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## Pharmacokinetics and distribution in interstitial and pulmonary epithelial lining fluid of danofloxacin in ruminant and preruminant calves

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The objective of this study was to compare active drug concentrations in the plasma vs. different effector compartments including interstitial fluid (ISF) and pulmonary epithelial lining fluid (PELF) of healthy preruminating (3-week-old) and ruminating (6-month-old) calves. Eight calves in each age group were given a single subcutaneous (s.c.) dose (8 mg/kg) of danofloxacin. Plasma, ISF, and bronchoalveolar lavage (BAL) fluid were collected over 96 h and analyzed by high-pressure liquid chromatography. PELF concentrations were calculated by a urea dilution assay of the BAL fluids. Plasma protein binding was measured using a microcentrifugation system. For most preruminant and ruminant calves, the concentration–time profile of the central compartment was best described by a two-compartment open body model. For some calves, a third compartment was also observed. The time to maximum concentration in the plasma was longer in preruminating calves (3.1 h) vs. ruminating calves (1.4 h). Clearance (CL/F) was 385.15 and 535.11 mL/h/kg in preruminant and ruminant calves, respectively. Ruminant calves maintained higher ISF/plasma concentration ratios throughout the study period compared to that observed in preruminant calves. Potential reasons for age-related differences in plasma concentration–time profiles and partitioning of the drug to lungs and ISF as a function of age are explored.

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### INTRODUCTION

Danofloxacin is a synthetic fluoroquinolone antibiotic licensed for use in cattle in the United States for the treatment of bovine respiratory disease (BRD) associated with *Mannheimia haemolytica* and *Pasteurella multocida*. It exerts efficacy against gram-negative and gram-positive bacteria by inhibiting bacterial DNA gyrase and topoisomerase, thereby blocking DNA replication and effectively killing the bacteria (Drlica, 2012).

Extra-label drug use of antibiotics in food producing species has led to concerns over an increase in the selection of resistant microbial strains, with the potential for horizontal gene transfer and for the spread of resistant strains to other animal and human pathogens (Martinez *et al.*, 2014). In situations when extra-label use is a necessity, it is essential that the prescribing practitioner select a drug that is appropriate for the targeted pathogen, the administered dose is safe, and the selected dosage regimen is consistent with achieving a systemic

drug exposure profile that is consistent with efficacy. Integral to defining an appropriate dosage regimen is an understanding of the pharmacokinetics (PK) of that drug in the targeted patient population and tissue compartments.

Danofloxacin is approved for use in ruminating calves but does not currently have an approval for veal or preruminating calves. While one may consider defining the dose in younger calves simply by adjustments based on body weight, such dose adjustments fail to recognize potential age-associated differences in drug PK. Therefore, the PK of danofloxacin in preruminant and ruminating calves needed to be examined.

Several reports have described the age-associated bovine PK for a variety of drugs. Considering the findings associated with sulfamethazine (Nouws *et al.*, 1986), phenylbutazone (Volner *et al.*, 1990), ceftiofur sodium (Brown *et al.*, 1996), and meloxicam (Mosher *et al.*, 2012), there appears to be a trend toward a lower total systemic clearance (CL) and larger volumes of distribution at steady-state ( $V_{ss}$ ) in neonatal calves as

compared to adults (approximately 6 months of age). However, these relationships may not be universal as the Vss of the fluoroquinolone enrofloxacin was suggested to be smaller in 1-day-old calves as compared to one-week-old calves (Kaartinen *et al.*, 1997).

Additional considerations of pharmacodynamics (PD) and disease progression need to be factored into dose evaluation for neonatal calves. As the immune system of these calves is not fully developed, it is preferable to use drugs with bactericidal activity. This is one of the reasons why fluoroquinolones such as danofloxacin are particularly attractive as they are known to exert concentration-dependent action (Lees & Shojae Aliabadi, 2002). Typically, fluoroquinolone efficacy has been linked to the extent of exposure over a 24-h period (area under the curve,  $AUC_{0-24\text{ h}}$ ) at steady-state relative the minimum inhibitory concentration (MIC) of the targeted pathogen ( $AUC/MIC$ ) and to the ratio of peak plasma drug concentrations ( $C_{\max}$ ) to the minimum inhibitory concentration ( $C_{\max}/MIC$ ) (Andes & Craig, 2002). Consistent with this pharmacokinetic/pharmacodynamic (PK/PD) relationship, danofloxacin has been shown to achieve high concentrations in lung tissues, with rapid penetration into the pulmonary fluids of calves with respiratory disease (Terhune *et al.*, 2005). However, the precise PK/PD target necessary to achieve the desired clinical outcome may not be identical for ruminating calves vs. preruminant calves. Potential reasons for such age-related differences include a dissimilarity in the partitioning of drug from the blood to the infection site or the possible need to achieve a greater microbicidal activity in the presence of the yet immature immune system (i.e., greater reliance on the drug vs. the host response).

The objective of this study was to compare the drug concentrations (free and total) in the plasma vs. concentrations in the ISF and PELF of healthy preruminating (3-week-old) and ruminating (6-month-old) calves. It is important to acknowledge that the extra-label use of fluoroquinolones in major food producing species is prohibited by law. However, it was selected for use in this study because it is approved for use in ruminating calves and has well-defined pharmacokinetic/pharmacodynamic (PK/PD) characteristics, and the importance of the use of peak concentration and extent of drug exposure for determination of its antimicrobial effects needed to be examined. By providing more data on the effect of age on PK parameters in preruminant calves, these results could provide the scientific foundation upon which to base assessments of potential future applications for the use of antimicrobial compounds for use in neonatal calves.

## MATERIALS AND METHODS

### Animals

This study was approved by the North Carolina State University Institutional Animal Care and Use Committee. Weaned and unweaned male Holstein calves were bought from the North Carolina State University Dairy herd. Eight unweaned

Holstein calves, 2–3 weeks of age, weighing between 41 and 53 kg were classified as preruminants. These calves were housed in individual hutches at the University dairy, were fed commercial milk replacer twice a day, and had free access to water and calf starter ration *ad libitum* throughout the study. Eight weaned calves, 6 months of age and weighing between 151 and 214 kg at time of study, were classified as ruminating calves. The calves were group housed indoors on a concrete floor bedded with wood shavings. Ruminant calves were maintained on grass hay and water *ad libitum* and were supplemented with grain. None of the calves had any previous history of disease or antibiotic administration and had normal physical examinations prior to start of the study. The extra-label use of fluoroquinolones is expressly prohibited in food producing animals in the United States, and these calves were not allowed to enter to human food supply at the completion of the study.

