The effects of early weaning on innate immune responses of Holstein calves¹

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ABSTRACT

The objectives of this study were to compare innate immune responses of calves weaned early (EW; $n = 23$; weaned at 23.7 ± 2.3 d of age) with those of conventionally weaned calves (CW; $n = 22$; weaned at 44.7 ± 2.3 d of age). All calves were fed 3.8 L of colostrum within 12 h of birth and were subsequently fed milk replacer twice daily. The weaning process began by withdrawal of the afternoon milk-replacer feeding. Milk was fully withdrawn, and the calf was considered completely weaned when it consumed 900 g of calf starter as-fed for 2 consecutive days. Blood samples were collected from all calves at 24, 27, 31, 45, 48, 52, and 66 ± 2.3 d of age. Early weaned calves took a variable amount of time to completely wean from milk replacer; therefore, data were also analyzed by comparing calves grouped by latency to completely weaned (fast = 1 to 5 d; intermediate = 6 to 8 d; slow = 15 to 17 d). Slow-EW calves weighed less than either the fast- or intermediate-EW calves before initiating weaning. At 27 d of age, circulating neutrophils were greater among EW calves than CW calves. Moreover, fast-EW calves had lower neutrophil:mononuclear cell ratios at 45 d of age than other EW calves. Slow-EW calves had lower TNF- α concentrations from whole blood stimulated with endotoxin at 27 and 31 d of age compared with fast- and intermediate-EW calves. All EW calves had decreased neutrophil L-selectin at d 27 and increased neutrophil L-selectin at 31 d of age. At 31 d of age, neutrophil β_2 -integrin was the greatest among the fast-EW calves. All EW calves had decreased neutrophil oxidative burst at 27 and 31 d of age. Three days after CW calves were weaned they had higher neutrophils, hematocrit percentages, and circulating cortisol than EW calves. In addition, 3 d after CW calves were weaned they had decreased neutrophil oxidative burst

responses to *Escherichia coli*. Weaning, irrespective of age, suppressed many innate immune responses. In addition, early weaning transiently suppressed L-selectin expression on neutrophils; however, the immunological significance in the context of the resistance to disease is unknown because EW calves likely had greater protection from passively derived immunoglobulins when they were weaned. Finally, calves with lower BW around 24 d of age may not be suitable for early weaning programs as evident in the suppressed secretion of TNF- α from whole blood cultures during the week following the initiation of weaning.

Key words: bovine, immune, nutrition, stress

INTRODUCTION

Prewaning growth is the most expensive growth that an animal will undergo during its life. It is common practice within the dairy cattle industry to restrict the consumption of milk or milk replacer to stimulate an earlier consumption of calf starter, which decreases the age at which calves can be completely weaned from milk. Data from Martin et al. (1959) indicate that calves were able to utilize volatile fatty acids at 3 wk of age. Furthermore, calves weaned as early as 3 wk had similar performance as calves weaned at either 5 or 7 wk (Winter, 1985). However, recent estimates from the USDA National Animal Health Monitoring and Surveillance (NAHMS) indicated that the average age at weaning was 8.2 wk of age, with large operations weaning calves at an older age, 9.1 wk (NAHMS, 2007). Therefore, it is common within the dairy industry to feed dairy calves restricted quantities of milk, but actually wean them at an older age. The early weaning of calves would greatly reduce calf-rearing costs by decreasing feed and labor expenditures.

Perhaps the dairy industry fails to wean calves earlier because it is assumed that weaning is stressful for calves. Changes in feeding strategies, including weaning, can be a potential stressor to beef calves (Arthington et al., 2003). Previous research has evaluated BW gain, rumen development, blood glucose concentrations, blood urea N concentrations, and fecal and respiratory scores as indicators of health when evaluating various weaning strategies (Burt, 1968; Anderson et al., 1987,

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1988; Heinrichs et al., 1990). It is not known, however, whether age at weaning influences the innate immunological responses to weaning in dairy calves. It is conceivable that if weaning dairy calves does decrease innate immune defenses regardless of age, then weaning at an earlier age may provide better immunity to disease because passively derived humoral immunity will be greater. Therefore, the objective of this experiment was to determine the influence of weaning during either wk 4 or wk 7 of life on innate immune responses of Holstein calves.

MATERIALS AND METHODS

Animals and Housing

The experiment was conducted in March and April 2010. All animal procedures were reviewed and approved by the Texas Tech University Animal Care and Use Committee. Forty-five Holstein bull calves (12 to 36 h after birth) were purchased from 2 local commercial dairies over a 7-d period. All calves were fed 3.8 L of pooled colostrum from each dairy within 12 h of birth. All calves were transported approximately 60 km to the Hilmar Cheese Calf Research Facility at Texas Tech University (New Deal, TX), and calves were housed with straw bedding in commercial polyethylene calf hutches (Agri-Plastics, Tonawanda, NY).

Feeding and Weaning Strategy

Before assigning calves to weaning strategy treatments, all calves were fed, on a percentage as-fed basis, 227 g of a 20% all-milk protein/20% fat from edible lard milk replacer (Herd Maker, Land O'Lakes Animal Protein Co., Shoreview, MN) twice daily at 0800 and 1600 h. After the first week, all calves were offered ad libitum access to a calf starter (Table 1) and water for the remainder of the study. The quantity of calf starter was adjusted daily for approximately 15% refusals. Twenty-eight days after the first group of calves was acquired all calves were randomly allotted to 1 of 2 weaning strategy treatments. The treatments were early weaned (**EW**; $n = 23$; weaned at 23.7 ± 2.3 d of age) or conventionally weaned (control) calves (**CW**; $n = 22$; weaned at 44.7 ± 2.3 d of age). The weaning process was initiated by restricting the intake of milk replacer to 50% by withdrawing the 1600 h feeding. From d 24 to 45 of age, CW calves continued to be fed milk replacer twice daily. Calves were designated as completely weaned when they consumed 900 g of calf starter as fed for 2 consecutive days. Calves were individually weighed and BW was recorded at arrival, 24, 31, 45, 52, and 66 ± 2.3 d of age. Fecal scores

