Consecutive changes in serum alkaline phosphatase isoenzyme 3 activities in Holstein heifers during the first 18 months of life

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ABSTRACT. This study investigated consecutive fluctuations in serum activities of bone-specific alkaline phosphatase (ALP) isoenzyme 3 (ALP3) in 11 clinically healthy Holstein heifers during the first 18 months of life. ALP3 activities at the first sampling time point after weaning (3 months) were significantly lower than those at multiple time points during the pre-weaning period. Those activities increased from a minimum at 3 months to a peak at 6 months during the post-weaning period. In the anthropometric data, daily body weight and wither height gains appeared to be below the public data at 4 months and 4–5 months, respectively. The data suggested that serum ALP3 activity can be used to monitor skeletal growth of heifers at weaning.

KEY WORDS: agarose gel electrophoresis, alkaline phosphatase isoenzyme, heifer, weaning

Replacement rearing of heifers is an essential part of dairy farm management, because approximately 25–35% of the herd is culled every year and must be replaced [15]. The assessment of nutritional plans is required for stable management of dairy farms. Therefore, heifers must be monitored throughout the rearing period to identify any deviations from pre-set rearing targets at an early stage [15]. Analysis of blood biochemistry is an indirect approach to infer the efficiency of replacement rearing of heifers [4].

A recently developed commercial agarose gel electrophoresis (AGE) kit can be used to analyze circulating alkaline phosphatase (ALP) isoenzymes in humans, and this off-the-shelf kit may be useful for veterinary practice. We recently reported that this AGE kit was useful for measuring ALP isoenzymes in newborn calves [17] and lactating cows [3]. In particular, the use of the kit with both protease- and protease and neuraminidase-treated sera can discriminate among three major alkaline phosphatase isoenzymes in bovine sera: a hepatic ALP isoenzyme derived from hepatic tissue (ALP2), a bone isoenzyme derived from osteoblasts (ALP3), and an intestinal isoenzyme derived from intestinal tissue (ALP5) [3]. Because the ultimate goal of replacement rearing of heifers is to reach first-calving age at a predetermined time with an optimal skeletal growth rate [15], we hypothesized that circulating ALP3 activities may be useful for the monitoring of their skeletal growth. Therefore, the present study determined consecutive fluctuations in serum activities of ALP3 in 11 clinically healthy Holstein heifers during the period from birth to 18 months.

The study included 11 female Holstein newborn singleton calves born at the Obihiro University of Agriculture and Veterinary Medicine (OUAVM) farm. They were separated from the dams immediately after birth, and fed good-quality colostrum defined by density >1,044 kg/m^3 [7] three times on the first day (6 *l* in total). From the second day, calves were fed 3 *l* milk replacer (Calf Top EX; Zenrakuren, Tokyo, Japan) twice a day until day 7. Then they were fed replacer using an automatic calf feeder (VARIO+ automatic feeder; Förster-Technik GmbH, Engen, Germany) with calf starter (Calf Manna; Futaba Feed, Shioya, Japan), followed by hay and water *ad libitum* until the time of weaning. The calves were fed 6 *l* milk replacer per day until 6 weeks, and it was

gradually decreased to 3 *l* per day by 7 weeks for calves with body weight (BW) >77 kg at 6 weeks. Subsequently, the calves were fed 3 *l* replacer until 8 weeks and then weaned. Calves with BW <77 kg at 6 weeks were fed 6 *l* milk per day until the BW exceeded 77 kg, at which point they were considered heifers.

After weaning (mean ± standard deviation [SD]: 9.6 ± 0.7 weeks, range: 8.8–10.9 weeks), the heifers moved through three groups in sequence as they gained weight (~150 kg, ~200 kg, and ~300 kg). They were fed concentrate (Shin-Yogyu-Green; HOKUREN Kumiai Shiryo, Sapporo, Japan), vitamins, and minerals depending on their growth, along with hay and water *ad libitum*. When the heifers reached BW >300 kg, they were moved into another group and fed total mixed ration containing grass silage, concentrate (Farm Aid 18; Snow Brand Seed Co., td., Sapporo, Japan), vitamins, and minerals, with hay and water *ad libitum*. They were conceived by artificial insemination at 13.4 ± 1.1 months of age (range: 11.8–15.3 months of age). The procedures of feeding management for the heifers in this farm during the experiment period was operated according to the guideline of Japanese Feeding Standard for Dairy Cattle [16].