### Drug administration and blood collection

All calves were weighed on a digital scale on the morning of the study commencement for determination of the administered dose. Approximately 24 h prior to start of the study, calves were restrained for intravenous catheter placement. The area where the catheter was to be placed was clipped and cleaned with alternating scrubs of chlorhexidine and isopropyl alcohol. Using sterile technique, a 14 G  $\times$  3.25 mm catheter (Angiocath<sup>TM</sup>, BD, Franklin Lakes, NJ, USA) was inserted into the right jugular vein with an extension set and sutured to the skin using a 2-0 monofilament suture. Catheters were flushed three times a day using 6 mL of 10 units/mL of heparin saline. A single s.c. injection of danofloxacin (8 mg/kg) (Advocin<sup>TM</sup>; Zoetis, Florham Park, NJ, USA) was administered to each calf in the neck per label instructions. Blood samples were taken from the jugular vein and were transferred to lithium heparinized tubes at 0 (pretreatment), 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, and 96 h postadministration of danofloxacin. These samples were stored on ice until centrifugation at approximately 3500 g for 20 min to collect plasma. The plasma samples were stored at  $-80\text{ }^{\circ}\text{C}$  until analysis.

### Interstitial fluid collection

All calves were implanted with s.c. ultrafiltration interstitial fluid (ISF) probes on the side of the neck opposite site of s.c. injection (BASI Inc, West Lafayette, IN, USA). Each probe contained three semipermeable loops connected to a nonpermeable tube that extended outside the animal and attached to a 3 mL no additive plastic vacutainer tube. This tube provided negative pressure for fluid collection through small pores in the probe membranes. These pores allowed for the movement of water, electrolytes, and low molecular weight molecules ( $<30\text{ }000\text{ Da}$ ) to pass into collection tube. This pore size excludes large molecules such as proteins, protein bound drugs, and cells. Probes were placed with calves under sedation with xylazine [Rompun<sup>®</sup> Injectable, Shawnee Mission, KS, USA

(20 mg/mL; Bayer Animal Health Division] at a dose of 0.05–0.1 mg/kg in the cervical neck muscles. Probes were placed 24 h before the start of the trial to allow them adequate time to equilibrate. One probe was inserted into each calf subcutaneously in the area cranial to the scapula. All probes were placed through a small stab incision using an introducer needle provided by the company. The ISF was collected at 0 (pretreatment), 2, 4, 8, 10, 12, 24, 48, 72, and 96 h after s.c. administration of danofloxacin. As each ISF sample represents fluid collection over a certain amount of time (i.e., not instantaneous sample), a lag time was calculated based on the length of the tube and fluid collected over time for each sample. The fluid collected was frozen at  $-80^{\circ}\text{C}$  until analysis.

#### Lung fluid collection

To determine drug concentrations in PELF, a bronchoalveolar lavage (BAL) was performed using a method described previously (Poulsen & McGuirk, 2009). Briefly, BALs were performed in all preweaned calves using a sterilized, flexible 10 French  $\times$  36 inch catheter with a 3-cc balloon cuff and in ruminating calves, a 24 French  $\times$  59 inch catheter was used (Mila International, Inc. Medical Instrumentation for Animals, Florence KY). At each time point, the calf was restrained and the head and neck of the calf were extended to facilitate passage of the sterile BAL catheter. The BAL catheter was introduced into the ventral meatus of the nose through which it was advanced down the trachea until it was wedged in a terminal bronchus. Repeated coughing was used as an indicator of appropriate placement. In the wedged position, the balloon cuff was inflated to create a seal and the catheter was held firmly in place while the guide wire was removed. At each time point, 100 mL of sterile saline was infused into the lungs. Immediately after the infusion, negative pressure was applied to aspirate fluid. The volume of fluid that was retrieved ranged from 0 to 55 mL of clear to mildly turbid foamy fluid. The fluid sample was placed into a sterile collection tube, the total amount recorded and placed on ice until centrifugation. The BAL samples were centrifuged at 300 *g* for 10 min, and supernatant fluid was separated from cell pellet and frozen at  $-80^{\circ}\text{C}$  until analysis.

#### Drug analysis

Plasma and BAL fluid were analyzed by high-performance liquid chromatography (HPLC) with fluorescence detection following solid-phase extraction (SPE). The ISF was injected directly onto the HPLC. Danofloxacin was extracted from plasma and BAL supernatant using a method described previously by Davis *et al.* (2007). Briefly, a 3 mL/60 mg HLB cartridge (Waters Corporation, MA) was conditioned and equilibrated with 2 mL of methanol followed by 2 mL of water. A 500  $\mu\text{L}$  of plasma sample was added to cartridge followed by a wash step using 2 mL of 95:5 water:methanol. The drug was then eluted into glass tubes with 1 mL of methanol followed by another 1 mL of methanol. The eluted sample was

evaporated to dryness under nitrogen at  $40^{\circ}\text{C}$  and reconstituted in 500  $\mu\text{L}$  of mobile phase [water:methanol with 0.1% trifluoroacetic acid (85:15, v:v)] for plasma and 250  $\mu\text{L}$  for BAL samples for HPLC analysis.

The extracted plasma and BAL samples were analyzed using a Waters XBridge C18 3.5  $\mu\text{m}$  (4.6  $\times$  100 mm) HPLC column (Waters Corporation, MA) at 800  $\mu\text{L}/\text{min}$  with an injection volume of 25  $\mu\text{L}$ . Danofloxacin was detected using fluorescence (W2475) at an excitation wave length of 280 nm and an emission wave length of 400 nm. Validation standards were prepared over a linear range for each matrix (plasma, ISF, sodium chloride 0.9%, mobile phase) and were used to construct calibration curves. These standards were validated over the range 0.001–5.0  $\mu\text{g}/\text{mL}$  in fortified (spiked) blank plasma, BAL, and ISF with danofloxacin (reference drug standard was provided by Zoetis) to validate the HPLC analysis. Over the validated range, the percent coefficient of variation (%CV) for inter- and intraday averaged 10.7% with an average recovery of 95.9%. The limit of quantification (LOQ) was 5 and 10 ng/mL, and the limit of detection (LOD) was 1 and 5 ng/mL for plasma and BAL and ISF, respectively.

#### Danofloxacin Concentrations in PELF

Estimation of the amount of PELF sampled by BAL fluid was performed using the urea dilution method as described previously in cattle (Giguère *et al.*, 2011). Urea nitrogen concentrations in serum (Urea<sub>SERUM</sub>) and in BAL fluid (Urea<sub>BAL</sub>) were determined by use of a urea test kit (Urea test kit; Sigma Chemical, St Louis, MO, USA) and the absorbance values measured by use of a spectrophotometer. The volume of PELF ( $V_{\text{PELF}}$ ) in BAL fluid was derived from the following equation:

$$V_{\text{PELF}} = V_{\text{BAL}} \times \left( \frac{\text{Urea}_{\text{BAL}}}{\text{Urea}_{\text{SERUM}}} \right)$$

in which  $V_{\text{BAL}}$  is the volume of recovered BAL fluid. The concentration of danofloxacin in PELF (DANO<sub>PELF</sub>) was derived from the following relationship:

$$\text{DANO}_{\text{PELF}} = \text{DANO}_{\text{BAL}} \times \left( \frac{V_{\text{BAL}}}{V_{\text{PELF}}} \right)$$

in which DANO<sub>BAL</sub> represented the measured concentration of danofloxacin in BAL fluid.