Table 1. Calf starter ingredient and chemical compositions

Item	Amount
Ingredient, % of DM	
Steam-flaked corn	67.3
Soybean meal, 48% CP	13.3
Cottonseed meal	11.3
Molasses	5.3
Calf mineral/vitamin premix ¹	2.8
Chemical composition	
DM, %	88.2
CP, %	18.9
ME, Mcal/kg	3.06

¹Premix contained (DM basis): 52.6% limestone; 39.9% soybean meal; 6% salt; 0.451% zinc sulfate; 0.4% selenium selenite 0.2%; 0.267% manganese oxide; 0.18% vitamin E, 500 IU/g; 0.157% copper sulfate; 142 mg/kg vitamin A, 1,000 kIU/g; 12.5 mg/kg ethylenediamine dihydroiodide; 8.7 mg/kg cobalt carbonate.

were collected multiple times daily by 2 independently trained observers and classified according to Ballou and DePeters (2008).

Blood Collection and Analysis

Nine milliliters of peripheral blood were collected via jugular venipuncture immediately before assignment of treatments (24 d of age), as well as 27, 31, 45, 48, 52, and 66 ± 2.3 d of age. Samples had to be collected into 2 blocks on consecutive days to accommodate logistics of running the immune response assays. The block (A or B) was randomly assigned to calves within their treatment (block A: EW, $n = 12$ CW, $n = 11$; block B: EW, $n = 11$, CW, $n = 11$). Blood was collected into 2 evacuated blood collection tubes (6 and 3 mL) containing heparin and immediately placed on ice. Within 1 h after collection, the 3-mL Vacutainer from each calf was analyzed for hematocrit, total leukocyte counts, and differential analyses of neutrophils and mononuclear cells using a Cell Dyn 3700 with automated 50-sample loader and vet-package software (Abbott Laboratories, Abbott, IL). The neutrophil:mononuclear cell ratio (**N:M**) was calculated. The Cell Dyn settings did not distinguish between lymphocytes and monocytes; therefore, the N:M ratio was determined rather than the neutrophil:lymphocyte ratio. Plasma was collected after centrifugation at $1,250 \times g$ and stored at -80°C until analyzed for cortisol, glucose, urea nitrogen, and haptoglobin concentrations as described previously (Hulbert et al., 2011). The intraassay coefficients of variations were 5.8, 3.8, 4.4, and 1.8% for plasma cortisol, glucose, urea nitrogen, and haptoglobin concentrations, respectively. The interassay coefficients of variations were 7.2, 4.0, 5.1, and 1.8% for plasma cortisol, glucose, urea nitrogen, and haptoglobin concentrations, respectively.

Ex Vivo Innate Immunological Responses

The ex vivo innate immune responses evaluated were described in a companion paper (Hulbert et al., 2011). Briefly, the simultaneous phagocytic (**PG**) and oxidative burst (**OB**) capacities of neutrophils in response to an enteropathogenic *Escherichia coli*, isolated from the spleen of a septicemic calf, were analyzed by dual-color flow cytometry. Data are reported as the percentage of neutrophils phagocytizing and producing an oxidative burst (OB+PG+) as well as the geometric mean fluorescence intensities of the FL-1 (oxidative burst) and FL-3 (phagocytosis). Additionally, both L-selectin (CD62L) and β_2 -integrin chain of CD11a, b and c heterodimers (CD18) expression on circulating neutrophils was determined by flow cytometry. Data are reported as the geometric mean fluorescence intensity. Finally, whole blood was diluted with RPMI and cocultured at a final concentration of 1 $\mu\text{g/mL}$ of LPS (*E. coli* O111:B4; Sigma-Aldrich, St. Louis, MO) for 24 h. Supernatant concentrations of tumor necrosis factor (TNF)- α were quantified using a commercially available ELISA (DY2279E; R&D Systems Minneapolis, MN). The intra- and interplate coefficients of variations were 3.2 and 4.1%, respectively.

Statistical Analysis

Data were analyzed in 2 separate sets: (1) EW calf weaning (24 to 45 d of age) and (2) CW calf weaning (45 to 66 d of age). For all data sets, baseline measurements taken immediately before assigning weaning strategy treatments were tested as covariates in all statistical models. If the covariate was not significant, it was removed from the model. For all metabolic and immune response data, excluding data analyzed by flow cytometry, a linear, mixed model with the fixed effects of time, treatment, and the interactions of treatment $\times$ time and block $\times$ treatment $\times$ time was fitted. The random effect was calf tested within treatment $\times$ block.

To account for daily variation in flow cytometry settings, before statistical analyzing all the flow-cytometry data, the data from EW calves in data set 1 were divided by the block mean from the CW calf data. For data set 2, data from CW calves were divided by the block mean from EW calves that completely weaned within 5.9 ± 1.24 d. All flow cytometry data are presented as the percentage of respective calf means. The fixed effects of time and the interaction of time $\times$ block were fitted. The random effect was calf nested within block.

