

Perioperative pharmacokinetics and pharmacodynamics of meloxicam in emus (*Dromaius novaehollandiae*) of different age groups using nonlinear mixed effect modelling

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Abstract

Meloxicam is a widely used nonsteroidal anti-inflammatory drug in avian species. However, variability in pharmacokinetic (PK) and pharmacodynamic (PD) parameters in birds warrants species-specific studies for dose and dosing interval optimization. We performed a perioperative PK study of meloxicam (0.5 mg/kg, intravenously) on emus of three different age groups: 3 chicks (5 weeks old, 3.5 kg), 4 juveniles (26 weeks old, 18.8 kg) and 6 adults (66 weeks old, 38.8 kg). A two-compartment population PK model including weight as a significant covariate on clearance and central volume of distribution (V1) best fitted the data. The typical values (20 kg bird) for clearance and V1 were 0.54 L/kg/h and 0.095 L/kg. Both parameters significantly decreased with increasing weight/age. Meloxicam potency and selectivity for COX-1 and COX-2 were measured in whole blood assays (TxB₂ production endpoint). Meloxicam was partially selective in emus (IC₅₀ COX-1:COX-2 = 9.1:1). At the current empirical dose (0.5 mg/kg/24 hr), plasma meloxicam concentration is above IC₅₀ of COX-2 for only 2 hr. PK/PD predicted dose required for 80% COX-2 inhibition over 24 hr were 3.4, 1.4 and 0.95 L/kg/day in chicks, juveniles and adult emus, respectively. The safety, therapeutic efficacy and practicality of modifying the daily dose or dose interval should be considered for dose recommendations in emus.

KEYWORDS

anaesthesia, analgesia, birds, NonSteroidal Anti-Inflammatory Drugs, whole blood assay

1 | INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are an important component of pain management in veterinary species, with meloxicam being widely available in clinical practice. They are indicated as antipyretics, analgesics and anti-inflammatories for both short- and

long-term use. These therapeutic benefits of NSAIDs relate to their inhibition of prostaglandin endoperoxide synthase (cyclooxygenase, COX). These eicosanoids function with other mediators to cause various physiological and pro-inflammatory effects (Kumar et al. 2009).

Two main isoforms of COX exist. COX-1 is constitutively present in many healthy tissues and contributes to physiological functions

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including gastroprotection, renal protection and haemostasis. Inhibition of this isoform by NSAIDs relates to their adverse effects of gastric ulceration and renal toxicity (Herschman, 1996; Warner et al., 1999). COX-2 is an inducible isoform expressed in response to injury or inflammation and is associated with the therapeutic benefits of NSAIDs. However, COX-2 has physiological effects on salt and water excretion and therefore COX-2 selectivity does not signify renal safety (Horl, 2010; Weir, 2002). Increased understanding of the differing roles of COX-1 and COX-2 has prompted a need for research into the selectivity of existing NSAIDs and the development of COX-2 specific NSAIDs.

There are marked species differences in the expression and activity of COX, conferring variation in selectivity, potency and safety of NSAIDs (Brideau et al., 2001). This can result in sub-optimal dose and dose frequency when choosing a dose empirically in veterinary species. For effective NSAID therapy, pharmacokinetic (PK) and pharmacodynamic (PD) studies performed on the target species with specific NSAIDs are required to ensure adequate analgesia with minimal side effects (Lees et al., 2004). There is minimal research into the PD and PK of NSAIDs in birds (Goessens et al., 2016; Houben et al., 2016), and none published with emus as the target species. A study by Baert and De Backer (2003) investigated the PK of three NSAIDs in five bird species noted the large variability in plasma clearance and half-life of meloxicam between species, with ostriches clearing the drug especially rapidly. As a similar large ratite species, emus may exhibit similar PK of meloxicam and the empirical dose reported may be too low in this species.

Variability of PK parameters between individuals can result in differing plasma concentration–time courses despite administration of the same dose per unit body weight. This is due to intrinsic variation within a population of factors including genetics, disease status, age and concurrent drug therapy (Buclin et al., 2009). Within paediatric populations, there are notable differences in physiology compared to adults, which alter PK. These included increased relative size of the liver and kidneys and therefore clearance, as well as varying body fat and extracellular fluid composition resulting in increased volume of distribution (Rane, 1992). Hepatic maturation also brings changes in enzyme expression and activity which can result in altered metabolite production (Batchelor & Marriott, 2015). These differences should be considered when adjusting doses for paediatric individuals as scaling dose directly by weight may not be appropriate. In juvenile animals, similar changes in PK parameters have been reported, which can be linked to maturational changes in kidney and liver function, as well as in body water/fat composition (Gasthuys et al., 2017; Millecam et al., 2019). In this respect, growing conventional piglets have been recently studied as surrogate large animal model in paediatric drug research (Millecam, et al., 2019).