#### Plasma protein binding

Pooled plasma was collected from five healthy calves that have not received danofloxacin. Plasma aliquot of each age of calf was spiked with danofloxacin, generating samples in triplicate with concentrations of 0.01, 0.1, 1.0, and 10.0  $\mu\text{g}/\text{mL}$  (Davis *et al.*, 2007). All samples were allowed to stand at room temperature for 30 min. A 1 mL sample of each standard was added to the microcentrifugation device and was centrifuged at 4000 *g* for 10 min. The ultrafiltrate was analyzed using HPLC

without extraction to determine unbound concentration and protein binding of danofloxacin was then determined. Non-specific binding of danofloxacin was determined to be minimal in the microcentrifugation device and filter.

#### Pharmacokinetic analysis

Unless otherwise indicated, all PK parameter values are expressed relative to the unbound danofloxacin concentrations taking into account the nonlinearity in protein binding as a function of drug concentrations.

Plasma concentrations of danofloxacin were analyzed using a computer software program (Phoenix<sup>®</sup> WinNonlin/NLME, Version 1.3 Certara, USA Inc. Princeton, NJ, USA). Nonlinear mixed-effects modeling of danofloxacin concentrations was performed. The selected model was parameterized by clearance and estimated volume of distribution and absorption rate constant. Model selection was based on goodness-of-fit plots, statistical significance between models using lowest Akaike information criteria (AIC) values obtained in Phoenix<sup>®</sup> software, and coefficient of variation (CV%) of the parameter estimates. An examination of covariates was performed to determine whether there were any factors that may explain the variability in Cl/F, V/F, and/or Ka. Covariates investigated included age, weight, and physiological status (preruminant vs. ruminant). No covariates were found to significantly improve model predictions for any parameter for danofloxacin. A Student's *t*-test from compartmental values for age differences in the two groups of calves was performed to confirm the results obtained from NLME.

Compartmental modeling was performed to determine PK parameters in each age group. To select between a one-, two-, or three-compartment open body model, the AIC and the highest  $R^2$  values were evaluated. A two-compartment model was determined to provide the best fit and therefore was the model used to describe the danofloxacin concentration–time curve for each calf. Parameter estimates were obtained by minimizing the sums of the weighted deviations using a weighting factor of  $1/Y_i^2$ , where  $Y_i$  is the observed plasma concentration at time  $i$ . For the selected model,  $R^2$  values ranged from 0.81 to 0.99 for preruminant calves and from 0.87 to 0.98 for ruminant calves.

Noncompartmental analysis (NCA) was also performed to generate estimates of the area under the curve (AUC), maximum plasma concentrations ( $C_{\max}$ ), time to  $C_{\max}$  ( $T_{\max}$ ) (plasma and tissue fluid), and the terminal elimination half-life ( $t_{1/2}$ ) (plasma). The AUC was estimated from time 0 to the last measured concentration (as defined by the LOQ) using the log-linear trapezoidal method. Values of  $t_{1/2}$  were generated using  $0.693/\lambda_z$  where  $\lambda_z$  is the slope of the log-linear portion of the terminal depletion phase (terminal portion of the curve out to 96 h for preruminant calves and 72 h for ruminant calves. Slopes were also estimated from  $T_{\max}$  to h 24 postdose to explore the potential difference for bias in the half-life estimate due to the slower input rate preruminating vs. ruminating calves.

All PK parameters were reported as a mean  $\pm$  SD. As the  $t_{1/2}$  was estimated as  $0.693/\text{mean } \lambda_z$ , the  $t_{1/2}$  values are reported as harmonic mean  $\pm$  standard deviation (SD). The SD was estimated using the Delta method<sup>1</sup> where:

$$SD = HM^2 \times \sqrt{\frac{\sum_{i=1}^n (H_i - MH)^2}{n - 1}}$$

And where MH is the mean of the harmonic values (MH) for

$$\frac{\sum \frac{1}{x_i}}{n}$$

$H_i$  is the harmonic value ( $\lambda_z$ ) and HM is the harmonic mean being report ( $t_{1/2}$ ).

ISF/plasma concentration ratios at each time point were determined for each calf using the concentration of ISF (unbound drug) to the total (bound and unbound) and unbound danofloxacin plasma concentration. Statistical analyses were performed on the mean PK parameters of preruminant vs. ruminant calves using SigmaPlot (Systat Software, Inc, San Jose, CA, USA); *P*-values of  $\leq 0.05$  were considered statistically significant. The normality assumption was tested for each variable set with the Shapiro–Wilk *W*-test, which is the preferred method for testing the normality of data when the sample size is small (Ghasemi & Zahediasl, 2012). NLME modeling of the PELF data was unsuccessful as the data were sparse.

Due to missing values and the sparse nature of the dataset, PELF was evaluated using a noncompartmental analysis from the perspective of concentrations as a function of sampling times and the corresponding averages of those concentrations at each time point for the ruminant and preruminant calves. NLME modeling of the PELF data was unsuccessful as the data were sparse.

## RESULTS

There were no adverse reactions noted from placement of ultrafiltration devices, catheters, procedures, or from the danofloxacin administration. Plasma was obtained at each time point. The 96-h sample for ruminant calves was mislabeled and could not be matched with an individual calf and was excluded from analysis. Therefore, ruminant calves profiles were truncated to 72 h postdose. Over some sampling intervals, fluid was not collected into the ISF probe or the probes were pulled out by the calves. Therefore, ISF data are missing

<sup>1</sup>Feiveson AH (2005). What is the delta method and how is it used to estimate the standard error of a transformed parameter?: Explanation of the delta method, <http://www.stata.com/support/faqs/statistics/delta-method/>

at these time points. There was no significant binding of danofloxacin to the ultrafiltration probe.

Several challenges were encountered during efforts to capture the BAL fluid. Irrespective of age, BAL fluid volumes collected ranged from 0 to 55 mL. At the 2-h sample for preruminant calves, the lavage yielded no lung fluid for three of the calves. Greater success was achieved at the 12- and 24-h time points where fluid could not be aspirated from only one preruminant calf each of these time points. The variability of fluid return with the BAL procedure in the preruminant calves could be attributed to anatomical differences and the corresponding difficulties in achieving proper placement of the catheter in the terminal bronchus.

