Plasma concentrations of substance P and cortisol in beef calves after castration or simulated castration

Johann F. Coetzee, BVSc, PhD; Brian V. Lubbers, DVM; Scott E. Toerber, BS; Ronette Gehring, BVSc, MMedVet; Daniel U. Thomson, DVM, PhD; Bradley J. White, DVM, MS; Michael D. Apley, DVM, PhD

Objective—To evaluate plasma concentrations of substance P (SP) and cortisol in calves after castration or simulated castration.

Animals—10 Angus-crossbred calves.

Procedures—Calves were acclimated for 5 days, assigned to a block on the basis of scrotal circumference, and randomly assigned to a castrated or simulated-castrated (control) group. Blood samples were collected twice before, at the time of (0 hours), and at several times points after castration or simulated castration. Vocalization and attitude scores were determined at time of castration or simulated castration. Plasma concentrations of SP and cortisol were determined by use of competitive and chemiluminescent enzyme immunoassays, respectively. Data were analyzed by use of repeated-measures analysis with a mixed model.

Results—Mean ± SEM cortisol concentration in castrated calves (78.88 ± 10.07 nmol/L) was similar to that in uncastrated control calves (73.01 ± 10.07 nmol/L). However, mean SP concentration in castrated calves (506.43 ± 38.11 pg/mL) was significantly higher than the concentration in control calves (386.42 ± 40.09 pg/mL). Mean cortisol concentration in calves with vocalization scores of 0 was not significantly different from the concentration in calves with vocalization scores of 3. However, calves with vocalization scores of 3 had significantly higher SP concentrations, compared with SP concentrations for calves with vocalization scores of 0.

Conclusions and Clinical Relevance—Similar cortisol concentrations were measured in castrated and control calves. A significant increase in plasma concentrations of SP after castration suggested a likely association with nociception. These results may affect assessment of animal well-being in livestock production systems. (Am J Vet Res 2008;69:751–762)
ated with castration. However, applying those results to livestock in typical production settings is confounded by the fact that handling alone can stimulate a major cortisol response.\textsuperscript{3,14} This may be further complicated by the diurnal rhythm of endogenous cortisol secretion reported in several studies.\textsuperscript{15-17} These factors substantially diminish the use of plasma cortisol measurement in assessing animal well-being in typical livestock production environments.

Substance P consists of 11 amino acids and is a prototypic neuropeptide for > 50 neuroactive molecules, which include CGRP, neuropeptide Y, and endothelin.\textsuperscript{18,19} It has been determined that SP regulates the excitability of dorsal horn nociceptive neurons and can be detected in areas of the neocortex involved in the integration of pain, stress, and anxiety.\textsuperscript{20} A lack of SP and CGRP in the dorsal root ganglia of naked mole rats can selectively reduce the response to painful stimuli.\textsuperscript{21,22} Plasma concentrations of SP have been used to evaluate muscle pain, spinal cord disease, soft tissue injury, and the efficacy of therapeutic agents for humans with osteoarthritis, headache, and fibromyalgia.\textsuperscript{20,23-24} In 1 study,\textsuperscript{19} plasma concentrations of SP were 27-fold higher in human patients with soft tissue injury, compared with concentrations in healthy control subjects.

Cortisol concentrations decrease significantly after cats are handled and receive periodontal treatment, whereas plasma concentrations of SP remain high.\textsuperscript{25} These data suggest that measurement of plasma concentrations of SP may enable clinicians and researchers to discriminate between a stressful event culminating in increases in plasma concentrations of cortisol and more specific nociceptive stimuli resulting in increases in plasma concentrations of SP. On the basis of the aforementioned reports, we hypothesized that SP could potentially be a more specific measure of pain in cattle following castration than would the plasma cortisol response. The specific objective of the study reported here was to compare plasma concentrations of cortisol and SP in beef calves following surgical castration.

**Materials and Methods**

**Animals**—Ten Angus-crossbred calves were used in the study. Calves were 4 to 6 months old and weighed approximately 250 kg. Calves were acquired from a livestock commission company in Kansas in April 2008. Scrotal circumference (measured by use of a tape measure specifically manufactured for that purpose\textsuperscript{c}) for each calf ranged from 22 to 30 cm prior to study commencement. On arrival at our facility, the calves received a single dose of a 7-way clostridial vaccine (administered IM), and a single injection of florfenicol (40 mg/kg, SC). Calves also were provided amprolium in the drinking water (10 mg/kg) for treatment and prevention of coccidiosis during the period of intensive housing in the study. The protocol for this study was approved by the Institutional Animal Care and Use Committee at Kansas State University (protocol No. 2472).