This study hypothesizes that there are significant differences in the PK of meloxicam between emus of three different age groups and the empirical dosage regime may be too low in this species. This will be achieved through the following objectives: (a)

investigate the effect of age on the main parameters after a single IV bolus by performing PK analysis of meloxicam for the three age groups under general anaesthesia, (b) compute the potency of meloxicam against COX-1 and COX-2 from whole blood assays through PD analysis and (c) calculate the duration of COX-2 and COX-1 inhibition and define an ideal dosage regime and anaesthetic protocol in this species.

2 | MATERIALS AND METHODS

2.1 | Study population & anaesthetic

The emus in this study were anaesthetised during a biomechanical ratite study for surgical implantation of bone strain gauges and electromyogram wires (Cuff et al., 2019; Main & Biewener, 2007). The study was approved by the Royal Veterinary College Clinical Research (CRERB approval number: CR2018-006-2) and Ethics and Welfare Research boards. It was carried out under the Animal (Scientific Procedure) Act (PPL70/7122).

Thirteen emus (*Dromaius novaehollandiae*) in three age groups were sourced from a UK farm population. The chicks ($n = 3$) were aged 4–6 weeks, weighing 3.45 kg (range 3.35–3.5 kg); the juveniles ($n = 4$) were aged 24–28 weeks, weighing 18.75 kg (17–20 kg); and the adults ($n = 6$) were aged 64–68 weeks, weighing 38.75 kg (36–42 kg). The emus were premedicated with xylazine (3 mg/kg for juveniles and adults, 1.5–3 mg/kg for chicks (Chanazine®, 10%, ChanneleAnimal Health Ltd) and ketamine (15–20 mg/kg, Ketaset®, 100 mg/ml, Fort Dodge Animal Health) by intramuscular injection into the epaxial lumbar musculature. General anaesthesia was induced by mask with 4%–5% isoflurane (Isoflo® 100% w/w, Zoetis UK Ltd) in oxygen. After tracheal intubation, general anaesthesia was maintained with isoflurane in oxygen to effect. Butorphanol was administered intravenously at 0.5 mg/kg (Torbugesic®, 10 mg/ml, Fort Dodge Animal Health) via a right jugular catheter placed after induction (Triple-lumen 5.5Fr jugular catheter, Arrow, Teleflex Medical Europe Ltd). For this study, 0.5 mg/kg meloxicam (Metacam®, 5 mg/ml solution for injection for dogs and cats, Boehringer Ingelheim Ltd, UK) was administered intravenously at time zero. The dose was empirical due to lack of data in this species (Baert & De Backer, 2003).

2.2 | Pharmacokinetics of meloxicam (perioperative)

2.2.1 | Blood sampling

Blood samples were collected for anaesthetic monitoring from another lumen of the right jugular catheter in heparin tubes prior to meloxicam administration ($t = 0$) and at predetermined times following ($t = 2, 5, 10, 20, 40, 60, 90, 120, 180, 240$ min) or until the end of surgery. The total sampled volume did not exceed 10% of each individual's blood volume. Samples were centrifuged (2,400 g for 5 min) to separate plasma and stored at -80°C until analysis.

2.2.2 | Determining plasma concentration of meloxicam

The method for determining plasma concentrations of meloxicam using validated high-pressure liquid chromatography with ultra-violet detection (HPLC-UV) was based on Baert and De Backer (2003). Sample preparation involved addition of 100 µl plasma to 100 µl methanol, 250 µl water, 25 µl internal standard (piroxicam, 10 µg/ml in methanol), 150 µl 0.1 M hydrochloric acid and 5 ml diethylether. Samples were extracted for 20 min, then centrifuged at 2,400 g for 5 min, the organic layer was transferred and evaporated under nitrogen at 40°C. The remaining residue was re-dissolved in 200 µl of the mobile phase (80 water/20 acetonitrile, v/v) and vortexed, and 50 µl was injected into the HPLC-UV system (see Appendix 1), with a mobile phase consisting of 0.1% acetic acid in water and acetonitrile in gradient elution. The flow rate was 0.5 ml/min. This method was validated prior to analysis. A calibration curve was created by spiking blank plasma with known concentrations of meloxicam, ranging from 0.075 to 5.0 µg/ml. Selectivity was demonstrated by injecting blank emu plasma samples not containing meloxicam. The limit of quantification (LOQ) was 75 ng/ml. The within-run and between-run accuracy and precision fell within specified ranges (Council Directive 96/23/EC, 2002; VICH, 2012).

