assistance of David Eagles, Kerry Goodwin, Bridget Pekin, Keanne Santos, Jereme Spiers, Natasha Steiger, Sam Van De Wakker and the DAF staff at Brian Pastures Research Facility.

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