**Housing and husbandry**—Calves were initially housed in typical dry-lot confinement facilities for 3 days after arrival at our facility. Thereafter, calves were acclimated and housed separately in indoor stalls (13.40 m\textsuperscript{2}) in the veterinary medical teaching hospital for 5 days prior to study commencement. Stalls were separated by solid walls, and each had a head gate to enable animal restraint. During the acclimation period, each calf was restrained for at least 15 to 30 min/d by use of the head gate and a rope halter. For the entire housing period, calves had unlimited access to water and grass hay and were fed a diet formulated for newly arrived cattle at beef feedlots, which consisted of whole corn, wheat middlings, dry distillers grain, soybean hull pellets, cottonseed hulls, molasses, and a protein-vitamin-mineral mixture.

**Jugular catheterization**—Approximately 48 hours before study commencement, each calf was restrained. The area over the right jugular vein was clipped and disinfected with 70% isopropyl alcohol and povidone iodine. The catheter site was infiltrated with approximately 0.5 mL of a 2% lidocaine solution, and a small skin incision was made to facilitate insertion of a 14-gauge × 140-mm catheter, which was sutured to the skin with 2-0 nylon suture.\textsuperscript{a} Catheter patency was maintained by use of a heparinized saline (0.9% NaCl) flush solution (3 U of heparin sodium/mL of saline solution).

**Assignment to groups**—Calves were blocked in pairs on the basis of the scrotal circumference measured prior to study commencement. Calves were ranked by ascending scrotal circumference and assigned a computer-generated random number. In each pair, the calf with the highest random number was assigned to the castration group, whereas the other calf was designated as an uncastrated control calf (n = 5 calves/group). Mean ± SEM scrotal circumference was 25.80 ± 1.36 cm for the castration group and 26.40 ± 0.86 cm for the control group.

**Castration and simulated castration**—The study commenced at 7 AM. Castration or simulated castration was performed at 3-minute intervals. All castrations were performed by a single experienced veterinarian (BVL) to minimize variation.

The scrotum of each calf was washed with dilute chlorhexidine disinfectant. Castration was performed via an open surgical technique without the provision of local anesthesia. This was consistent with standard industry practices used at many intensive production facilities in the United States. After the scrotum was washed with disinfectant, it was incised with a sharp Newberry castrating knife.\textsuperscript{a} The testes and spermatic cords were exteriorized by blunt dissection, and the scrotal fascia was stripped from each testis. A Henderson castration tool was clamped across the entire spermatic cord immediately proximal to a testis. The tool was attached to a 6.0-V cordless variable-speed hand drill with a 3/8-inch chuck. The drill was used to rotate the clamped spermatic cord at a slow to moderate speed in a clockwise direction; after an initial 5 to 6 revolutions, the drill speed was increased in accordance with the manufacturer's instructions until the torsion resulting in removal of the testis (approx 10 revolutions). The
tightly torsed sealed segment of the cord then retracted into the abdomen. The same procedure was used to remove the second testis.

For each calf in the control group, the effect of manipulation associated with castration was simulated. The testes within the scrotum were firmly grasped, and ventral traction was applied for approximately 20 seconds.

**Behavioral scoring**—Behavioral changes in response to castration or simulated castration were assessed by assigning a score for vocalization and changes in attitude or temperament at the time of the procedure. Vocalization was scored on a scale of 0 to 3 (0, no vocalization; 1, snorting or grunting; 2, momentary vocalization; and 3, continuous vocalization during and immediately after testicular manipulation). Attitude was also scored on a scale of 0 to 3 (0, unchanged from premanipulation behavior; 1, head shaking, kicking, or tail flicking; 2, momentary escape behavior [eg, lunging against the head gate], head shaking, and kicking; and 3, violent escape behavior [eg, repeated lunging against the head gate, head shaking, and kicking] throughout testicular manipulation).

**Collection of blood samples**—Blood samples for baseline cortisol and SP determination were collected in syringes via the jugular catheter at 24 and 12 hours before and immediately prior to castration or simulated castration (time of castration or simulated castration was designated as time 0). Additional samples were collected immediately after and 10, 20, 30, and 45 minutes and 1, 1.5, 2, 2.5, 3, and 4 hours after castration or simulated castration. Blood samples were immediately transferred to 6-mL evacuated tubes that contained potassium EDTA and lithium heparin and stored on ice for up to 30 minutes before processing. Blood samples were centrifuged for 15 minutes at 1,500 × g. Plasma was then harvested, placed in cryovials, and frozen at −70°C until analysis. All samples were analyzed within 16 days after sample collection.

**Cortisol analysis**—Plasma cortisol concentrations were determined as described elsewhere by use of a solid-phase competitive chemiluminescent enzyme immunoassay and an automated analyzer system. Laboratory technicians who analyzed the samples were not aware of the identity of the sample source (ie, treatment group). A minimum sample volume of 100 µL was used in each assay well. The reported calibration range for the assay was 28 to 1,380 nmol/L, and sensitivity was 5.5 nmol/L.