### Plasma

Although drug samples at the 96 h could not be included in the analysis for the ruminating calves, the use of the compartmental analysis enabled an analysis of values to time infinity in all subjects. To insure that assessments of CL/F and Vd/F are not biased by the loss of the area defined by the 72- to 96-h trapezoid, these estimates could not be reliably compared on the basis of data generated by the NCA, but were extrapolated out to 96 h for both groups of calves in the compartmental analysis.

Age-associated differences were observed in several PK parameters. The AUC and  $T_{max}$  values tended to be greater in the preruminant as compared to ruminating calves (Table 1). Using the compartmental analysis, K01 (absorption rate constant) values tended to be lower in preruminants vs. ruminants (Tables 1 and 2). Despite the observed differences in mean values as a function of age, the  $AUC_{0-96}$  was not statistically significant between the two groups, which likely reflect a high degree of variability and the limited number of study subjects per age group. The elimination rate constant (K10) tended to be smaller in the preruminants (slower elimination). The rate of drug distribution from the peripheral compartment back into plasma (K21) values tended to be smaller (slower partitioning back into the plasma) in the ruminating vs. preruminating calves. As discussed later, these findings may be useful in explaining the observed relationship between ISF/plasma concentrations in ruminants vs. preruminants. Compartmental parameter rate estimates in the individual ruminant and preruminant calves are depicted in Appendix (Figure S2).

After s.c. administration, the maximum unbound danofloxacin concentration in plasma  $C_{max}$  values, as generated via our compartmental analysis, achieved a value of 1.79  $\mu\text{g/mL}$  for preruminant and 2.12  $\mu\text{g/mL}$  in ruminant calves. The corresponding mean  $T_{max}$  values were 3.125 and 1.44 h, respectively. The mean terminal elimination  $t_{1/2}$  was estimated as 20.36 h for preruminant calves and 15.31 h for ruminant calves (Table 1). These differences were not statistically significant. To ascertain whether this difference may have been biased by drug input rates, slopes were also estimated from  $T_{max}$  to 24 h. In this case, the profile depletion rates were similar in preruminating and ruminating calves, with

**Table 1.** Plasma pharmacokinetic parameters in preruminating vs. ruminating calves

Parameter	Preruminating calves		Ruminating calves	
	Mean	SD	Mean	SD
$AUC_{0-96}$ ( $\text{h}^*\mu\text{g/mL}$ )	23.23	10.07	15.17	1.83
Cl/F ( $\text{mL/h/kg}$ )	385.15	132.15	535.11	66.83
$C_{max}$ ( $\mu\text{g/mL}$ )	1.79	0.43	2.12	0.53
$t_{1/2}$ (h)	20.36	2.46	15.31	4.97
$T_{max}$ (h)	3.13*	1.25	1.44*	1.11
Vz/F ( $\text{mL/kg}$ )	4125.01	3842.5	2693.8	1709.3

Mean pharmacokinetic parameters from two-compartment model after single S.C. injection of 8 mg/kg danofloxacin in 3-week-old vs. 6-month-old calves. Half-life parameter is reported as harmonic mean.

\*Indicates significantly different by *t*-test ( $P < 0.05$ ).

**Table 2.** K01 (absorption rate constant) for preruminant and ruminant calves from compartmental analysis

Calf no.	Preruminant (1/h)	Ruminant (1/h)
1	0.73	1.1
2	0.52	19.5
3	0.90	2.8
4	0.91	1.5
5	2.2	24.3
6	0.81	1.5
7	0.69	24.5
8	0.89	10.8

**Table 3.** Noncompartmental analysis to determine the terminal half-life after injection of 8 mg/kg danofloxacin in 3-week-old vs. 6-month-old calves. Reported half-life was calculated using the equation  $0.693/\lambda_z$  value for the first distribution phase after  $C_{max}$  is reached for each group of calves

Calf no.	Preruminant (4–24 h)	Ruminant (1–24 h)
1	7.43	5.44
2	10.16	4.62
3	7.07	4.75
4	6.02	4.64
5	5.29	4.00
6	4.23	4.45
7	5.66	4.08
8	4.64	4.38

corresponding rather than the terminal elimination phase, the  $t_{1/2}$  estimates ranging from 4.23 to 10.16 h in the preruminant calves and 4.0 to 5.44 h in the ruminant calves (Table 3). These values were substantially less than the slopes observed in the terminal depletion phase.

A nonlinear plasma protein binding was noted, with protein binding decreasing as concentration increased, ranging from 67 to 17% bound as danofloxacin concentrations varied from 0.01 to 10  $\mu\text{g/mL}$  (Table 4). Importantly, at 10  $\mu\text{g/mL}$ , the preruminant binding was substantially lower than that of the

ruminant calves.  $K_d$  and  $B_{max}$  values were estimated using the plasma protein binding data in preruminant and ruminant calves using a graphic software (GraphPad Software, Inc, La Jolla, CA, USA). The relationship between bound and free concentrations in plasma was best described by a saturable binding model using the following equation:

$$C_{bound} = B_{max} \frac{C_{free}}{C_{free} + K_D}$$

where  $B_{max}$  is the maximum amount of drug that is bound to in the plasma (maximum binding capacity) and  $K_D$  is the equilibrium dissociation constant which reflects the equilibrium concentration at which the drug meets half-maximal binding capacity.  $B_{max}$  and  $K_D$  values showed differences in preruminant and ruminant calves.  $B_{max}$  value was estimated to be  $6.09 \mu\text{M}$  ( $2.176 \mu\text{g/mL}$ ) and  $21.66 \mu\text{M}$  ( $7.740 \mu\text{g/mL}$ ) and  $K_D$  values of  $5.39 \mu\text{M}$  ( $1.925 \mu\text{g/mL}$ ) and  $24.88 \mu\text{M}$  ( $8.892 \mu\text{g/mL}$ ) in preruminant and ruminant calves, respectively. The unbound fraction was calculated using the free concentration,  $B_{max}$  and  $K_D$  values using the formula previously described (Toutain & Bousquet-Melou, 2002). The unbound fraction was not significantly different between the two groups (Table 4).

#### Interstitial fluid data

Using an *in vivo* ultrafiltration technique to collect ISF in repeated samples allowed for the monitoring of unbound drug disposition over time and was less invasive than tissue biopsies. Although some samples were missed due to occlusion of the probe or the calf movement around the hutches, these devices were well tolerated and collected between  $0.05 \mu\text{L}$  and  $>2 \text{ mL}$  of fluid for drug analysis.

The mean ( $\pm$ SD) plasma and ISF samples from both groups of calves are shown in Fig. 1 ( $n = 8$  calves in each group). A comparison of ISF and plasma (bound and unbound) concentrations is shown in Fig. 2 for each group of calves. The  $T_{max}$  for ISF fluid occurred later than in plasma irrespective of calf age. Whether considering the ratios of ISF to total or free

plasma danofloxacin concentrations, the ISF/plasma concentration ratios tended to be higher in ruminant calves as compared to that seen in the preruminant calves (Fig. 3).