An additional analysis was performed on the EW calf data for data set 1. Early weaned calves were classified according to the latency of the calf to be fully weaned

(removal of all milk replacer after consumption of 900 g of calf starter on 2 consecutive days). Early weaned calves were separated into 3 discrete groups including 1 to 5 d (fast); 6 to 8 d (intermediate); and 15 to 17 d (slow) to completely wean. All non-flow-cytometry immune data were fitted to a linear model with the fixed effects of group (fast, intermediate, or slow), time, and the interactions of group $\times$ time, and group $\times$ time $\times$ block. Again, all flow cytometry data for the EW group were first divided by the block mean from the CW calf data to account for daily variation in flow cytometry settings. A linear model with the fixed effects of group, time, and the interactions of group $\times$ time and group $\times$ time $\times$ block was fitted.

All data were analyzed by REML ANOVA using the MIXED procedure of SAS (v.9.2, SAS Inst. Inc., Cary, NC). The ante-regressive (1) covariance structure for the within-subject measurement was used for all models. Repeated data were tested for normality of the residuals by evaluating the Shapiro-Wilk statistic using the UNIVARIATE procedure of SAS (v.9.1.3, SAS Inst. Inc.). Data that were not normally distributed were log-transformed before mixed model analysis. Pairwise differences were performed at each time using a sliced-effect multiple comparison approach with a Tukey-Kramer adjustment. Least squares means ($\pm$ SEM) are reported throughout. A treatment difference of $P \leq 0.05$ was considered significant, and $P \leq 0.1$ was considered a tendency.

RESULTS

Performance

Two CW calves died and were removed before analyzing the data. Before assigning treatments, all EW calves had similar BW, ADG, milk replacer intake, and calf starter intake to CW calves ($P > 0.43$; Table 2). Although EW calves were eating more calf starter than CW calves from 24 to 45 d of age ($P < 0.01$; Table 2), they were consuming less total ME ($P < 0.01$; Table 2) and were more efficient at utilizing ME for live BW gain than CW calves ($P < 0.01$; Table 2). After CW calves were weaned, they tended ($P = 0.07$; Table 2) to have lesser ADG than EW calves from 45 to 52 d and from 45 to 66 d of age. In addition, during this period, EW calves were consuming more calf starter and had greater intakes of total ME ($P < 0.01$; Table 2).

When EW calves were grouped by latency to completely wean, slow-EW calves weighed less ($P < 0.05$) than either the intermediate- or fast-EW calves before weaning (41.7 , 46.3 , and 48.7 ± 2.04 kg for slow-, intermediate-, and fast-EW calves, respectively). The ADG

from 31 to 45 d of age and total intake of ME from 24 to 45 d of age were less in slow-EW calves than either the intermediate- or fast-EW calves ($P < 0.01$; Table 2). Intake, performance, or efficiency of ME for BW gain did not differ among EW calves from 45 to 66 d of age ($P > 0.10$; Table 2).

Overall performance, from birth to 66 ± 2.3 d of age, was not different between EW and CW calves or among calves with varying latencies to weaning ($P > 0.17$; Table 2). As expected, EW calves consumed less milk replacer and more calf starter than CW calves. In addition, EW calves that required more time to completely wean also consumed more milk and less calf starter than EW calves that completely weaned more quickly (Table 2).

Blood Parameters

No treatment $\times$ time, group $\times$ time, treatment, or group effects were observed for total leukocyte counts from 24 to 45 d of age ($P > 0.10$; Table 3). All calves had a decrease in total leukocyte counts at 27 d of age ($P > 0.01$; Table 3). Neutrophil:mononuclear cell ratios were elevated among all EW calves at 27 d of age compared with CW calves (log-transformed $P = 0.05$; Figure 1A). Slow-EW and intermediate-EW calves had greater N:M at 45 d of age compared with fast-EW calves (log-transformed $P < 0.01$; Figure 1B). The CW calves had greater N:M ratios than EW calves on 48 d of age, which was 3 d after they were weaned (log-transformed $P = 0.05$; Figure 2). At 52 d of age, all

Table 2. Performance data for control (CW) and early weaned (EW) calves, as well as early weaned calves grouped by latency to be completely weaned¹