Cortisol was extracted from pooled bovine plasma by use of diethyl ether to yield cortisol-free plasma to validate the immunoassay. Briefly, blank plasma was fortified with 5 concentrations of cortisol (obtained from a stock solution of 0.5 g/dL) that spanned the expected analytic range of the assay. Each spiked sample was then analyzed in triplicate. The coefficient of variation for triplicate samples at each spiked concentration ranged from 3% to 11%. The linear regression line for the 3 points at each of the 5 concentrations had a correlation coefficient of 0.99.

**SP analysis**—Plasma SP concentrations were determined by use of a commercial competitive immunoassay kit. Samples were subjected to solid-phase extraction by use of C-18 cartridges. This immunoassay used a polyclonal antibody against SP that competitively bound to SP in the test sample or to an alkaline phosphatase molecule that was covalently attached to an SP molecule. The concentration of SP in the sample was inversely proportional to the intensity of color generated after incubation, as determined at 405 nm on a microplate reader. The analytic range of the assay reported by the manufacturer was 9.75 to 10,000 pg/mL, and sensitivity was 8.04 pg/mL.

The SP immunoassay was validated for bovine plasma by use of calibration samples fortified with a stock solution of SP dissolved in assay buffer. Bovine plasma was spiked with 3 concentrations (low, middle, and high) that spanned the expected analytic range of the assay. Each spiked sample was then analyzed in triplicate in accordance with manufacturer instructions. Briefly, 1 mL of sample was transferred into separate 13 × 100-mm glass culture tubes and acidified by addition of an equivalent volume of 1.0% aqueous TFA followed by vortexing at medium intensity. Acidified samples were centrifuged at 3,000 × g for 10 minutes to separate precipitate from plasma. Concurrently, a 24-port vacuum manifold was used to prepare solid-phase extraction cartridges for use. Equilibration of the solid-phase extraction cartridges was achieved by successive washings with 1 mL of high-performance liquid chromatography–grade methanol containing 0.1% TFA (1 mL) and distilled water containing 0.1% TFA (1 mL). After cartridges were loaded with the acidified samples, each was washed 6 times with 3-mL aliquots of distilled water containing 0.1% TFA. The SP-containing fractions were then collected during elution by use of methanol containing 0.1% TFA. Eluents were evaporated to dryness by use of a vacuum centrifuge under nitrogen gas at 37°C. Dry samples were stored at −20°C prior to competitive immunoassay, which was performed within 12 to 16 hours after extraction.

The competitive immunoassay was conducted by adding 50 µL of assay buffer to the nonspecific-binding and zero-standard wells, in duplicate. Remaining wells on each plate were filled with 50 µL of appropriately diluted sample. Thereafter, 50 µL of assay buffer was added to the nonspecific-binding wells, whereas the remaining wells received 50 µL of conjugate followed by 50 µL of antibody. Plates were then incubated at 22°C on a plate shaker for 2 hours at approximately 500 revolutions/min. Following incubation, contents of each plate were discarded, and wells were washed 3 times (400 µL of wash solution for each wash).

After washing, wells were emptied, and plates were tapped on a paper towel to remove remaining wash buffer. Then, p-nitrophenyl phosphate substrate solution (200 µL) was added to each well, which was followed by incubation for 1 hour without shaking. Finally, 30 µL of stop solution was added to each well. Immediately after the addition of the stop solution, results for each plate were determined by use of a plate reader. Results for each test well were compared against results for blank wells, and the optical density was then measured at 405 nm with correction at 570 nm. Computer software was used to process the data by converting...
the net optical density by use of the following equation: net optical density of samples/net optical density of maximum binding wells. Resulting values were plotted versus the concentration of SP standards to create a standard curve.

The standard curve for SP was obtained by use of a 4-parameter logistic curve for concentrations from 9.76 to 10,000 pg/mL ($R^2$, 0.98). A validation curve derived from the spiked bovine plasma samples was constructed by plotting net optical density versus corresponding concentrations of SP. The coefficient of variation among triplicate bovine samples at each fortified SP concentration ranged from 6% to 22%. The linear regression line for the 3 points at each of the 3 concentrations had a correlation coefficient of 0.99.

Data analysis—Mean ± SEM values were calculated for cortisol and SP concentrations at each time point. Repeated-measures data were analyzed by use of a mixed-effects model allowing for unequal variances across time. The model incorporated treatment group, time, and the treatment by time interaction as fixed terms and calf as a random effect. Baseline cortisol and SP concentrations and scrotal circumference were included in the model as covariates. Similarly, results for repeated-measures analysis of cortisol and SP concentrations for each calf after castration or simulated castration were compared with vocalization and attitude scores measured at the time of castration. An autoregressive covariance structure was used for the repeated measures, whereby subject was the animal identification number. Estimation was performed by use of restricted maximum likelihood.