Where concentrations fell below the LOQ of the HPLC-UV method, samples were re-run using liquid chromatography-tandem mass spectrometry (LC-MS/MS). A UHPLC system was coupled to a Xevo TQ-S triple quadrupole mass spectrometer (see Appendix 1). Mobile phase comprised 0.01% formic acid in water and 0.01% formic acid in acetonitrile. A gradient elution was performed on a Hypersil Gold column (50 x 2.1 mm i.d., dp: 1.9 µm) with a precolumn of the same type. The mass spectrometer operated with positive electrospray ionization and multiple reaction monitoring (MRM) was performed. Transitions monitored were m/z 332.30 (precursor ion) → 95.00 (product ion) for piroxicam and m/z 352.30 → 115.00 for meloxicam. Retention times were 2.29 min for meloxicam and 2.07 min for piroxicam. The limit of quantification was 10 ng/ml.

2.2.3 | Pharmacokinetic analysis

We have used the principle of nonlinear mixed effect modelling for the analysis of this unbalanced dataset (6/ 4/ 3 birds) (Bon et al., 2018). The coding of covariates in the model resulted a single PK model supported by the data from 13 richly sampled emus, from 3 age categories.

General model

Pharmacokinetic analysis was performed with Phoenix NLME 8.1 (Certara USA). All concentrations below the LOQ of the LC-MS/MS were excluded from analysis (8 out of 96 samples). Noncompartmental analysis (NCA) was used to compute the

mean standard PK parameters for each age group (Gabrielsson & Weiner, 2012) and estimate the effect of the age categories on clearance (CL) and volume of distribution (V).

Pharmacokinetic modelling using nonlinear mixed effect identified the optimal base model and assessed variability between individuals. One- and two-compartment models were compared using the maximum likelihood estimation objective function value (OFV), expressed as the negative sum of the log of the likelihoods (-2LL), to rank models by goodness of fit. A multiplicative residual error was used for fitting plasma concentration data. As the two-compartment population PK model had a significantly lower -2LL, this model was used to assess the effect of covariates on parameters. Random effects were added progressively to the model for PK parameters until no further reduction of the -2LL could be obtained while keeping eta(η)-shrinkage of the basic model around 20% maximum. A model with random effect on CL, V and V2 fitted the data the best.

Covariate model

Weight was explored as a continuous covariate to explain the differences in CL and V between the three age groups. Weight was used instead of age as it was accurately measured for each individual and would allow greater distribution of data within groups. The model with the lowest -2LL with minimal compromise of η-shrinkage was with CL and V adjusted by the covariate weight, according to following parametrization (average weight 20 kg) in Equations (1) through (4):

$$CL = tvCL * \left(\frac{weight_{individual}}{weight_{average}} \right)^{dCLdweight} * \exp(\eta_{CL}) \quad (1)$$

$$V = tvV * \left(\frac{weight_{individual}}{weight_{average}} \right)^{dVdweight} * \exp(\eta_V) \quad (2)$$

$$CL2 = tvCL2 \quad (3)$$

$$V2 = tvV2 * \exp(\eta_{V2}) \quad (4)$$

where tv = typical value of the parameter for the population, η = individual's deviation from the typical value. The covariate terms $dCL_{dweight}$ and $dV_{dweight}$ adjust the population typical value CL and V to account for weight.

The final two-compartment population PK model with weight as a covariate for CL and V was simulated with 1,000 iterations in a visual predictive check (VPC) plot (diagnostic plots in Appendix 3).

2.3 | Pharmacodynamics of meloxicam in emu blood

2.3.1 | Whole blood assays

The method for performing the whole blood assay in emus was adapted from Giraudel et al. (2005). At the conclusion of the biomechanics study, blood was collected from 4 adult emus immediately

after euthanasia in heparinized tubes (20 Unit/ml), then divided into 500 µl aliquots to be used for either the COX-1 or COX-2 assay.

For the COX-1 assay, following incubation at 37°C for 1.5 hr, aliquots were spiked with concentrations of meloxicam 0.02–500 µM or saline as a positive control. After 4 hr, 50 µM calcium ionophore (A23187, Sigma Aldrich Co Ltd, Dorset, UK) was added to induce platelet activation and eicosanoid generation, in particular thromboxane (Tx)_{B₂}. Four hr later, the reaction was terminated by addition of 800 µM meclofenamic acid (Sigma Aldrich) to inhibit any secondary COX stimulation and chilling to 4°C. Plasma was separated off by centrifugation prior to freezing.