#### Pulmonary epithelial lining fluid

Average PELF concentrations from the BAL samples were reported in Table 5 and are seen graphically in Fig. 4. The maximum concentration in PELF was noted to occur at 2 h postadministration in both preruminant and ruminant calves, with estimated PELF concentrations far exceeding the concentrations seen in plasma and ISF. Greater variability in drug concentrations in PELF was seen in the preruminant calves as compared to ruminating calves.

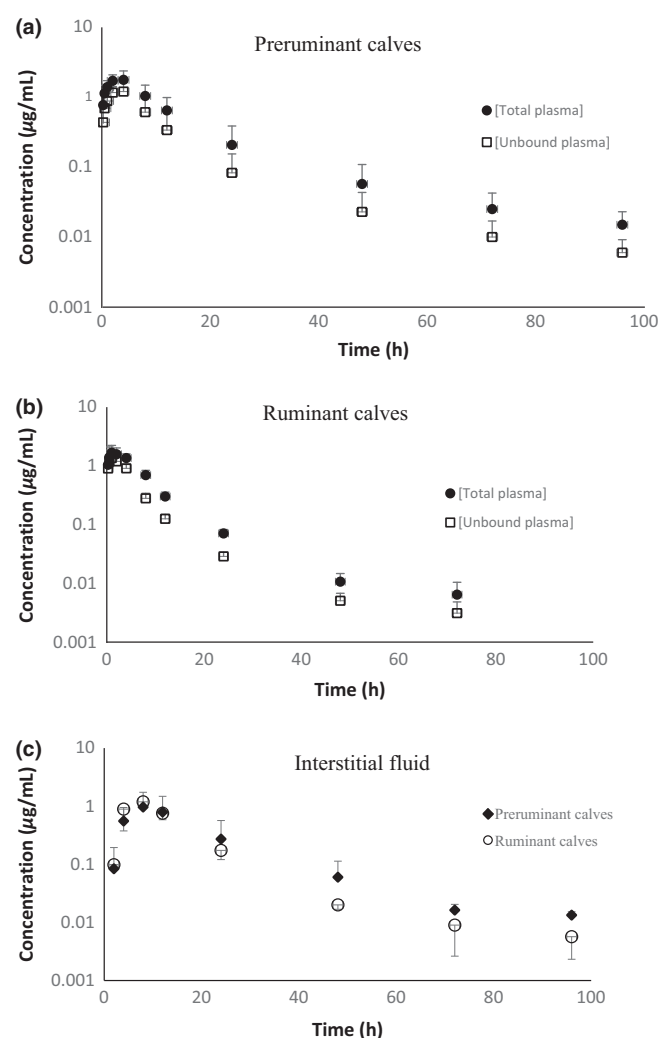


Fig. 1. Mean plasma (bound and unbound)  $\pm$  SD concentrations after single S.C. injection of 8 mg/kg danofloxacin in preruminant (a) and ruminant calves (b). (c). Average Interstitial fluid danofloxacin concentrations  $\pm$  SD.

Table 4. Average % Plasma protein binding and calculated % bound of danofloxacin using microcentrifugation

Concentration ( $\mu\text{g/mL}$ )	Preruminant		Ruminant	
	Mean	Calculated % bound	Mean	Calculated % bound
0.01	68.3	53	65.6	47
0.1	67.0	53	63.4	47
1.0	46.8	47	44.9	45
10.0	17.64	18	33.2	33

Percent plasma protein binding of danofloxacin spiked plasma from preruminant (3-week-old) and ruminant (6-month-old) calves and calculated % bound using the equation from (Toutain & Bousquet-Melou, 2002). Samples were spiked in triplicate at four concentration levels (0.01, 0.1, 1.0, and 10  $\mu\text{g/mL}$ ) and averaged.

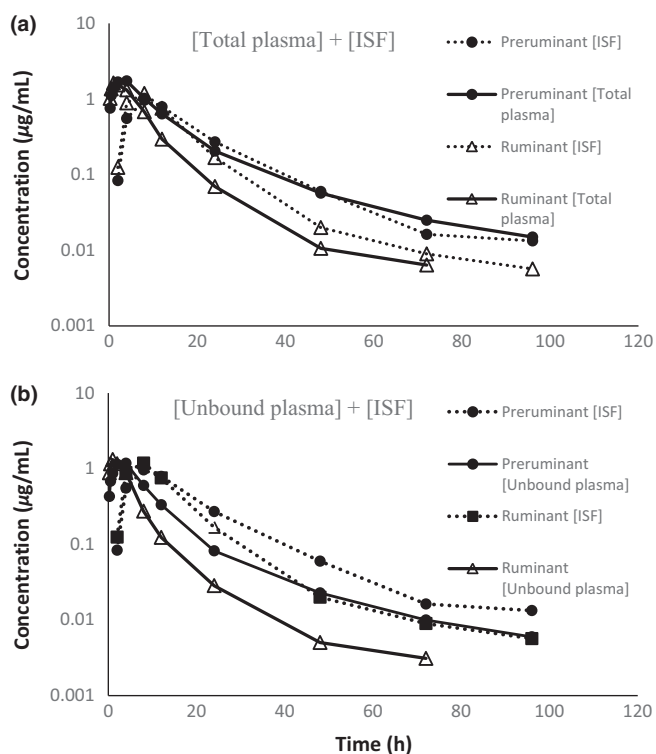


Fig. 2. Mean total plasma (a) and unbound (b) and ISF concentrations after single S.C. injection of 8 mg/kg danofloxacin in preruminant and ruminant calves.

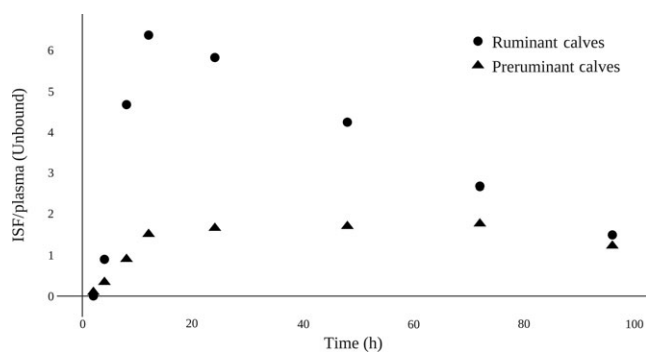


Fig. 3. ISF/Unbound plasma concentration ratios in ruminant and preruminant calves. Each point is a calculated geometric mean at each time point.