The AUC$_{\text{total}}$ (including the proportion of the curve above and below the baseline value), $C_{\text{min}}$, $C_{\text{max}}$, and times at which $C_{\text{min}}$ and $C_{\text{max}}$ were detected were determined by use of noncompartmental analysis, as described for cortisol in another study. The amount of time that the plasma cortisol concentration remained above and below the baseline concentration was also calculated by use of this model. These analyses were implemented by pharmacokinetic modeling software. Statistical analysis of noncompartmental data was conducted by use of a general linear model analysis with scrotal circumference as a covariate. Vocalization and attitude scores were compared by use of a generalized linear mixed-model analysis, assuming a Poisson response and with scrotal circumference as a covariate.

Results

Plasma cortisol concentrations—Mean ± SEM plasma cortisol concentration was 50.84 ± 10.99 nmol/L and 76.06 ± 11.97 nmol/L at 12 and 24 hours before castration, respectively. Mean peak cortisol concentrations of 112.20 ± 4.81 nmol/L for the castrated group and 112.92 ± 32.26 nmol/L for the control group were detected 30 minutes after castration or simulated castration. Thereafter, cortisol concentrations decreased to less than baseline values by 2 hours for the control group and by 2.5 hours for the castration group (Figure 1). A larger interindividual variation in cortisol

![Figure 1](https://example.com/figure1.png)

Figure 1—Mean ± SEM plasma cortisol concentration in beef calves (n = 5 calves/group) after surgical castration (black triangles) or simulated castration (white squares). Time of castration or simulated castration was designated as time 0. Concentrations did not differ significantly ($P = 0.64$; repeated measures ANOVA) between groups.
response was evident in the uncastrated control calves throughout the study. Mean plasma cortisol concentration in castrated calves for all time points was 78.88 ± 10.07 nmol/L, which was similar to the mean response of 73.01 ± 10.07 nmol/L in uncastrated calves. Repeated-measures analysis of the cortisol concentrations revealed no significant (P = 0.64) difference in cortisol concentrations between the castrated and uncastrated calves over time. The effect of time on mean cortisol concentrations also did not differ significantly (P = 0.84) between the 2 groups. There was not a significant (P = 0.82) effect of the covariate scrotal circumference on mean cortisol concentrations.

Noncompartmental model estimates for plasma cortisol concentrations—The predicted noncompartmental model estimates for cortisol were summarized (Table 1). Predicted mean ± SEM C_max for

![Graph](image_url)

Figure 2—Mean ± SEM plasma SP concentration in beef calves (n = 5/group) after surgical castration (black triangles) or simulated castration (control group), as determined by use of a noncompartmental model.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Castrated</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_min (nmol/L)</td>
<td>16.08 ± 4.96</td>
<td>18.80 ± 4.45</td>
<td>0.81</td>
</tr>
<tr>
<td>C_max (nmol/L)</td>
<td>136.58 ± 31.94</td>
<td>128.80 ± 9.06</td>
<td>0.72</td>
</tr>
<tr>
<td>T_min (h)</td>
<td>3.40 ± 0.25</td>
<td>4.00 ± 0.00</td>
<td>0.047</td>
</tr>
<tr>
<td>T_max (h)</td>
<td>0.53 ± 0.16</td>
<td>0.68 ± 0.25</td>
<td>0.56</td>
</tr>
<tr>
<td>Baseline (nmol/L)</td>
<td>70.66 ± 14.46</td>
<td>63.28 ± 12.36</td>
<td>0.57</td>
</tr>
<tr>
<td>AUC (h × nmol/L)</td>
<td>44.50 ± 99.98</td>
<td>137.87 ± 6.11</td>
<td>0.46</td>
</tr>
<tr>
<td>AUC_Above (h × nmol/L)</td>
<td>0.13 ± 34.93</td>
<td>74.46 ± 23.53</td>
<td>0.76</td>
</tr>
<tr>
<td>AUC_Below (h × nmol/L)</td>
<td>4.37 ± 32.27</td>
<td>63.41 ± 19.50</td>
<td>0.69</td>
</tr>
<tr>
<td>T_Above (h)</td>
<td>1.36 ± 0.42</td>
<td>1.94 ± 0.43</td>
<td>0.76</td>
</tr>
<tr>
<td>T_Below (h)</td>
<td>2.22 ± 0.35</td>
<td>2.16 ± 0.43</td>
<td>0.81</td>
</tr>
</tbody>
</table>

*Values determined by use of a general linear model with scrotal circumference as a covariate; values of P < 0.05 were considered significant. †Baseline concentration was determined from samples collected 24 and 12 hours before and immediately prior to castration or simulated castration.