Item	Treatment				Early weaned calves				
	CW	EW	Largest SEM	<i>P</i> -value	Fast	Int.	Slow	Largest SEM	<i>P</i> -value
n	20	23			8	9	6		
0 to 24 d of age									
Birth BW, kg	41.4	42.1	1.25	0.71	43.4	41.5	38.7	2.41	0.36
Total serum protein, g/dL	5.12	4.95	0.203	0.53	4.90	4.73	5.40	0.407	0.43
Fecal score ²	1.61	1.66	0.059	0.39	1.66	1.66	1.66	0.127	0.37
BW at 24 d age, kg	46.7	46.0	1.09	0.60	48.7 ^a	46.3 ^a	41.7 ^b	2.04	0.05
ADG, kg/d	0.17	0.17	0.025	0.95	0.18	0.19	0.13	0.043	0.45
Milk intake, kg of DM	8.8	8.8	0.02	0.67	8.8	8.8	8.8	0.20	0.36
Starter intake, kg of DM	2.4	2.7	0.29	0.45	3.3	2.3	2.4	0.57	0.26
Total ME, Mcal	51.5	52.5	0.86	0.43	54.5	51.3	51.6	1.56	0.31
ME:BW gain, Mcal:kg ³	10.5	11.3	0.82	0.54	10.3	9.9	12.1	1.69	0.60
24 to 45 d of age									
ADG 24 to 31 d, kg/d	0.62	0.52	0.070	0.32	0.47	0.46	0.68	0.136	0.41
ADG 31 to 45 d, kg/d	0.63	0.63	0.038	0.95	0.71 ^a	0.69 ^a	0.43 ^b	0.063	0.01
ADG 24 to 45 d, kg/d	0.62	0.59	0.023	0.35	0.63	0.62	0.62	0.051	0.19
Milk intake, kg of DM	8.8	3.2	0.20	0.01	2.3 ^a	3.3 ^b	4.2 ^b	0.41	0.01
Starter intake, kg of DM	12.3	15.6	0.76	0.01	19.1 ^a	15.2 ^b	11.8 ^b	1.18	0.01
Total ME, Mcal	82.8	65.0	1.74	0.01	70.8 ^a	64.4 ^b	58.1 ^b	2.47	0.01
ME:BW gain, Mcal:kg ³	6.4	5.2	0.14	0.01	5.2	4.8	5.5	0.29	0.29
Completely weaned age, d	—	33.7	0.78	—	29.5 ^a	33.3 ^b	40.0 ^c	0.9	0.01
45 to 66 d of age									
ADG 45 to 52 d, kg/d	0.86	0.99	0.055	0.07	0.97	1.05	0.97	0.057	0.42
ADG 52 to 66 d, kg/d	0.59	0.66	0.051	0.29	0.62	0.69	0.67	0.089	0.79
ADG 45 to 66 d, kg/d	0.68	0.77	0.038	0.07	0.74	0.81	0.77	0.059	0.58
Milk intake, kg of DM	1.3	—	—	—	—	—	—	—	—
Starter intake, kg of DM	29.6	35.8	1.07	<0.01	36.9	35.6	34.7	2.16	0.75
Total ME, Mcal	99.1	111.9	3.45	<0.01	115.3	111.3	108.6	6.75	0.75
ME:BW gain, Mcal:kg ³	7.4	7.1	0.33	<0.01	7.5	6.8	6.85	0.49	0.39
Completely weaned age, d	46.4	—	0.8	—	—	—	—	—	—
0 to 66 d of age									
ADG, kg/d	0.48	0.50	0.020	0.49	0.51	0.52	0.45	0.036	0.29
Milk intake, kg of DM	18.9	12.0	0.24	<0.01	11.0 ^a	12.1 ^{ab}	13.0 ^b	0.42	0.01
Starter intake, kg of DM	41.9	54.1	1.89	<0.01	59.2 ^a	53.1 ^{ab}	48.8 ^b	2.99	0.05
Total ME, Mcal	230.3	229.4	5.09	0.91	240.5	227.1	218.3	8.67	0.17
ME:BW gain, Mcal:kg ³	7.4	7.1	0.26	0.39	7.3	6.7	7.4	0.47	0.40

^{a-c}Means within a row with different superscripts differ significantly, $P < 0.05$.

¹Latency to be completely weaned was confirmed when milk replacer was completely withdrawn after 2 consecutive days of consuming 900 g of calf-starter on an as-fed basis. Early weaned calves were grouped into 3 categories: fast (1 to 5 d), intermediate (Int.; 6 to 8 d), and slow (15 to 17 d).

²Significance reported from treatment $\times$ time interaction.

³ME:BW gain was calculated by dividing the total Mcal of ME by BW gain for a given period.

Figure 1. Neutrophil:mononuclear cell ratios from 24 to 45 d of age for (A) control (CW) and early weaned (EW) calves and (B) EW calves grouped by latency to completely wean. Control calves were not weaned and were fed milk-replacer twice daily. Latency to be completely weaned was confirmed when milk replacer was completely withdrawn after 2 consecutive days of consuming 900 g of calf-starter on an as-fed basis. Early weaned calves were divided into 3 categories: fast (1 to 5 d), intermediate (6 to 8 d), and slow (15 to 17 d). *Treatment $\times$ time effect log-transformed $P < 0.05$.

calves had decreased N:M ratios ($P < 0.05$; Table 4), and at 66 d of age, all calves had decreased hematocrits ($P < 0.01$; Table 4).

Figure 2. Neutrophil:mononuclear cell ratios from 45 to 66 d of age for control (CW) and early weaned (EW) calves. Control calves were weaned by removal of 50% of milk replacer at 44.7 ± 0.5 d of age, whereas 17 calves (EW) began the weaning process at 23.7 ± 0.5 d of age and were completely weaned within 5.9 ± 0.3 d. Complete weaning was confirmed when milk replacer was completely withdrawn after 2 consecutive days of consuming 900 g of calf-starter on an as-fed basis. **Treatment $\times$ time effect log-transformed $P < 0.01$.

No treatment $\times$ time, group $\times$ time, treatment, or group effects for cortisol concentrations at 24 d to 45 d of age ($P > 0.10$; Table 3); however, all calves (CW and EW) had increased cortisol at 31 and 45 d of age ($P < 0.01$; Table 3). On d 48 and 66 of age, circulating cortisol was greater among CW calves than EW calves ($P = 0.05$; Figure 3).

Hematocrits were less in CW calves than in EW calves at 31 d of age ($P < 0.05$; Figure 4A). Fast- and slow-EW calves had lower hematocrits than intermediate-EW calves at 45 d of age ($P < 0.05$; Figure 4B). Similar to EW calves after weaning, CW had greater hematocrits at 48 and 52 d of age than EW calves ($P < 0.05$; Figure 5).

Table 3. Peripheral blood measurements for all 43 calves from 24 to 45 d of age¹

Item	Age, ² d				Largest SEM	P-value		
	24	27	31	45		Time	Treatment	Treatment $\times$ Time
WBC, ³ $\times 10^6$ /mL	8.1	7.8 ^a	8.6 ^b	8.4 ^b	0.3	0.01	0.92	0.25
N:M ⁴	0.6	0.5	0.51	0.44	0.05	0.69	0.25	0.19
Hematocrit, %	32.6	32.6 ^a	32.2 ^a	30.2 ^b	0.3	<0.01	0.23	0.13
Cortisol, ng/mL	24.3	22.7 ^a	35.2 ^b	40.3 ^c	2.9	<0.01	0.31	0.59
TNF- α , ⁵ pg/mL	1,132.7	1,362.8 ^a	1,669.0 ^b	2,104.3 ^c	110.4	<0.01	0.48	0.4
Haptoglobin, OD ⁶ $\times 100$	1.99	1.88	1.72	1.94	0.3	0.47	0.19	0.85

^{a-c}Means within a row with different superscripts differ significantly, $P < 0.05$.