Blood samples were withdrawn from the jugular vein immediately before the first colostrum feeding (0), at 1, 2, 3, 4, 6, and 8 weeks (0.25, 0.5, 0.75, 1, 1.5 and 2 months) during the pre-weaning period, and at 3, 4, 5, 6, 8, 10, 12, 15, and 18 months during the post-weaning period. The blood samples were withdrawn into 5 m*l* plain vacuum collection tubes (Venoject II; Terumo, Tokyo, Japan), centrifuged, and then serum was stored at −60°C prior to analyses. BW was determined using a weight scale (EziWeigh5i Weigh System; Datamars Inc., Temple, TX, USA) before the first colostrum feeding (0) and at 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, and 18 months. Wither height (WH) was measured at 2 weeks and 1, 2, 3, 4, 5, 6, 8, 10, and 12 months. Daily BW and WH gains were calculated by dividing the difference between two consecutive measurements by the number of days.

The study protocol and study design were approved by the OUAVM Laboratory Animal Care and Use Committee (approval Nos. 28-158 and 29-123), under the jurisdiction of the Science Council of Japan.

Total ALP (t-ALP) activity in serum was measured spectrophotometrically using a LabAssay ALP kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Agarose gel electrophoresis (AGE) was performed using a QuickGel ALP agarose gel kit (J713; Helena Laboratories Japan, Saitama, Japan), QuickGel ALP (bone-type) reagent (J871; Helena Laboratories Japan), and an automatic electrophoresis system (Epalyzer-2; Helena Laboratories Japan), as described previously [3]. Briefly, control sera (5139; Helena Laboratories Japan) containing extract of bovine liver or intestine tissue were used as references for ALP2 and ALP5, respectively. Each serum sample (60 μl) was subjected to two treatments before electrophoresis; one half (30 μl) was mixed with a 300 U/m*l* protease cocktail (4 μl) and distilled water (2 μl) (P-treated serum), while the remainder (30 μl) was mixed with the protease cocktail (4 μl) and a separator solution containing neuraminidase (2 μl) (PN-treated serum). After electrophoresis (23 min at 230 V and 15°C), the gels were stained and scanned as densitometric images. The P-treated serum showed a distinct ALP5 fraction emerging on the cathode side and a fraction containing poorly separated ALP2 and ALP3 on the anode side, whereas the PN-treated serum showed a definite ALP2 fraction on the anode side and a poorly resolved fraction of overlapping ALP3 and ALP5 on the cathode side. The relative percentages of the ALP2 and ALP5 fractions were determined by the optical absorbance of the bands. The percentage of the ALP3 fraction was assessed by subtracting the percentage of the ALP5 fraction from that of the overlapping ALP3 and ALP5 fraction in the PN-treated serum. The absolute activity (U/*l*) of each isoenzyme was calculated from the t-ALP activity measured spectrophotometrically.

All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R Commander designed to add statistical functions frequently used in biostatistics [12]. The numerical data are expressed as means ± SDs. First, the values were checked for normality of the distribution by Kolmogorov–Smirnov normality test. The parameters were analyzed by one-way repeated-measures ANOVA and Holm *post hoc* tests to compare the values between pre-weaning and post-weaning periods and to evaluate the changes in values within the pre-weaning or post-weaning periods. In all analyses, $P<0.05$ was taken to indicate statistical significance.

Figure 1 shows the changes in serum levels of t-ALP and ALP3 activities during the pre-weaning (0–8 weeks) and post-weaning (3–18 months) periods. ALP3 was the largest fraction accounting for 64.1–77.5% of t-ALP activity, whereas ALP2 and ALP5 accounted for 19.0–31.4% and 3.5–6.6% of t-ALP activity, respectively. The activities of t-ALP at six time points (1, 2, 3, 4, 6, and 8 weeks) during the pre-weaning period were significantly higher than those at either of eight time points (3, 4, 5, 8, 10, 12, 15, and 18 months) during the post-weaning period ($P<0.01$–0.05). ALP3 activities at four time points (1, 4, 6, and 8 weeks) during the pre-weaning period were significantly higher than those at either of seven time points (3, 4, 8, 10, 12, 15, and 18 months) during the post-weaning period ($P<0.01$–0.05).