T_min = Time at which C_min was detected. T_max = Time at which C_max was detected. AUC_Above = Proportion of the curve above the baseline value. AUC_Below = Proportion of the curve below the baseline value. T_Above = Duration for AUC_Above. †Below = Duration for AUC_Below.
cortisol for the castrated calves was 128.80 ± 9.06 nmol/L at 0.68 hours after castration. This was lower than, but not significantly different from, the mean predicted \( C_{\text{max}} \) for cortisol for the control calves of 136.58 ± 31.94 nmol/L at 0.53 hours after simulated castration. The time after castration at which \( C_{\text{min}} \) was estimated for the castrated calves was significantly (\( P = 0.047 \)) greater than that estimated for the uncastrated control calves. Mean integrated cortisol response represented by AUC \( \text{Total} \) was 137.87 ± 6.11 (h \times nmol)/L and 144.50 ± 39.98 (h \times nmol)/L for the castrated and control group, respectively. For the castrated group, mean AUC \( \text{Total} \) could be partitioned into 74.46 ± 23.53 (h \times nmol)/L above the model baseline value and 63.41 ± 19.50 (h \times nmol)/L below the baseline value. These data did not differ significantly from those for the uncastrated control group in which the mean AUC above the predicted baseline value was 60.13 ± 34.93 (h \times nmol)/L and mean AUC below the predicted baseline value was 84.37 ± 32.27 (h \times nmol)/L. On the basis of results for the model, the cortisol concentration remained above baseline concentrations for a mean of 1.84 ± 0.43 hours in the castrated group and 1.38 ± 0.42 hours in the uncastrated control group. There was not a significant effect of scrotal circumference for any of the noncompartmental variables.

**Plasma SP concentrations**—During SP analysis, samples collected at 24 hours before and 10 minutes and 1.5 and 3 hours after testicular manipulation in 1 control calf had > 150 µL of residual moisture in the test tube after evaporation. These data were omitted from the final analysis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Castrated</th>
<th>( P ) value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C_{\text{min}} ) (pg/mL)</td>
<td>88.68 ± 23.92</td>
<td>303.98 ± 119.73</td>
<td>0.12</td>
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<tr>
<td>( C_{\text{max}} ) (pg/mL)</td>
<td>691.38 ± 71.83</td>
<td>888.92 ± 235.44</td>
<td>0.48</td>
</tr>
<tr>
<td>( T_{\text{min}} ) (h)</td>
<td>2.22 ± 0.65</td>
<td>1.06 ± 0.45</td>
<td>0.19</td>
</tr>
<tr>
<td>( T_{\text{max}} ) (h)</td>
<td>1.75 ± 0.93</td>
<td>1.72 ± 0.75</td>
<td>0.90</td>
</tr>
<tr>
<td>Baseline (nmol/L)</td>
<td>507.46 ± 92.22</td>
<td>506.42 ± 76.89</td>
<td>0.98</td>
</tr>
<tr>
<td>AUC ( \text{Total} ) (h \times pg/mL)</td>
<td>39.99 ± 151.05</td>
<td>675.68 ± 114.97</td>
<td>0.83</td>
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<tr>
<td>AUC ( \text{Above} ) (h \times pg/mL)</td>
<td>5.26 ± 59.98</td>
<td>389.20 ± 156.14</td>
<td>0.14</td>
</tr>
<tr>
<td>AUC ( \text{Below} ) (h \times pg/mL)</td>
<td>44.73 ± 162.65</td>
<td>289.48 ± 199.44</td>
<td>0.40</td>
</tr>
<tr>
<td>( T_{\text{Above}} ) (h)</td>
<td>0.73 ± 0.31</td>
<td>1.71 ± 0.73</td>
<td>0.29</td>
</tr>
<tr>
<td>( T_{\text{Below}} ) (h)</td>
<td>2.67 ± 0.49</td>
<td>1.69 ± 0.82</td>
<td>0.39</td>
</tr>
</tbody>
</table>

*See Table 1 for key.*
Figure 5—Mean ± SEM plasma cortisol concentrations in 10 beef calves after surgical castration or simulated castration, based on the attitude score recorded at the time of the procedure (time 0). Concentrations did not differ significantly ($P = 0.66$; repeated-measures ANOVA) among attitude scores. Attitude was scored on a scale of 0 to 3 (0, unchanged from premanipulation behavior; 1, head shaking, kicking, or tail flicking; 2, momentary escape behavior [eg, lunging against the head gate], head shaking, and kicking; and 3, violent escape behavior [eg, repeated lunging against the head gate, head shaking, and kicking] throughout testicular manipulation).

Figure 4—Mean ± SEM plasma SP concentrations in 10 beef calves after surgical castration or simulated castration, based on vocalization score recorded at the time of the procedure (time 0). Concentrations differed significantly ($P = 0.033$; repeated-measures ANOVA) among vocalization scores. See Figure 3 for remainder of key.
sis because of the dilution effect on the SP concentration in those samples.

Mean ± SEM plasma SP concentrations were 452.69 ± 92.12 pg/mL and 556.71 ± 107.67 pg/mL at 24 hours before castration and the time point immediately before castration, respectively. Mean plasma SP concentrations for the castrated calves were greater than those measured for control calves at all time points, except at 90 minutes after castration. Mean peak plasma SP concentration of 731.91 ± 172.08 pg/mL was recorded at 45 minutes after castration (Figure 2). Pronounced within- and between-calf variations in SP concentration were evident throughout the study period, especially for the castrated group. Mean SP concentration for all time points after castration or simulated castration was 506.43 ± 38.11 pg/mL for the castrated calves, which was significantly (P = 0.042) higher than the concentration of 386.42 ± 40.09 pg/mL for the uncastrated control calves. There was not a significant (P = 0.44) effect of time on SP concentrations between the 2 groups. However, there was a significant (P = 0.048) effect of the covariate scrotal circumference on mean SP concentrations.