¹Age was 23.7 to 44.7 ± 2.3 d of age.

²Baseline measures (age 24 d) served as a covariate in the models; therefore, least squares mean differences are only compared among d 27, 31, and 45.

³Total circulating leukocyte count.

⁴Neutrophil:mononuclear cell ratio; log-transformed P -values.

⁵Tumor necrosis factor- α secreted from whole blood stimulated with $1 \mu\text{g/mL}$ LPS for 24 h.

⁶Optical density; root-transformed P -values.

Secretion of TNF- α in LPS-Stimulated Whole Blood Cultures

No treatment $\times$ time or treatment effects were observed for TNF- α concentrations in the supernatant fraction from LPS-stimulated whole blood between EW and CW from 24 to 45 d of age ($P > 0.10$; Table 3); however, all calves had gradual increases in TNF- α concentrations from LPS-stimulated whole blood over time ($P < 0.01$; Figure 6A). Slow-EW calves had lower TNF- α concentrations from LPS-stimulated whole blood at 27 and 31 d of age compared with fast- and intermediate-EW calves ($P < 0.05$; Figure 6B). All calves had decreased TNF- α concentrations at 48 d of age, 3 d after CW calves were weaned ($P < 0.01$; Table 4), but no block effect was observed for TNF- α concentrations at 48 d of age (data not shown; $P > 0.10$).

Expression of Neutrophil Adhesion Molecules

The EW calves had decreased neutrophil L-selectin at 27 d of age, 3 d after EW calves were weaned, but they had increased neutrophil L-selectin at 31 d of age ($P < 0.01$; Table 5). When EW calves were grouped by latency to completely wean, neutrophil L-selectin was decreased only among the fast- and slow-EW calves at 27 d of age ($P < 0.01$; Figure 7A). Neutrophil β_2 -integrin was elevated in fast-EW calves at 31 d of age, which was 7 d after they were weaned ($P < 0.05$; Figure 7B). No changes in neutrophil adhesion expression among CW were observed after they were weaned at 45 d of age ($P > 0.10$; Table 6).

Neutrophil Phagocytosis and Oxidative Burst in Response to *E. coli*

Among all EW calves, the percentage of OB+PG+ neutrophils increased on 27 d of age, which was 3 d after initiating weaning, but was decreased on 45 d of age ($P < 0.01$; Table 5). All EW calves had decreased neutrophil OB intensity at 27 and 45 d of age ($P < 0.01$; Table 5). No changes in neutrophil PG intensity in EW calves were observed after they were weaned ($P > 0.10$; Table 5). Furthermore, at 24 to 45 d of age, no differences were observed in neutrophil OB or PG response to *E. coli* between fast-, intermediate- and slow-EW calves ($P > 0.10$; data not shown). The percentage of OB+PG+ neutrophils decreased among CW calves on 48 d of age, which was 3 d after CW calves were weaned ($P < 0.05$; Table 6). In addition, CW calves also tended ($P = 0.06$) to have decreased neutrophil PG intensity at 48 d of age, followed by an increase at 52 d of age (Table 6).

Table 4. Peripheral blood parameters for all 43 calves from 45 to 66 d of age¹

Item	Treatment (Trt)		Largest SEM	Age, d				Largest SEM	P-value		
	CW	EW		45	48	52	66		Trt	Time	Trt $\times$ Time
N	20	23		43	43	43	43				
WBC ² $\times 10^6$ /mL	8.2	8	0.4	8.3 ^a	8.5 ^a	8.2 ^a	7.5 ^b	0.3	0.6	0.04	0.47
N:M ³	0.46	0.38	0.03	0.42 ^a	0.42 ^a	0.34 ^b	0.50 ^c	0.04	0.06	0.02	0.04
Hematocrit, %	29.3	28	0.4	30.3 ^a	29.6 ^a	28.5 ^a	27.9 ^b	0.3	0.03	<0.01	0.21
Cortisol, ng/mL	22.3	19.9	1.7	38.7 ^a	14.8 ^b	12.6 ^b	18.3	1.9	0.46	<0.01	0.4
TNF- α , ⁴ pg/mL	1,766	1,853	122	2,150 ^a	1,026 ^b	2,020 ^a	2,042 ^a	200.1	0.44	0.05	0.07
Haptoglobin, OD ⁵ $\times 100$	1.46	1.35	0.11	2.27 ^a	1.66 ^a	1.56 ^a	0.99 ^b	0.13	0.59	<0.01	0.13

^{a-c}Means within a row with different superscripts differ significantly, $P < 0.05$.

¹Control calves (CW) were weaned at 44.7 ± 2.3 d of age; early weaned (EW) calves were weaned at 23.7 ± 2.3 d of age.

²Total circulating leukocyte count.

³Neutrophil:mononuclear cell ratio; log-transformed P -values.

⁴Tumor necrosis factor- α secreted from whole blood stimulated with 1 μ g/mL LPS for 24 h.

⁵Optical density; root-transformed P -values.

Figure 3. Plasma cortisol concentrations 45 to 66 d of age for control (CW) and early weaned (EW) calves. Control calves were weaned by removal of 50% of milk replacer at 44.7 ± 0.5 d of age (indicated by white arrow), whereas 17 calves (EW) began the weaning process at 23.7 ± 0.5 d of age and were completely weaned within 5.9 ± 0.3 d. Complete weaning was confirmed when milk replacer was completely withdrawn after 2 consecutive days of consuming 900 g of calf-starter on an as-fed basis. *Treatment $\times$ time effect, $P < 0.05$.