During the pre-weaning period in the 11 heifers, the t-ALP activities showed bimodal peaks at 1 and 6 weeks (1139.2 ± 297.6 and 1136.9 ± 263.4 U/*l*, respectively) and a minimum value at 3 weeks (775.6 ± 149.3 U/*l*; $P<0.01$–0.05 vs. the bimodal peaks). ALP3 activities showed a similar pattern of fluctuation to t-ALP, with bimodal peaks at 1 and 6 weeks (883.0 ± 266.6 and 822.0 ± 240.1 U/*l*, respectively) and a minimum value at 3 weeks (495.9 ± 133.3 U/*l*; $P<0.01$–0.05 vs. the bimodal peaks).

During the post-weaning period, the t-ALP activities showed a minimum (423.4 ± 222.8 U/*l*) at 3 months, increased to a peak (785.2 ± 179.2 U/*l*; $P<0.05$ vs. the minimum) at 6 months, and then remained almost constant until 18 months. Similarly, ALP3 activities also showed a minimum (289.6 ± 188.9 U/*l*) at 3 months, increased to a peak (567.3 ± 171.5 U/*l*; $P<0.05$ vs. the minimum) at 6 months, and then remained constant thereafter.

Figure 2 shows the changes in BW, WH, and daily BW and WH gains in 11 heifers. BW was 41.1 ± 4.4 kg at birth (immediately before the first colostrum feeding), which increased to 556.5 ± 32.8 kg at 18 months. WH was 81.6 ± 3.0 cm at 2 weeks, which increased to 131.3 ± 3.6 cm at 12 months. The changes in these anthropometric measurements were equivalent to or somewhat
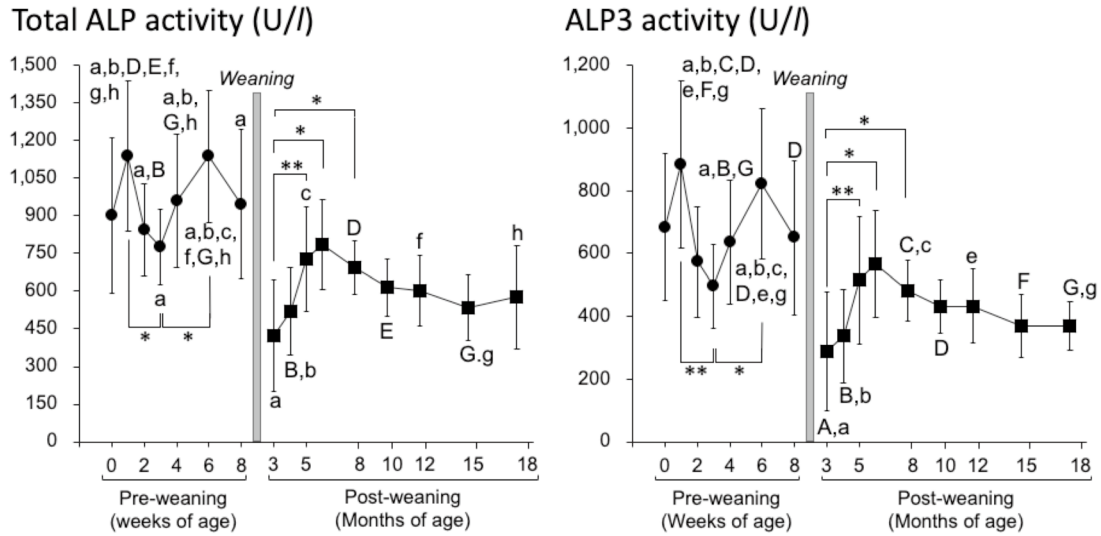