Noncompartmental model estimates for plasma SP concentrations—The predicted noncompartmental model estimates for SP were summarized (Table 2). Predicted mean C_{\text{max}} for SP for the castrated calves was not significantly greater than the predicted mean C_{\text{max}} for SP for the control calves. Furthermore, the mean integrated SP response represented by AUC_{\text{total}} was similar between the 2 groups. A larger proportion of the integrated SP response was represented by the AUC above the model baseline value, compared with the AUC below the baseline, for castrated calves; however, these values did not differ significantly. Furthermore, results for the model revealed that the SP response remained above the baseline value for a longer period for the castrated group than for the uncastrated control group, although these values also did not differ significantly. There was not a significant effect of scrotal circumference on any of the noncompartmental variables.

Behavioral scores—Four of 5 uncastrated control calves had scores of 0 for vocalization and attitude after testicular manipulation, whereas all the castrated calves had scores indicative of a change in attitude or vocalization after castration. Two castrated and 1 control call snorted or grunted when the testes were manipulated (vocalization scores of 1), whereas the remaining 3 castrated calves vocalized continuously during and immediately after castration (vocalization score of 3). One castrated and 1 control calf had head shaking, kicking, or tail flicking after testicular manipulation (attitude score of 1), whereas 1 castrated call had momentary escape behavior at the time of castration (attitude score of 2). The 3 remaining castrated calves all had violent escape behaviors throughout the procedure (attitude score of 3). The 3 remaining castrated calves all had violent escape behaviors throughout the procedure (attitude score of 3). Overall, there was a significant (P = 0.046) difference in attitude scores between castrated and control calves, whereas there was not a significant (P = 0.032) difference in vocalization scores between groups.
Distributions of cortisol and SP responses were compared with vocalization and attitude scores (Figures 3–6). Mean ± SEM cortisol concentration in calves with a behavioral score of 0 ranged from 130.73 ± 31.32 nmol/L at 45 minutes after castration to 18.63 ± 5.9 nmol/L at 240 minutes after castration. This was not significantly different from the mean cortisol concentration in calves with a behavioral score of 3, which ranged from 116.33 ± 6.88 nmol/L at 30 minutes after castration to 20.63 ± 7.6 nmol/L at 240 minutes after castration. Repeated-measures analysis revealed no significant differences for vocalization score (P = 0.17) and attitude score (P = 0.66).

Mean ± SEM plasma SP concentration in calves with a behavioral score of 0 ranged from 231.58 ± 56.17 pg/mL at 120 minutes after castration to 477.33 ± 120.94 pg/mL at 30 minutes after castration. In contrast, mean SP concentration in calves with a behavioral score of 3 at the time of castration ranged from 268.17 ± 152.19 pg/mL at 150 minutes after castration to 861.72 ± 278.38 pg/mL at 45 minutes after castration. Repeated measures analysis revealed that these values differed significantly for vocalization score (P = 0.033) but not for attitude score (P = 0.091). Over the course of the study, there was an overall increase in SP response for calves that had a behavioral score of 0 at the time of castration, compared with the response for calves that had a behavioral score of 3 at the time of castration.

**Discussion**

The study reported here was conducted to compare plasma cortisol and SP concentrations in calves following surgical castration. Our hypothesis was that an increase in plasma SP concentration may be a more accurate indicator of nociception associated with castration than would an increase in plasma cortisol concentration. Analysis of our results indicated that significantly (P = 0.042) higher mean SP concentrations were detected in castrated calves over the course of the study. Furthermore, plasma SP concentration measured for the duration of the study was significantly (P = 0.033) higher in calves that vocalized during the procedure (score 3) than for calves that did not vocalize (score 0). A similar pattern (although not significant [P = 0.091]) was detected for attitude scores. In contrast, mean cortisol response in castrated and unaesthetized (control) calves was not significantly different over the course of the study. The mean cortisol response was also similar irrespective of whether calves vocalized (P = 0.17) or had aversive behavior (P = 0.66) during the procedure. These findings contradict results in other studies7–12 in which it was suggested that an increase in plasma cortisol concentration correlates with the nociceptive response after castration. To our knowledge, this is also the first report in which investigators have evaluated the use of SP concentrations to quantify nociceptive responses in cattle after castration.