DISCUSSION

Earlier weaning of dairy calves may decrease labor and feed costs; therefore, the objective of this study was to determine the effects of weaning during either wk 4 or 7 of age on innate immune responses of Holstein calves. Before assigning weaning treatments, BW, ADG, milk intake, starter intake, and fecal scores did not differ among EW and CW calves. Furthermore, ADG did not differ between EW and CW calves from 24 to 45 d of age; however, EW calves consumed more starter but less total ME (Mcal) than CW calves during this period. Therefore, EW calves were more efficient at utilizing ME for BW gain. The exact reason for this observation is not known, but could be due to more efficient utilization of ME for maintenance or growth. Body composition was not determined in the current study; therefore, differences in composition of the BW gain cannot be excluded. Once CW calves were weaned at 45 d of age, they tended to have lesser ADG from 45 to 66 d of age, which can likely be attributed to the CW calves consuming less calf starter and total ME during this period. These data contrast with those of Heinrichs et al. (1990), in which greater calf starter intakes were observed from 5 to 7 wk of age among calves that were weaned at 4 versus 7 wk of age, but no differences in calf starter consumption from 8 to 24 wk of age were observed. Overall performance, from birth until 66 d of age, was not different between CW and EW calves, which is in agreement with Hill et al. (2009). In addition, the overall performance of calves in the present study was consistent with Hill et al. (2009).

When EW calf data were grouped according to their latency to completely wean, slow-EW calves had lesser

BW initially and at 24 d of age and had lesser ADG from 31 to 45 d of age than either intermediate- or fast-EW calves. Nonetheless, neither feed efficiency nor utilization of ME for BW gain was different among the groups of EW calves from 24 to 45 d of age. The slow-EW calves were slower to wean; however, they were consuming a similar amount of ME for BW gain. Because the fact that calves were completely weaned at a fixed intake of starter regardless of metabolic BW, the slow-EW calves were over 40 d old when they were completely weaned. Therefore, the additional stressor of having the remaining milk bottle withdrawn occurred 1 wk after either the fast- or intermediate-EW calves had their remaining milk replacer withdrawn. In contrast to the EW calves, CW calves were much less variable in latency to wean, as most of the CW were consuming sufficient starter by the time weaning was initiated at 45 d of age. The CW calves experienced a more abrupt weaning, whereas many of the EW calves experienced a "step-down" weaning.

Hematocrit percentages were influenced by weaning, regardless of age at weaning. Hematocrits were greater among EW calves than CW calves at 27 d of age, which

Figure 4. Hematocrit percentages from 24 to 45 d of age for (A) control (CW) and early weaned (EW) calves and (B) EW calves grouped by latency to completely wean. Control calves were weaned by removal of 50% of milk replacer at 44.7 ± 0.5 d of age (indicated by white arrow), whereas 17 calves (EW) began the weaning process at 23.7 ± 0.5 d of age and were completely weaned within 5.9 ± 0.3 d. Complete weaning was confirmed when milk replacer was completely withdrawn after 2 consecutive days of consuming 900 g of calf-starter on an as-fed basis. *Treatment (or group) $\times$ time effect, $P < 0.05$.

Figure 5. Hematocrit percentage at 45 to 66 d of age for control (CW) and early weaned (EW) calves. Control calves were weaned by removal of 50% of milk replacer at 44.7 ± 0.5 d of age (indicated by white arrow), whereas 17 calves (EW) began the weaning process at 23.7 ± 0.5 d of age and were completely weaned within 5.9 ± 0.3 d. Complete weaning was confirmed when milk replacer was completely withdrawn after 2 consecutive days of consuming 900 g of calf-starter on an as-fed basis. Treatment $\times$ time SLICE effect, * $P < 0.05$; ** $P < 0.01$.

was 3 d after EW calves were weaned. Similarly to EW calves, CW calves had higher hematocrits compared with EW calves 3 and 7 d after they were weaned. The increased hematocrit percentages were likely due to the reduction of water intake from milk replacer; however, the changes in hematocrits were within the normal range for Holstein calves and thus these calves were not considered dehydrated (Moonsie-Shageer and Mowat, 1993).

Plasma concentrations of the acute phase protein haptoglobin were not influenced by weaning strategy; however, plasma concentrations of haptoglobin among all calves were higher from 24 to 45 d of age and subsequently decreased linearly from 45 to 66 d of age, which suggests that calves were generally less stressed at that age (Arthington et al., 2003). Plasma cortisol concentrations during these periods further indicate that all calves were generally less stressed after 48 d of age.

Circulating cortisol is the most commonly measured value to assess the stress response of animals (Von Borell, 2001). Plasma concentrations of cortisol from 24 to 45 d of age did not differ between EW and CW calves; however, CW calves had greater plasma cortisol concentrations at 48 and 66 d of age, which were 3 and 21 d after they were weaned, respectively. The higher plasma cortisol concentrations in CW calves after weaning could be because they experienced a more abrupt weaning than all EW calves or because of age differences in the responsiveness of the hypothalamo-pituitary-adrenal axis. Data on the influence of weaning on plasma cortisol concentrations are equivocal. Higher plasma concentrations of cortisol were reported by some (Arthington et al., 2003; Loberg et al., 2008; Carroll et

al., 2009), with no changes reported by others (Lefcourt and Elsasser, 1995; Hickey et al., 2003).