In the COX-2 assay, aliquots were pretreated with 10 µg/ml aspirin in dimethyl sulfoxide (Sigma Aldrich) to inhibit COX-1 (Giraudel et al., 2005) and incubated at 37°C for 1.5 hr. Samples were spiked with concentrations of meloxicam from 0.005–200 µM or saline as a positive control. After 0.5 hr, lipopolysaccharide (LPS *Escherichia coli* serotype O26:B6, Sigma Aldrich) was added to samples to 100 µg/ml to induce COX-2 activity in blood monocytes. The reaction was terminated at 8 hr as for the COX-1 assay.

2.3.2 | Measurement of eicosanoids in plasma by LC-MS/MS

The following eicosanoids were analysed in the plasma samples, 13,14-dihydro-15-keto-prostaglandin(PG)A₂, PGB₂, PGD₂, 6-keto-PGF_{1α}, 6,15-diketo-13,14-dihydro-PGF_{1α}, PGF_{2α} and derivatives (15-keto-PGF_{2α}, 13,14-dihydro-15-keto-PGF_{2α}), PGE₂ and derivatives (15-keto-PGE₂, 13,14-dihydro-15-keto-PGE₂ or PGEM, bicyclo-PGE₂), prostacyclin derivatives (6-keto-PGF_{1α}, 6,15-diketo-13,14-dihydro-PGF_{1α}), PGJ₂ and derivatives (15-deoxy-D12,14-PGJ₂, D12-PGJ₂) and Tx_{B₂} and derivative (11-dehydro-TxB₂) (Welsh et al., 2007).

Samples were prepared by transferring 50 µl of plasma to an extraction tube, adding 25 µl internal working solution (25 ng/ml), 25 µl methanol and 150 µl of water. Samples were vortexed with 25 µl 0.1 M hydrochloric acid. 3 ml n-hexane/ethyl acetate (1/1, v/v) was added for extraction over 25 min. Following centrifugation for 10 min at 1,800 g, the supernatant was transferred and evaporated under a nitrogen stream (40 ± 5°C). The dry residue was re-dissolved in 125 µl of methanol in water (1/9, v/v) and filtrated using a Millex PVDF 0.22 µm syringe filter, then transferred to an autosampler vial. 10 µl aliquots were injected onto the same LC-MS/MS system

as mentioned above. Chromatography was performed on an Acquity UPLC® BEH C18 column (50 × 2.1 mm i.d., dp: 1.7 µm), using a gradient elution with 0.05% acetic acid in water (A) and acetonitrile (B) as mobile phases. The flow rate was 0.3 ml/min. The mass spectrometer was operated in the positive electrospray ionization mode, and two MRM transitions were monitored per component, as shown in Appendix 2. The method was validated, and the following parameters were evaluated: linearity, within-run precision and accuracy, between-run precision and accuracy, limit of quantification and carry-over and specificity. The limit of quantification was between 0.2 and 0.5 ng/ml, depending on the prostanoid.

2.3.3 | Pharmacodynamic modelling

Inhibition of Tx_{B₂} production by meloxicam was used as a marker of both COX-1 and COX-2 activities and expressed as percentage of the control value (meloxicam concentration = 0 µM). Four curves for COX-1 inhibition of Tx_{B₂} and one of COX-2 inhibition could be used for PD modelling of the four birds, using Phoenix NLME.

COX-1 inhibition was initially modelled as a naïve average of the 4 birds with additive residual error with the Hill equation (Equation 5):

$$I = I_0 + \frac{I_{\max} * C^\gamma}{IC_{50}^\gamma + C^\gamma} \quad (5)$$

Where I = percentage inhibition of COX-1 activity compared with control, I₀ = baseline inhibition, I_{max} = maximal possible inhibition, IC₅₀ = concentration of meloxicam producing 50% inhibition of COX-1 activity, C = total concentration of meloxicam and γ (gamma) = slope of the concentration–effect curve. A population-based PD model with additive residual error using a Laplacian algorithm was used to investigate inter-individual variability. Models with random effect on single and multiple parameters were compared with identify the one with the lowest –2LL and shrinkage. Simulation with 500 iterations of the final model was performed to produce a VPC plot (diagnostic plots in Appendix 4).

As only one curve of COX-2 inhibition was produced, this curve was modelled without random effect, using an I_{max} model with additive residual error. Models were compared with varying γ and with γ equal to 1. The simple I_{max} model was selected as it had the lowest Akaike Information Criterion (AIC).