Castration is considered a necessary management practice in livestock production,26 although concerns related to pain during castration were published as early as 1929.20 Surgical removal of the testes and irreparable damage to the testes by stricture of the blood supply are the most common methods of physical castration.20,31 In a review32 of several studies, it was concluded that all methods of castration induce physiologic, neuroendocrine, and behavioral changes that indicate a stress response. Despite this, a survey33 of Canadian veterinarians and their use of analgesics revealed that only 6.9% of beef calves and 18.7% of dairy calves ≤ 6 months old and approximately 20% of beef calves and 33% of dairy calves > 6 months old received an analgesic at the time of castration. The authors of that survey cited a lack of approved, long-acting, and cost-effective analgesics with established withdrawal times in Canada as 1 explanation for why analgesics were not used routinely to alleviate pain after castration.

Several organizations, including the National Cattlemen’s Beef Association and the AVMA, have stated that pain and physiologic stress resulting from castration should be minimized. The National Cattlemen's Beef Association suggests that calves weighing > 227 kg should be castrated by use of a bloodless castration technique, such as an elastic banding device, unless pain management is used (eg, local anesthesia).34 The AVMA supports the use of procedures that reduce or eliminate the pain of dehorning and castrating of cattle.35 Available methods of minimizing pain and stress include application of local anesthetics and the administration of analgesics. Although the use of local anesthetics at the time of castration is a regulatory requirement in several European countries,26,32,35 there currently are no analgesic drugs approved for the alleviation of pain in livestock in the United States.36 The FDA Center for Veterinary Medicine guidance for the development of effectiveness data for NSAIDs indicates that validated methods of pain assessment must be used for a drug to be indicated for pain relief in the target species.37 This requirement explains the lack of analgesic drugs approved for pain relief in livestock in the United States because there currently are no validated methods of pain assessment in food-producing animals.

Cortisol response has been widely used to assess well-being in farm animals. Acute cortisol response has also been used to characterize nociceptive responses because the magnitude, duration, or integrated plasma cortisol concentration (ie, AUC) corresponds with the predicted noxiousness of a particular stimulus.4,7–12,27,38 In 1 study,39 it was reported that mean peak cortisol concentrations of 129 nmol/L (converted from 46 ng/ mL)22 were detected at 30 minutes after surgical castration in 5.5-month-old calves. This concurs with the Cmax of 128.80 ± 9.06 nmol/L at 0.68 hours after castration determined by use of noncompartmental analysis in the study reported here. However, calves in the unaesthetized (control) group in our study had a numerically greater model Cmax of 136.58 ± 31.94 nmol/L at 0.53 hours after castration, which suggested that the magnitude of cortisol response was not specifically associated with nociception. An additional contradiction with other studies4,12 was that the AUC0–t = Cmin + Cmax, and amount of time that plasma cortisol concentrations remained above and below baseline values in our study were not significantly different between castrated and unaesthetized control calves.

The limitations of measuring cortisol concentrations to directly quantify nociceptive response have
been acknowledged in the literature, although specific reports describing these deficiencies are lacking. Although peak cortisol response correlates with the noxiousness of a procedure, interpretations at the lower and upper extent of the response range are less predictive. Studies13,14,15,26 revealed that for the lower extent of the response range, tail docking with a ring and tail docking with a docking iron cause similar cortisol responses to those resulting from control handling in older lambs. For the upper extent of the response range, several studies,13,14,26 as well as the study reported here, failed to detect an increase in cortisol response that is proportional to the severity of a procedure. Analysis of these data suggests the existence of a ceiling for the plasma cortisol response that may be elicited by handling alone.14,27 This implies that an increase in plasma cortisol response after handling and castration is not proportional to the nociceptive stimulus.

In 1 study,9 plasma SP concentrations were up to 27-fold greater in human patients with soft tissue injury than in healthy control subjects, whereas there was a 4-fold increase in CGRP concentrations, and plasma neuropeptide Y and endothelin concentrations were unchanged. In that study, the median SP concentration was 401.94 pg/mL (interquartile range, 106.23 to 940.42 pg/mL) in patients with soft tissue injury and 7.45 pg/mL (interquartile range, 4.25 to 48.66 pg/mL) in healthy control subjects (a molecular weight of 1,329,398 g/mol was used to convert pmol/L to pg/mL). This is consistent with the results of the study reported here in terms of the castrated group, which had a median SP concentration of 441.10 pg/mL (interquartile range, 307.60 to 569.80 pg/mL). In contrast, calves in the control group had a much greater median SP concentration of 337.35 pg/mL (interquartile range, 195.31 to 491.22 pg/mL) than was reported for the human subjects. However, SP concentrations in serum of control humans ranged from 12.25 to 397 pg/mL,10 which is similar to the values detected in the calves of our study.

The pronounced intersubject variability reported in other studies and the study reported here could be explained by evidence that suggests plasma SP concentrations in humans may be increased in response to stress.13 However, in contrast to plasma cortisol concentrations, there is no evidence of a ceiling effect or circadian rhythm associated with endogenous SP production. Furthermore, it has been reported19 that sample preparation and the choice of analytic method for SP quantification may yield significantly different values, which may also contribute to the variability of SP values in the literature. Additional research is required to examine the extent to which plasma SP concentrations may increase in calves in response to the stress of handling and refine the diagnostic test to reduce variability.