Fig. 1. Changes in serum activities (mean ± SD) of total alkaline phosphatase (ALP) and ALP isoenzyme 3 (ALP3) during the pre-weaning (0–8 weeks; ●) and post-weaning (3–18 months; ■) periods in 11 Holstein heifers. Superscript letters indicate significant differences in mean values between the pre-weaning and post-weaning periods: [A,B,C,D,F,G] $P<0.05$, [a,b,c,e,g] $P<0.01$. Asterisks indicates significant differences in mean values from the minimum during each period of pre-weaning (3 weeks) or post-weaning (3 months): $*P<0.05$, $**P<0.01$.
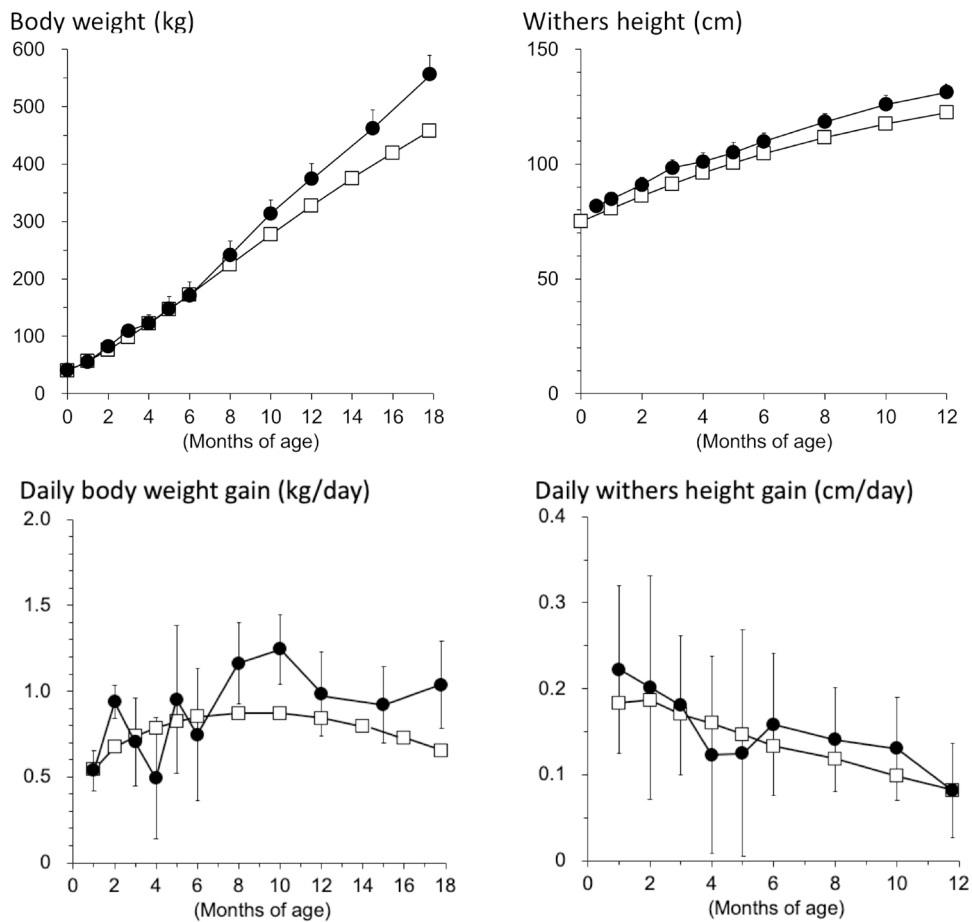

Fig. 2. Changes in body weight (BW), wither height (WH), and daily BW and WH gains of 11 Holstein heifers (●). For comparison, the standard values of mean BW, WH, and daily BW and WH gains of Holstein heifers in Japan obtained from the public data of the Holstein Cattle Association of Japan (http://hcaj.lin.gr.jp/) are also shown (□).

higher than the public data on BW (40.0 and 458.0 kg, respectively) and WH (75.1 and 122.4 cm, respectively) of heifers in Japan (http://hcaj.lin.gr.jp). The daily BW gain of our heifers fluctuated around the values of the public data until 6 months, and thereafter showed consistently higher values than the public data until 18 months. In particular, the daily BW gain at 4 months (i.e., the value obtained by dividing the difference in BW between 3 and 4 months by the number of days) appeared to be below the public data. The daily WH gain gradually declined in a manner similar to the public data until 12 months, except at 4 and 5 months when the values dropped markedly.