## DISCUSSION

Danofloxacin is approved in the United States for the treatment of bovine respiratory disease in calves. It is not approved for use in cattle intended for dairy production or for calves to be processed for veal. In the United States, extra-label use of fluoroquinolone antibiotics is prohibited by law (Davis *et al.*, 2009). Although the calves used in this study were Holsteins, these steers were not meant for veal production. They were all ultimately shipped to a feedlot to be finished as beef. Danofloxacin was chosen in this study as part

Table 5. Pulmonary epithelial lining fluid concentrations in preruminant vs. ruminant calves

Sample time (Postinjection)	Preruminating calves		Ruminating calves	
	Mean	SD	Mean	SD
2 h	5.4	6.3	3.6	1.7
12 h	3.1	3.6	0.36	0.06
24 h	0.62	0.86	0.44	0.36

Mean  $\pm$  SD in PELF determined from bronchoalveolar lavage after single S.C. injection of 8 mg/kg danofloxacin in ruminant and preruminant calves.

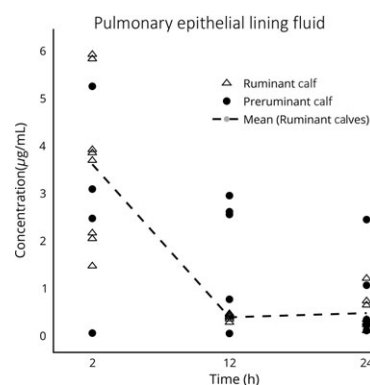


Fig. 4. Average danofloxacin concentrations in ruminant calves PELF. Dashed line represents the average PELF concentrations from ruminant calves. Two data points were omitted from analysis from calf 7.

of a larger project to compare the PK of different antimicrobials in both healthy and diseased calves of varying ages to examine what differences may be present related to age and physiologic development.

## Half-life comparisons

Danofloxacin is labeled to be given once s.c. at 8 mg/kg for the treatment of bovine respiratory disease. Past studies on the PK parameters of danofloxacin in 2- to 8-month-old calves have reported  $t_{1/2}$  values ranging from 2.65 to 7.4 h when administered at 1.25 mg/kg intravenous (Friis, 1993; Giles *et al.*, 1991; McKellar *et al.*, 1999; Shojaee Aliabadi & Lees, 2003). The reported mean plasma  $t_{1/2}$  in this study was considerably longer than these other reported values. Using NCA, the  $t_{1/2}$ , for the first distribution phase (from 4 to 24 h for preruminant calves and 1–24 h for ruminant calves) was very similar to those values reported in previous studies, indicating that the observed difference between our results and those reported by other investigators primarily reflected our ability to capture a second depletion phase. Most of previous studies reported total (unbound + bound) concentration, which may miss significant changes as a function of age in clearance, volume of distribution, and other parameters. As only unbound drug is free to move across barriers and is active to fight infections, reporting



unbound concentrations is a more clinically significant when determining drug concentrations at the active sites.

All calves used in this study remained clinically normal throughout the study, but it has been noted previously that disease status may also affect the  $t_{1/2}$  of danofloxacin. Apley and Upson (1993) reported that crossbred steer calves with acute pneumonia had a  $t_{1/2}$  of approximately 6.3 h (Apley & Upson, 1993). Estimates of  $t_{1/2}$  can be influenced by several physiological and experimental factors, including the experimental design (such as sampling time) and the composite effects of drug distribution and free drug clearance (Toutain & Bousquet-Melou, 2004). The ramifications of such differences as it pertains to unbound drug concentrations at the site of infection need to be considered from the perspective of the etiology of any age- or disease-associated change in drug PK.

#### Absorption comparisons

While there were no statistically significant differences in the observed preruminant vs. ruminant danofloxacin  $C_{\max}$  values, there was a significant difference ( $P < 0.006$ ) in the time to maximum concentration ( $T_{\max}$ ). Preruminant calf peak concentrations were observed between 2 and 4 h in all but one calf. In contrast, the corresponding ruminant  $T_{\max}$  values ranged between 0.5 and 1 h in all but one ruminating calf. The significant difference noted between these two age groups of calves following s.c. injection was reflected in the fitted absorption rate constants ( $K_{01}$ ). From the NLME model, the reported  $K_{01}$  values were around 2.18/h. Considering some of the physiological changes that occur during the maturation of neonatal humans (Batchelor & Marriott, 2013), the larger  $K_{01}$  values observed in the older calves could be a function of such maturation-induced differences as higher body temperature, body composition (lower tissue fluid volumes), and a greater local tissue blood flow as compared to that of neonates (Evans *et al.*, 1975). With respect to the latter point, pediatric human patients have been shown to have relatively lower density of skeletal muscle capillaries than that in adults. This may have also contributed to the lower bioavailability of drugs after intramuscular and s.c. routes of administration in neonates (Carry *et al.*, 1986). An increase in vascular perfusion to certain muscle groups increase with age and may impact drug absorption rates (Greenblatt, 1976).

#### Plasma protein binding

Initially, protein binding was thought to potentially contribute to some of the kinetic differences observed between preruminant vs. ruminant calves. As only the unbound fraction can reach the site of action, the distribution and availability are greatly influenced by the degree of protein binding of certain drugs. Frequently, the unbound fraction of many drugs is higher in human neonates and infants as compared to that of adults because of their lower concentration of binding proteins (Kearns *et al.*, 2003; Grandison Monica & Boudinot, 2000).

Coupling this difference in protein with a large extracellular fluid volume may account for the larger drug volume of distribution occasionally observed in neonates.

In the current study, within the range of concentrations observed in the plasma (0.01–1.0  $\mu\text{g/mL}$ ), only small differences in plasma protein binding were observed as a function of age. Therefore, plasma protein binding is unlikely to have had a significant impact on the age-associated PK differences observed in this study. This conclusion is supported by examining the similarity in the relationship between total vs. free drug concentration–time profiles in preruminant vs. ruminating calves (Fig. 1). Similar estimates of danofloxacin protein binding were reported in buffalo calves (36% at concentrations ranging from 0.0125  $\mu\text{g/mL}$  to 1  $\mu\text{g/mL}$ ). (Sappal *et al.*, 2009). A strong nonlinear binding has already been reported by Friis *et al.* (Friis, 1993) not only in plasma, but also in bronchial and nasal secretions.

Although average protein binding was similar in the linear range of 0.01–1  $\mu\text{g/mL}$ , percent protein bound tended to be higher in ruminant calves at 10  $\mu\text{g/mL}$ . The origin of these differences was not investigated; however, in human pediatric patients, a decrease in gestational age was directly proportional with a decrease in binding capacity of albumin, but did not affect the binding affinity to albumin (Bender *et al.*, 2007). There was a lack of fit of the algorithm used to estimate  $B_{\max}$  and  $K_D$  at lower concentrations which was noticed in the predicted and observed values for both groups (Table 4). This could reflect that the danofloxacin binding may be better defined by a different equation than was used. Differences were noted between the two age groups suggests a binding capacity and efficiency changes as a function of age but the validity of such assumptions is the subject of ongoing research. Although plasma protein binding of fluoroquinolones is usually associated with albumin, there is some evidence that this group of drugs may bind to other binding proteins, including  $\alpha$ 1-acid glycoprotein (Barbato *et al.*, 2007). In calves, increase in  $\alpha$ 1-acid glycoprotein concentrations are seen over the first 30 days of life, which could explain differences noted in  $B_{\max}$  and  $K_D$  values in these two age groups (Tothova *et al.*, 2015). When unbound concentrations tend toward the number of available binding sites, protein binding becomes concentration dependent (i.e., saturable binding). Clinical differences in PK were not observed in this study. In future studies, alternative algorithms for describing the relationship between drug concentration and free fraction should be explored.