Parameter	Chicks (n = 3)	Juveniles (n = 4)	Adults (n = 6)	Statistical significance
CL (L/kg/hr)	1.406** [1.23–1.53]	0.43 [0.38–0.46]	0.37** [0.26–0.63]	p = .014**
V (L/kg)	0.212* [0.174–0.360]	0.107 [0.092–0.190]	0.112* [0.047–0.140]	p = .047*

Note: The pharmacokinetic parameter estimates were determined by noncompartmental analysis. Data presented as median [min–max]. Figures with the same superscripts indicate significant differences (Kruskal–Wallis test).

TABLE 1 PK parameters of meloxicam in emus of three different age groups (Chicks n = 3, Juveniles n = 4, Adults n = 6) after administration of a single IV bolus of 0.5 mg/kg

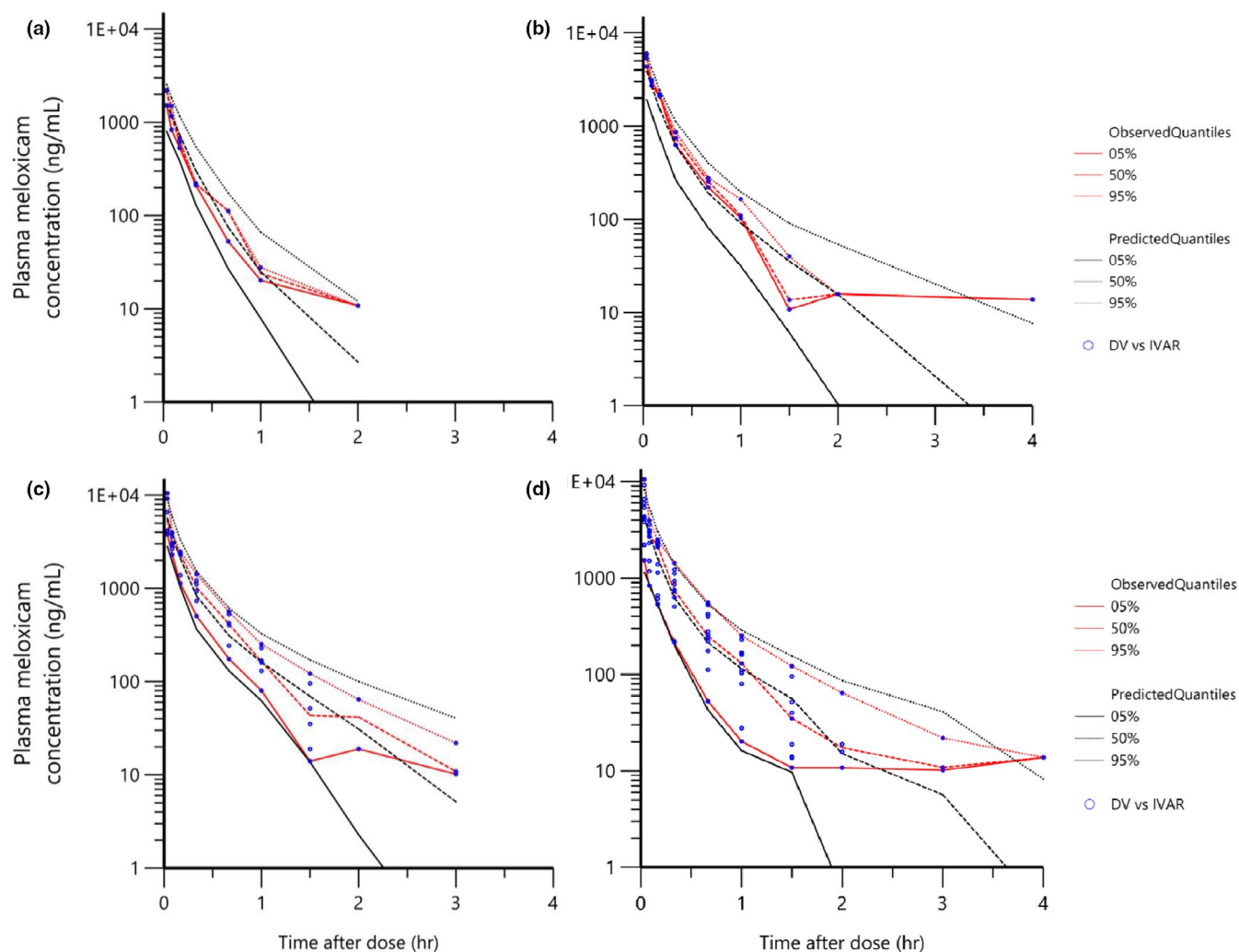


FIGURE 1 Plasma concentration ($\mu\text{g/L}$) versus time (hr) profiles describing the pharmacokinetics of meloxicam after a single IV bolus (individual observations as blue dots and 5, 50, 95% observed percentiles) stratified visual predictive check (5, 50, 95% predicted percentiles) obtained after 1,000 iterations from the final 2-compartment population PK model (Visual Predictive Check). Top left: chicks, top right: juveniles, bottom left: adults, bottom right: all birds [Colour figure can be viewed at wileyonlinelibrary.com]