It is noteworthy that castrated calves had greater interindividual variation in SP response than did uncastrated control calves, whereas the opposite was evident for the plasma cortisol response. One explanation is that stress associated with handling alone induces a uniformly lower and less variable SP response in uncastrated calves because they are not exposed to a nociceptive stimulus. However, the intensity and extent of sympathetic and HPA stimulation associated with handling alone vary among calves because this probably is related to genetics, previous episodes of handling, and individual stress tolerance. On the other hand, the increase in plasma SP concentrations associated with castration in calves may be highly variable as a result of differences in the rate or mechanism of SP release and the timing of sample collection. However, nociception associated with castration is uniformly distressing to calves, which results in a more homogeneous and consistent sympathoadrenal and HPA stimulation within all subjects up to a proposed maximal ceiling response.14,27 The overall effect was greater variability in the SP response in castrated calves, compared with the response in control calves, whereas a more consistent, less variable cortisol response was detected after castration, compared with the response after handling alone.

Studies in which plasma SP concentrations have been measured to evaluate pain in veterinary species are limited to a report in elk after antler removal13 and another report in cats after dental treatment.25 Plasma SP and cortisol concentrations were monitored 4 times during the elk study26 to determine whether either of them could be used to predict pain in elk after antler removal. Substance P concentrations were measured by use of a commercial immunoassay kit following extraction of samples, as was conducted in our study. No significant differences in SP or cortisol concentrations between the groups were detected at any of the 4 time points. However, SP concentrations increased after velvet antler removal, although the difference was not significant.

Plasma concentrations of cortisol and SP were measured before and after periodontal treatment in cats in 1 study.25 Mean ± SEM cortisol concentration decreased from 41.1 ± 4.39 ng/mL before handling and treatment to 37.1 ± 3.99 ng/mL after periodontal treatment. In contrast, mean plasma SP concentrations were increased from a pretreatment value of 118 ± 30 pg/mL to a post-treatment value of 209 ± 43 pg/mL. Analysis of these data suggests that assessment of plasma SP concentrations may help to discriminate between stressful events that cause transient increases in plasma cortisol concentrations and more painful events that culminate in a prolonged increase in plasma SP concentrations. This conclusion is supported by the results for the study reported here in which there was an increase in plasma SP concentrations in calves with vocalization and attitude scores of 3, compared with results for calves that did not have aversive behavior. However, cortisol responses in the same calves were almost identical in animals with behavioral scores of 0 and 3. Analysis of these data indicates that assessment of plasma SP concentrations, when measured in conjunction with plasma cortisol concentrations, may be a useful tool to discriminate between painful and stressful procedures.

Potential limitations of our study include the fact that there were only a limited number of calves enrolled in the study and that samples were only collected for 240 minutes after castration. Although our sample size was relatively small and substantial variation in SP concentrations was detected, the data were sufficient to
reveal significant differences in SP concentrations between groups when unequal variances were accounted for in the statistical model. In other studies in which plasma cortisol response was evaluated, investigators typically used 8 to 10 animals/treatment group, whereas only 5 calves/group were used in the study reported here. The difference in mean (± 95% confidence interval) cortisol response between castrated and uncastrated control calves over the course of the study was 5.87 ± 22.15 nmol/L. This suggests that to detect a significant difference between the castration and control groups, the difference in mean response would need to be at least 28 nmol/L. This would have required a substantially larger sample size to reveal a significant difference between groups.

Despite the relatively short period of sample collection after castration, we were able to characterize an increase in cortisol concentrations during the first 30 minutes, which was followed by a substantial decrease in cortisol concentrations to below baseline values from 150 to 240 minutes after castration. It could be argued that the duration of this study was insufficient to fully characterize the SP response. To the authors’ knowledge, this is the first attempt to characterize the SP response in cattle after a painful stimulus. Future studies may reveal that fewer samples are required to determine SP concentrations before and after a nociceptive event. This would represent a substantial benefit over determination of plasma cortisol concentrations because intensive sample collection for at least 60 minutes is required to characterize a peak cortisol response. Assuming sampling frequency can be reduced, then the confounding stress response associated with intensive handling will also be minimized.

In the study reported here, we were unable to detect an increase in plasma cortisol response specifically associated with castration that has been described in the literature. However, a significant increase in plasma SP concentration was detected after castration, but additional research will be required to characterize this response. Considered together, these results suggest that simultaneous determination of plasma concentrations of SP and cortisol may be useful to differentiate between acute stress attributable to handling and prolonged distress associated with nociception. These results may have important implications for the assessment of pain in farm animals and the development of novel science-based variables used to assess animal well-being in livestock production systems. Further characterization of objective pain measurements is needed for the development of less painful management practices and effective analgesic drug regimens.

References