Many similarities were observed in the alterations in innate immune responses following weaning, regardless of age at weaning. Three days after initiating weaning, N:M ratios were increased in both EW and CW calves. Total leukocyte counts did not suggest that the change in the ratio was a result of infection. Peripheral neutrophilia is commonly observed following many stressors, including weaning (Murata et al., 1987; Buckham Sporer et al., 2007; Gupta et al., 2007). When EW calves were grouped by latency to completely wean, fast-EW calves had lower N:M ratios at 31 and 45 d of age than either intermediate- or slow-EW calves. Other researchers have reported decreased neutrophil percentages in circulation from Holstein calves from birth to 42 d of age (Mohri et al., 2007). Fast-EW calves already had milk completely withdrawn when these samples were taken; therefore, they may have experienced the final removal of the milk replacer sooner and had already coped with this stressor compared with intermediate-EW and slow-EW calves.

Figure 6. Tumor necrosis factor- α secretion from LPS-stimulated whole blood from 24 to 45 d of age for (A) control (CW) and early weaned (EW) calves, and (B) EW calves grouped by latency to completely wean. Control calves were weaned by removal of 50% of milk replacer at 44.7 ± 0.5 d of age (indicated by white arrow), whereas 17 calves (EW) began the weaning process at 23.7 ± 0.5 d of age and were completely weaned within 5.9 ± 0.3 d. Complete weaning was confirmed when milk replacer was completely withdrawn after 2 consecutive days of consuming 900 g of calf-starter on an as-fed basis. *Treatment $\times$ time effect, $P < 0.05$.

Figure 7. Adhesion molecule expression of (A) neutrophil L-selectin and (B) neutrophil β_2 -integrin from 24 to 45 d of age for early weaned calves grouped by latency to completely wean. Adhesion molecules were measured originally by the geometric mean fluorescence of FL-1, but data are presented as a percentage of the control calves' day and block means. Control calves were not weaned and were fed milk replacer twice daily. Latency to be completely weaned was confirmed when milk replacer was completely withdrawn after 2 consecutive days of consuming 900 g of calf-starter, as-fed basis. Early weaned calves were divided into 3 categories: fast (1 to 5 d), intermediate (6 to 8 d), and slow (15 to 17 d). **Treatment $\times$ time effect, $P < 0.01$.

In addition to altered N:M ratios following weaning, both EW and CW calves had altered neutrophil phagocytosis or oxidative burst to *E. coli* 3 d after their respective weaning. The intensity of the oxidative burst was decreased in EW 3 d after weaning. In contrast, CW calves tended to have a decrease in the intensity of phagocytosis 3 d after they were weaned. These data are similar to those of Lynch et al. (2010), where beef

calves that were abruptly weaned from dams and milk had decreased percentages of neutrophil phagocytizing 2 d after weaning followed by a return to preweaning percentages 7 d after weaning. In that study, however, weaning had no effect on the percentage of neutrophils producing an oxidative burst (Lynch et al., 2010). The effect that stressors have on neutrophil phagocytosis and oxidative burst responses is complex and likely dependent on the severity of the stressor and how quickly the animal is able to acclimate to it. For example, when 5-mo-old Holstein calves were exercised and subjected to cold stress, neutrophils produced less superoxide (Henricks et al., 1987); however, when Holstein bull calves were challenged with hydrocortisone, no differences in either neutrophil phagocytosis or oxidative burst capacities were observed (Pang et al., 2009). Weaning stress, regardless of age, in Holstein calves suppressed neutrophil phagocytic or oxidative burst capacities transiently, and within 7 d of weaning had returned to preweaning activities.

Stress and glucocorticoids have been reported to down-regulate the expression of L-selectin and β_2 -integrin on circulating neutrophils in lactating cows (Burton et al., 1995). Adhesion molecules play a critical role in leukocyte adhesion to vascular endothelium and migration from peripheral circulation into sites of infection (Tempelman et al., 2002). L-Selectin interacts with carbohydrate moieties on glycoproteins and glycolipids of vascular endothelium, allowing the neutrophil to roll along the blood vessel walls, acting as "surveillance" for inflammatory signals. Neutrophil L-selectin expression was decreased among EW calves at 27 d of age, which was 3 d after weaning was initiated. No changes in adhesion molecule expression were observed after CW calves were weaned. In agreement with the current findings, neutrophil L-selectin expression from beef calves was decreased 2 d after abrupt weaning and iso-

Table 5. Flow cytometric neutrophil function of early weaned calves¹ (n = 23)

Item ²	Age, ³ d				Largest SEM	P-value Time
	24	27	31	45		
L-selectin, GMFI	95.1	71.2 ^a	123.3 ^b	104.9 ^{bc}	13.3	0.01
β_2 -integrin, GMFI	91.5	91.1	109.3	89.8	8.6	0.15
OB+PG+, % ⁴	100.8	103.5 ^a	100.2 ^{ab}	96.2 ^b	1.7	0.01
OB, GMFI	94.6	80.2 ^a	97 ^b	89.1 ^b	3.7	<0.01
PG, GMFI	97.7	107	109.6	100.9	6.5	0.34

^{a-c}Means within a row with different superscripts differ significantly, $P < 0.05$.

¹All flow cytometry measures are presented as the percentage of control calf day and block means.

²L-selectin, β_2 -integrin, oxidative burst (OB), and phagocytosis (PG) were measured as geometric mean fluorescence intensity; OB and PG are log-transformed P -values.

³Baseline measures (age 24 d) served as a covariate in the models; therefore, least squares mean differences are only compared among 27, 31, and 45 d of age.

⁴Percentage of total neutrophils that produced both OB and PG.