2.4 | Statistics

The statistical significance of differences between PK parameters computed by NCA in each age group was tested with a Kruskal-Wallis test ($p < .05$) using GraphPad Prism (version 8.1.1, GraphPad Software, La Jolla California, US).

3 | RESULTS

3.1 | Pharmacokinetics of meloxicam

3.1.1 | Noncompartmental analysis

Mean PK parameters determined by NCA are summarized in Table 1. Meloxicam CL and V (per unit body weight) were significantly higher in chicks than adult emus ($p < .05$).

3.1.2 | Population-based two-compartmental PK model

Figure 1 describes the relationship between meloxicam concentration (observed and fitted) and time in different age groups (VPC plot obtained with 1,000 iterations). It was not possible to predict beyond 2 hr for the chicks and beyond 3 hr for the adults due to lack of data above the LOQ. Hence, the predictions beyond 3 hr are based solely on the data from juveniles and may not be representative of the population. All birds cleared the drug relatively rapidly, with chicks having the lowest area under the curve (AUC) due to an especially high CL.

Table 2 describes PK parameters obtained by the population-based two-compartment model investigating the effect of the covariate weight on CL and V. Inter-individual variability (IIV) could be reported for CL, V and V2, where it was the highest (IIV = 58%).

Table 2 here.

The covariate body weight had a significant effect on CL and V. By replacing the parameters in the Equations 1 and 2 with the values

Parameter	tv	CV%	[95% CI]	IIV	η Shrinkage
tvCl (L/kg/hr)	0.541	6.9		14%	19.8%
tvV (L/kg)	0.095	14.8		20%	38.6%
tvCl2 (L/kg/hr)	0.165	31.2		–	–
tvV2 (L/kg)	0.059	12.0		58%	17.4%
Covariate weight on Clearance	–0.506	–10.5	[–0.61 to –0.40]	–	–
Covariate weight on Volume	–0.555	–19.1	[–0.77 to –0.34]	–	–
Stdev0	0.291	7.5		–	–

Note: CL clearance, V central volume of distribution, CL2 inter-compartmental clearance and V2 peripheral volume of distribution. Parameters are expressed as the population typical value (tv), coefficient of variation (CV%), (95% CI) Confidence interval of the covariate, Inter-individual variation (IIV) and η -shrinkage. Stdev0 = residual error.

TABLE 2 PK parameters of meloxicam in emus as determined by a two-compartment population pharmacokinetic model with first order elimination with Body Weight as a covariate