Based upon the PR and R plasma concentrations noted in this study, assessments of the potential impact of protein binding on danofloxacin PK should be limited to a range of 0.01–1.0  $\mu\text{g/mL}$ . Based upon the *in vitro* data generated within this concentration range, there were no differences in the free fraction as a function of age. For this reason, so long as the danofloxacin blood levels remain within this concentration range, age-associated differences in protein binding should have negligible influence on danofloxacin clearance of distribution kinetics.

### Clearance and volume of distribution comparisons

The CL/F values of danofloxacin did not differ significantly between ruminant and preruminant calves. Assuming bioavailability (F) was very similar, clearance values (578 mL/kg/h) determined in 11- to 13-week-old calves were similar to those reported in the current study (Sarasola *et al.*, 2002). At this concentration and dose range, the clearance does not appear to change, implying that a change in dose would not change PK parameters. Although significance was not seen when comparing 3-week-old vs. 6-month-old calves, maturation differences of clearance values in younger calves (less than three weeks of age) may impact the intrinsic clearance and elimination of danofloxacin.

In urine, unchanged danofloxacin accounted for 88–94% of total drug, while the desmethyl metabolite accounted for the remainder (Pfizer Inc, 1989a). At birth, both phase I (primarily oxidation) and phase II (conjugation) metabolic enzymes in the liver may be immature, as well as lack of functional tubular transporters in the kidneys. Maturation of different elimination pathways (including liver and kidney) may impact drug metabolism and clearance and may explain why the impact of age on the reported mean half-lives vary depending on the class of drugs studied (Reiche *et al.*, 1980; Nouws *et al.*, 1983). The increase in glomerular filtration rate (GFR) in neonates during the first few weeks of life is mainly due to an increase in renal blood flow, which would increase the clearance values of drugs eliminated by renal pathways as calves mature (Baroni *et al.*, 2008).

Statistical significance was not achieved in the age comparison of CL/F, Vd/F, or of  $t_{1/2}$  values. It is likely that the lack of statistical significance was largely attributable to study size. If the study was powered differently (contained a larger number of study subjects), statistically significant differences in these parameters may have been detected.

### Interstitial fluid/plasma ratio comparisons

The observed PK differences in neonatal vs. mature calves led to a question on how these age-related differences may influence the relative concentration vs. time profiles (free or total) in the ISF or PELF vs. plasma. With respect to the ISF, this relationship was described both by the total or free danofloxacin concentration vs. time profiles in plasma. ISF/plasma ratios were examined before and after correcting the plasma concentrations for protein binding, taking into account the nonlinearity of plasma protein binding reported in both groups of calves (Fig. 3). As the total plasma drug concentrations exceed the free drug concentrations, in plasma, the ISF/plasma ratios are greater when considering the ISF concentrations (which are free values) to the free vs. to the total plasma drug concentrations (Fig. 3). Although the depletion-phase ISF concentrations were consistently higher than the plasma concentrations for both age groups, after an initial lag time, the ISF concentrations tended to more closely follow the total plasma concentration vs. time profile in preruminants than it did in ruminants (Fig. 2).

The observation that the best fit was a two-compartment model is inconsistent with the suggested presence of a deep compartment in some ruminant calves. Upon examining the data, although there is a suggestion of a change in slope (which would be consistent with a third, or deep, compartment), there were an insufficient number of time points to adequately characterize that final depletion component. However, based upon ISF data and visual examination of the profiles, the authors believe that a deep compartment was in fact present.

Differences were noted in the shapes of the ratios of ISF/plasma in preruminants vs. ruminants as a function of time (Fig. 3). For the preruminants, the ratios peaked at 24 h but then tended to remain relatively consistent through the remaining sampling times as compared to ruminant calves. In contrast, the ratios continued to decline over the duration of sampling period in ruminant calves. There are several factors should be considered when trying to understand the observed ISF/plasma relationships:

- The method used for capturing the ISF reflects an averaging of concentrations over a collection period. Accordingly, when estimated at the time of fluid sampling, the measured concentration will exceed the actual concentration of drug in the ISF at any given moment in time. Thus, the use of ultrafiltration tends to overestimate ISF concentrations at a point in time. However, with the longer interval over which the ISF samples were collected toward the terminal portion of the curve, the 'averaging' effect would be expected to increase and not decrease the ISF/plasma ratios over time (i.e., a magnification of the experimental error). It is unlikely that this confounding factor was responsible for the observed decrease in the ISF/plasma ratios as a function of time in ruminants.
- The concept of rate-limiting factors needs to be considered. In this regard, the trend toward slower CL/F (and K10) and faster K21 values in preruminant as compared to that in ruminants can lead to a greater similarity in ISF vs. plasma concentrations in preruminant vs. ruminating calves. More explanation on calculating transfer rate differences is discussed further in the Appendix S1.
- A potential physiological basis for this difference in shapes of the ruminant vs. preruminant ISF/plasma ratio profiles may be related to the higher amount of body fat in the older animals. As danofloxacin is a lipophilic drug, it could more easily distribute into the body fat of older animals. The body fat could then serve as a 'deep compartment' whose effects would be evident primarily at the very low drug concentrations. Adipose tissue in cattle has been shown to express  $\alpha$ 1-glycoprotein, which may bind to danofloxacin in the tissues (Rahman *et al.*, 2015). This is consistent with the smaller K21 values seen in the ruminating vs. preruminating calves. When this deep compartment becomes the rate-limiting factor in the elimination of drug from the central compartment, the depletion of drug from the plasma (CP) becomes slower than was observed at earlier time points. Simultaneously, with regard to the ISF, with the K12

being similar to that of the K21 in ruminating calves, the ISF concentrations temporarily declines at a rate slightly faster than that of the blood (as was seen on the average values plotted in Fig. 1). In view of this proposed scenario, the decrease in ISF/plasma would not be as evident in the preruminants both because of slower K10 values relative to other intercompartmental rate constants and because of the lower amount of body fat would limit the influence of this potential 'deep compartment'. With these similar K12 values but smaller K21 values in ruminating vs. preruminating calves, one might expect that the danofloxacin Vd/F values would have been larger in the older calves. However, similar values were observed as a function of age (with mean values of preruminants exceeding that of the ruminating calves). It is likely that this apparent inconsistency is attributable to the magnitude of variability observed across individual calves.