Table 6. Flow cytometric neutrophil function of control weaned calves (n = 20)¹

Item ²	Time relative to age, d				Largest SEM	P-value
	45	48	52	66		Time
L-selectin, GMFI	111.2	96.3	112.2	108.2	11.8	0.67
β_2 -integrin, GMFI	106.7	116.1	100.5	95.4	10.8	0.22
OB+PG+, % ³	106.0 ^a	99.9 ^b	101.6 ^b	101.1 ^b	1.4	<0.01
OB, GMFI	120.9	109.7	117.2	113.8	6.3	0.30
PG, GMFI	108.6	97.2	106.0	99.4	5.5	0.06

^{a,b}Means within a row with different superscripts differ significantly, $P < 0.05$.

¹All flow cytometry measures are presented as percentage of early weaned (EW) calf day and block means. Data from the slow-EW calves were excluded before calculating the day and block means for the EW calves.

²L-selectin, β_2 -integrin, oxidative burst (OB), and phagocytosis (PG) were measured as geometric mean fluorescence intensity (GMFI). OB and PG are log-transformed P -values.

³Percentage of total neutrophils that produced both OB and PG.

lation from their dams (Lynch et al., 2010). Similar to the EW calf neutrophil L-selectin in the current study, the down-regulated L-selectin expression among beef calves in the Lynch et al. (2010) study was transient and returned to preweaning values within 7 d of weaning. In addition, both transportation in young Belgian Blue-Friesian bull calves (Buckham Sporer et al., 2007) and parturition in Holstein cows (Weber et al., 2001), transiently decreased the expression of L-selectin on neutrophils. In contrast, neutrophil L-selectin expression was not altered when Holstein bull calves were either challenged with hydrocortisone or castrated (Pang et al., 2009). Therefore, as discussed previously, the influence of a stressor on innate immune responses is likely a function of the severity of the stressor and how quickly the animal is able to acclimate to the stressor. In the present study, when EW calves were grouped by latency to be completely weaned, only the fast- and slow-EW calves had reduced L-selectin expression at 27 d of age. The reason why intermediate-EW calves did not have decreased L-selectin expression in the current study is not known.

Unlike neutrophil L-selectin expression, the expression of β_2 -integrin was not suppressed during weaning. The β_2 -integrin adhesion molecules allow for hyper-adhesion and subsequently, transmigration through vascular endothelium to the site of inflammation (Arnaout, 1990; Burton et al., 1995). If cells are unable to increase integrins and migrate to inflammatory sites, then chronic and even fatal infections can occur (Burton et al., 1995; Lee and Kehrli, 1998). Stress and glucocorticoids or their analogs have been reported to decrease β_2 -integrin expression on neutrophils (Burton et al., 1995). Expression of LPS-induced β_2 -integrin on neutrophils from dairy cows was decreased by in vitro stimulation with a combination of glucocorticoid and catecholamine analogs (Diez-Fraile et al., 2000). Similarly, β_2 -integrin expression on neutrophils de-

creased with increasing doses of dexamethasone (Filep et al., 1997). In Brahman bulls, β_2 -integrin expression on neutrophils was decreased 24 h after initiating a repeated handling and transportation stress; however, 72 h later, β_2 -integrin expression was increased (L. E. Hulbert, J. A. Carroll, N. C. Burdick, R. D. Randel, M. S. Brown, and M. A. Ballou, unpublished data). In the current study, neutrophil β_2 -integrin was increased in fast-EW calves at 31 d of age, which was 7 d after initiating weaning. Compensatory or rebound effects on neutrophil molecule expression were observed in cattle following stressors. Belgian Blue-Friesian bull calves (233 ± 3.0 kg of BW and 282 ± 4 d of age) transported for 9 h had depressed neutrophil L-selectin gene expression at 4.5 h after transport followed by a greater peak at 9.75 h (Buckham Sporer et al., 2007). Similarly, Holstein bull calves (407 ± 70.1 kg) injected with glucocorticoids had down-regulated neutrophil L-selectin followed by a rebound above baseline measures when the corticoid was removed (Tempelman et al., 2002). The exact immunological significance of the elevated β_2 -integrin expression on neutrophils of fast-EW calves is not known, but it is conceivable that these calves would be more resistant to infection in this period.

Concentrations of TNF- α from LPS-stimulated whole did not differ between EW and CW in blood at any age; however, when EW calves were grouped by latency to completely wean, slow-EW calves had decreased secretion of TNF- α at 27 and 31 d of age compared with other EW calves. Monocyte-derived cells are the primary producers of TNF- α in LPS-stimulated whole blood (Finch-Arietta and Cochran, 1991; Carstensen et al., 2005). Stress, including weaning in pigs, generally suppresses TNF- α in LPS-stimulated whole blood (Kusnecov and Rossi-George, 2002; Carstensen et al., 2005). Human patients with low whole blood TNF- α responses had greater morbidity and mortality following surgery (Heagy et al., 2003). Therefore, slow-EW

calves might have an impaired ability to activate an inflammatory response and recruit effector leukocytes to sites of infection.

CONCLUSIONS

Weaning is a stressful event for calves at any age and could decrease resistance to disease. Weaning caused transient neutrophilia and suppressed neutrophil phagocytic and oxidative burst responses in all calves independent of age at weaning. Expression of neutrophil L-selectin was only suppressed in EW calves and the temporal response was similar to the observed neutrophilia and suppressed neutrophil phagocytic and oxidative burst responses, returning to baseline expression within 7 d after weaning. All calves had increased plasma concentrations of cortisol and haptoglobin from 24 to 45 d compared with those from 45 to 66 d of age, suggesting that older calves are generally less stressed. Slow-EW calves weighed less initially and before initiating early weaning, and they secreted less of the proinflammatory cytokine TNF- α in LPS-stimulated whole blood. Early weaned calves, and specifically slow-EW calves, had more innate immune responses suppressed during weaning; however, the immunological results in the current study in context of resistance to disease are not known because EW calves could have greater humoral protection from passively derived immunoglobulins.

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