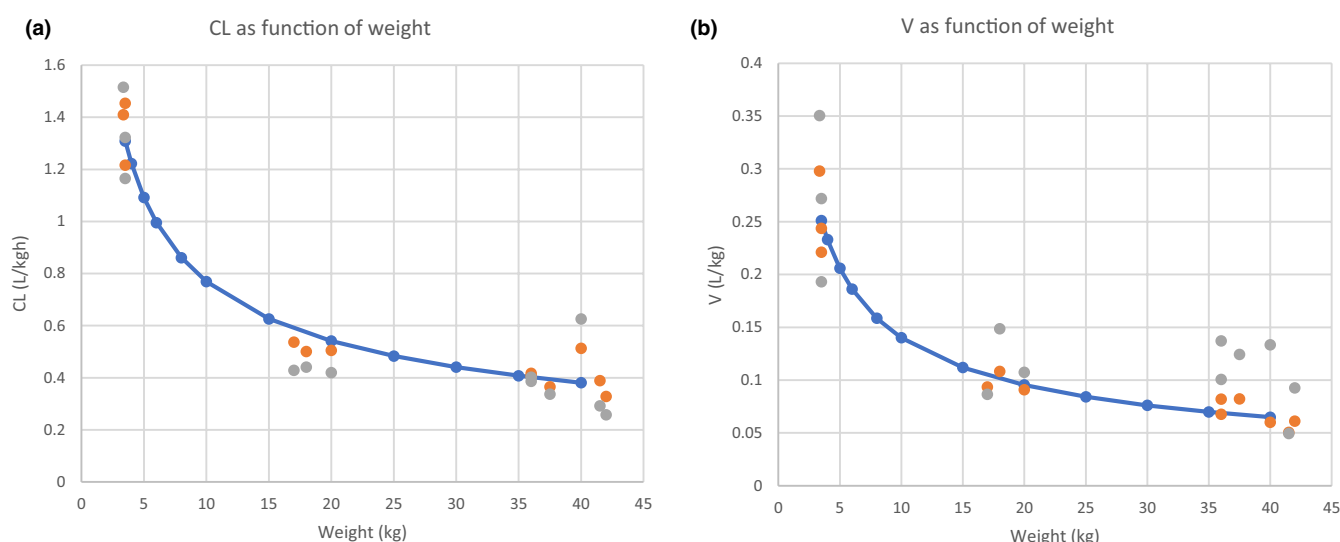


FIGURE 2 Relationships between meloxicam PK parameters and weight as predicted by population-based two-compartment PK modelling. Figure 2a (left) clearance of meloxicam and emu weight. Figure 2b (right) central volume of distribution of meloxicam and emu weight [Colour figure can be viewed at wileyonlinelibrary.com]

obtained in this model, Equations (6) and (7) describe the effect of body weight on CL (L/kg/hr) and V (L/kg) per unit weight:

$$CL = 0.541 * \left(\frac{\text{weight}}{20} \right)^{-0.506} \quad (6)$$

$$V = 0.095 * \left(\frac{\text{weight}}{20} \right)^{-0.555} \quad (7)$$

Individual clearances and central volume of distributions were plotted against weight range of the study population, as seen in Figure 2. The average CL (1.33 L/kg/h) and V (0.254 L/kg) were highest in the chicks (weight range: 3.35–3.5 kg) compared to the average CL (0.386 L/kg/h) and V (0.067 L/kg) in adults (weight range: 36–42 kg). In the juveniles, CL (0.563 L/kg¹) and V (0.10 L/kg) were intermediate between the extreme ages. As these parameters are

described per unit body weight, the absolute value in L/hr for CL and L for V can be calculated by multiplying by an individual's weight.

3.2 | Pharmacodynamic analysis

Pharmacodynamic parameters describing inhibition of Tx_{B2} production by COX-1 and COX-2 by meloxicam are summarized in Table 3, and concentration–inhibition curves are represented in Figure 3. Concentration–effect relationship (or lack of) for other prostanoid as summarized in Appendix 2.

The efficacy of meloxicam for COX-2 inhibition ($I_{\max} + I_0$) only reached 83.93% compared with 95.78% for COX-1. The models used produced a negative value of I_0 compared with the control (meloxicam concentration = 0 μ M) for both COX-1 and COX-2, indicating that at very low concentrations of meloxicam some Tx_{B2} stimulation had

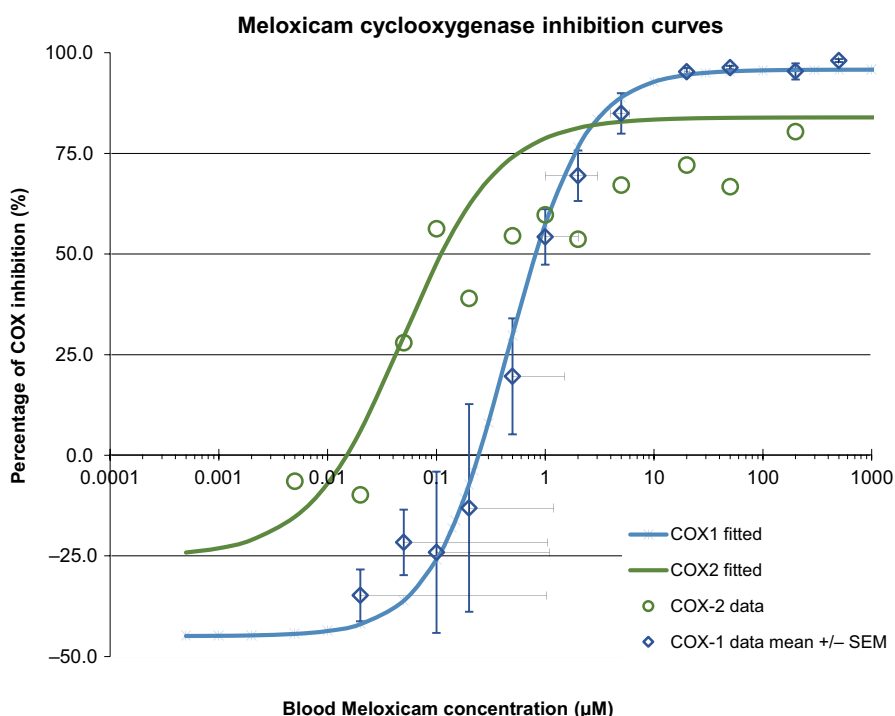
TABLE 3 Summary of pharmacodynamic parameters of COX-1 and COX-2 inhibition by meloxicam by whole blood assay. COX-1 results were obtained from a population-based PD model with additive residual error with random effect on IC_{50} and I_0 ($n = 4$). COX-2 result results were obtained from a simple I_{max} model with additive residual error with γ (slope gamma) equal to 1 ($n = 1$)