#### Potential PK/PD targets

Ultimately, one of the goals of this work is to facilitate the use of plasma concentration vs. time profiles as a mechanism for generating age-associated dose adjustments. However, this assumes that preruminant and ruminating calves have identical PK/PD targets. It is now recognized that the use of targeted plasma concentrations as the PK/PD metric for dose evaluation is best when it is linked to the outcome of clinical trials (Ambrose *et al.*, 2001). However, within veterinary medicine, practitioners often rely upon prior literature-based targets and an assumption that the plasma concentrations in normal healthy animals reflect the free (active) drug concentrations at the site of infection. Therefore, as part of this study, sampling of the PELF should relate to the concentration of free (active) drug in lung fluids. Past methods for determining lung concentrations in many veterinary species include biopsies, bronchial microsampling probes and bronchoalveolar lavages (BAL), and tissue homogenate (Winther, 2012; Giguère *et al.*, 2011; Menge *et al.*, 2012; Bidgood & Papich, 2005; Davis *et al.*, 2006, 2007; Messenger *et al.*, 2012; Warren *et al.*, 2014). Tissue homogenate represents a composite of cells, plasma, interstitial, pulmonary epithelial lining fluids, and tissue concentrations, which may falsely increase the estimates of drug concentration at the specific sites of infection and lead to incorrect clinical drug distribution conclusions (Mouton *et al.*, 2008). Lung homogenate does not allow for the differentiation between intracellular vs. extracellular drug concentrations, or the degree of binding that may have occurred between drug and pulmonary tissues (Gonzalez *et al.*, 2013). However, for many veterinary species, cost and availability for many of these techniques limit their use in PK studies.

The PELF is secreted extracellularly in the respiratory tract and is a potential site for bacterial colonization, making it an ideal target for evaluating the relationship between plasma PK/PD vs. target site exposure in pneumonia in calves and for designing dosage regimens for therapeutic purposes in calves. To obtain these data, a BAL technique was used to harvest PELF. The observed higher concentrations in PELF as

compared to plasma and ISF most probably reflect methodology-associated biases in PELF danofloxacin concentrations. In this regard, several limitations with this method have been described in literature, including under- and/or overestimation of drug concentrations in this matrix. The volume of PELF harvested from the BAL fluid is usually estimated using a dilution marker such as urea. When urea is used as a dilution marker, the dwell time of fluid infused in the airways during the BAL procedure may represent an important source of error and experimental variability (Baldwin *et al.*, 1992; Dargaville *et al.*, 1999). Several other limitations include overestimating drug concentrations in PELF due to potential 'contamination' of the fluid from cells, the dilution factor caused by repeated infusion of large amounts of fluid in the airway, and the problems with using the urea correction method to adjust for this dilution. Other studies performed in larger calves showed PELF concentration measurements are inherently highly variable and affected by the method of collection.

Lung microdialysis method may offer a better overall determining free drug concentration in the PELF (Kiem & Schentag, 2008). Previous studies using microdialysis have correlated free antibiotic concentrations in lungs with unbound concentration in muscle tissue (Marchand *et al.*, 2005). Moreover, recent evaluation of PELF concentrations of ciprofloxacin and enrofloxacin in mature calves showed a PELF/plasma ratio of about 40% based upon PELF values harvested via bronchial absorptive swabs (Foster *et al.*, 2016). Nevertheless, as any error-associated bias attributable to our method of PELF sampling would be expected to equally influence the data generated in all of the study animals, conclusions derived from the comparison of danofloxacin lung concentrations of age are expected to be valid.

When comparing the mean PELF concentrations in ruminants vs. preruminants, it would appear that the concentrations tended to be markedly higher in the neonatal calves. However, when examining the individual subject data (Fig. 4), it is evident that such conclusions may be incorrect. The range of values observed in the preruminating and ruminating calves was very large, and the reliability of the means was further compromised by missing data points, particularly in the preruminant treatment group. Therefore, the PELF values should be considered as only a rough approximation of pulmonary drug concentrations. Particularly in the preruminating calves where substantial technical difficulties were encountered, it may be necessary to identify some alternative procedure for estimating active drug concentrations in the lung.

The measurement of the unbound danofloxacin in ISF and PELF is ideal as most respiratory pathogens seen in calves are extracellular bacteria. By measuring the drug concentration in the active sites of infection, a more accurate conclusion of clinical efficacy can be determined. Many studies report the use of specific target ratios using AUC and  $C_{max}$  values to determine the clinical efficacy (Tessman & Giguère, 2011). The problem with extrapolating these ratios from ruminating calves to preruminant calves is that targets to achieve efficacy may vary between the two age groups. The host immune competency as well as bioburden at the site of action may differ between these

two groups, and more research is needed to determine age effects on clinical efficacy, especially considering younger calves may not have a fully mature immune system.

A limitation in the current study is relying on the use of the statistical *t*-test to determine the significance. As preruminant calves have a larger variability, there is the possibility that there was an underlying violation of homogeneity of variances across age groups for the parameters undergoing evaluation. Furthermore, given the large variability, the power to detect statistical differences was low. In this regard, a larger sample size for each group would have helped to achieve a better study power.

## CONCLUSIONS

Trends toward differences in CL/F, rate of drug absorption, Vd/F, and  $t_{1/2}$  were observed as a function of calf age. However, the lack of study power prohibited the detection of statistically significant differences. A deep or third compartment, similar to previous studies, was detected and was most evident in the ruminating calves. This contributed to age-associated differences between the relationships of ISF/plasma over time.

The ability to quantify drug concentrations out to 96-h post-dose and the successful utilization of *in vivo* ultrafiltration and BAL techniques provided the opportunity to obtain unique insights into potential factors responsible for age-associated differences in danofloxacin PK in calves. Although efforts to explain the source of these differences remain speculative, it has identified additional questions that need to be resolved. Specifically, if an objective is to use data derived from ruminating calves to support approvals in preruminating animals, it is evident that further examination is needed to determine whether there are differences in the impact of disease on drug PK and the relationship between drug exposure vs. response. In addition to clinical reasons for resolving these issues, the alteration of drug PK as a function of age and disease could impact withdrawal time considerations.

There are limited data in veterinary species addressing pediatric-specific PK data, and these results raise several questions about the physiological changes that may impact PK in animals of various age, as well as disease state. Further studies are warranted to compare concentrations in PELF and ISF of diseased vs. healthy calves and the impact of these relationships on drugs presenting with distributional characteristics that differ from those associated with the fluoroquinolones.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Appendix S1.** Graphs: Simulated/Observed ISF/plasma ratios and the relationship between  $C_P$  and  $C_T$ .

**Figure S2.** Compartmental parameter rate estimates in the individual ruminant and preruminant calves.

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