The present study examined consecutive changes in serum activities of t- ALP and ALP3 in 11 heifers during the first 18 months of life. Our data indicate that ALP3 is the primary contributor to t-ALP activity. Generally, serum t-ALP activity is higher in young animals than in adults [6], and ALP3 accounts for the major fraction in immature and/or growing animals, including kittens, puppies, foals, and calves [1, 6, 11, 17]; our results are consistent with these data.

ALP3 is considered an accurate marker of bone formation, which is the standard index for increased osteoblastic activity [1, 20] and extracellular mineralization [8]. In the present study, the activities of ALP3 as well as t-ALP showed bimodal peaks at 1 and 6 weeks during the pre-weaning period. Hatate *et al*. [10] reported that a large amount of ALP in the colostrum was transferred to the neonatal circulation of calves, leading to marked increases in plasma activities of t-ALP and bone-specific ALP [14] during the period from 10 to 30 hr after the first colostrum feeding. A similar increase in t-ALP activity was seen after ingestion of colostrum in 1- to 2-day-old kittens [6]. Therefore, this first peak of t-ALP and ALP3 at 1 week in our calves may have been due to the increases in levels of these enzymes after colostrum feeding.

Activities of t-ALP and ALP3 in our heifers showed a significant decline at the first sampling time point after weaning (3 months) compared to the values at multiple time points during the pre-weaning period. A decrease in serum t-ALP activity has also been reported in association with undernutrition in humans [9]. During the pre-weaning period, the weight of the reticulorumen increases from 38 to 67% of the total weight of the forestomach in calves [5]. Weaning alters the diet fed to the calves gradually from milk to solid food (hay and concentrate), which often leads to a risk of insufficient nutrient intake [18, 21]. Serum t-ALP activity is positively correlated with average feed intake [19] and feed efficiency [2] in growing cattle. In our heifers, daily BW and WH gains seemed to decrease at 4 and 4–5 months, respectively, suggesting a transient deceleration of active growth of the physique and skeleton soon after weaning (3–5 months). Therefore, the decline in t-ALP and ALP3 activities may be related to reduced bone formation due to insufficient feed intake and/or nutritional condition at the time of weaning.

After weaning, t-ALP and ALP3 activities in our heifers increased, peaking at 6 months and then mostly remained constant thereafter. The feeding time in post-weaned calves increases to a level equivalent to that in adults by 6 months [13]. The increases in t-ALP and ALP3 activities after weaning were considered to reflect activated bone formation due to the increase in food intake following prolongation of feeding time and BW gain.

The overall fluctuation of serum ALP3 activity in our heifers during the first 18 months of life appeared to show a peak at 6 weeks (1.5 months) followed by gradual decline, if the first transient rise at 1 week and the significant decrease at the 3 months were not existed. Dairy WH gain seem to be the highest at 1–2 months in our heifers and the public data (Fig. 2), suggesting the possibility of accelerated bone formation around 6 weeks. It was controversial whether colostral ALP3 suppressed an endogenous release of ALP3 from osteoblasts during the early period of pre-weaning, because this enzyme may facilitate extracellular mineralization [8, 10]. On the other hand, ALP3 activity in our heifers showed a significant decrease at the time of weaning (3 months), suggesting reduced bone formation. Weaning off milk is the major feeding transition for young heifers, which is particularly stressful for the animal and challenging for the producer [21]. In the present study, daily BW and WH gains appeared to be below the public data at 4 months and 4–5 months, respectively, which were consistent with the timing of weaning and herd migration. The declines of ALP3 activity may be specific blood biochemical indicators of the need to improve the techniques of weaning and herd migration in heifers. In conclusion, the present study suggested that the measurement of circulating ALP3 activity can be useful for the monitoring of skeletal growth of Holstein heifers. Further studies are needed to examine change in serum ALP3 activity specific to several nutritional and several disease conditions in cattle to develop useful diagnostic methodologies for veterinary clinicians.

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