Parameter	COX-2			COX-1			IIV	Shrinkage
	Units	Estimate	CV%	Estimate	CV%			
tvI_0	%	-25.2	-1.4	-44.9	-6.9			
tvI_{max}	%	109.2	0.38	140.7	2.2			
$tvIC_{50}$	(μM)	0.0499	0.5	0.452	34.4	65%	9%	
tvGamma	scalar	1	(fixed)	1.23	26.9	53%	7%	
stdev0		11.8	0.05	12.9	18.8			
-2LL		93.254		399.441				
AIC		101.254		417.441				

Note: I = percentage inhibition of COX-1 activity compared with control, I_0 = baseline inhibition, I_{max} = maximal possible inhibition, IC_{50} = concentration of meloxicam producing 50% inhibition of COX-1 activity, C = total concentration of meloxicam and gamma = slope of the concentration-effect curve. Stdev0 = residual error. IIV inter-individual variation and η -shrinkage eta-shrinkage (random effect). -2LL: negative sum of the log of the likelihoods

AIC: Akaike information criterion

FIGURE 3 Sigmoid curves of percentage inhibition COX-1 (blue diamonds for observed average % inhibition $\pm$ SEM from 4 birds and continuous blue line for fitted regression) and COX-2 (green circle and regression line) by meloxicam [Colour figure can be viewed at wileyonlinelibrary.com]



occurred. This effect was similarly noted by Giraudel, et al. (2005) for meloxicam and carprofen and by Kay-Mugford et al. (2000) for meloxicam, tolafenamic acid, carprofen and ketoprofen. The percentage inhibition was therefore rescaled (0%–100%), and the total meloxicam concentrations converted from μM to $\mu g/ml$ to calculate selectivity indices of meloxicam for COX-1 versus COX-2 (Table 4), as in Giraudel et al. (Giraudel, et al., 2005). Meloxicam was COX-2 selective with values of IC_{50} COX-1 of 158 $\mu g/ml$ and IC_{50} COX-2 of 17.5 $\mu g/ml$, yielding a ratio IC_{50} COX-1/COX-2 of 9:1. As the two inhibition curves were not parallel due to the higher sensitivity of meloxicam for COX-1 versus COX-2 ($\gamma = 1.232$ COX-1 and $\gamma = 1$ for COX-2), the ratios of COX-1:COX-2 inhibition were largest at IC_{20} and smallest at IC_{80} . Other IC ratios have been included to assess

clinically relevant levels of inhibition in terms of balancing therapeutic efficacy (high inhibition of COX-2, ideally more than 80%) with safety (low inhibition of COX-1, ideally less than 20%).

3.3 | Dose computation

The method for evaluating the empirical dose of meloxicam (0.5 mg/kg) was adapted from Giraudel, et al. (2005) using results of the PK/PD analysis. As PD values obtained with a whole blood assay account for drug protein binding, in vitro inhibitory concentrations are comparable with in vivo therapeutically relevant concentrations. When predicting doses using plasma clearance from PK analysis, a

TABLE 4 Indices describing selectivity of meloxicam in emus for COX-1 versus COX-2 as determined by interpolation of inhibition curves using the Hill equation $I = I_0 + (I_{\max} * C^{\gamma}) / ((I_{50}^{\gamma} + C^{\gamma}))$ using values obtained from PD analysis

Indices	COX-1 (µg/L)	COX-2 (µg/L)	Ratio COX-1:COX-2
IC ₂₀	51.5	4.4	11.8:1
IC ₅₀	158.4	17.5	9.1:1
IC ₈₀	486.7	70.1	6.9:1
IC _x :IC _y (COX1:COX2)			
IC ₁ :IC ₉₉			0.002:1
IC ₅ :IC ₉₅			0.092:1
IC ₁₀ :IC ₉₀			0.169:1
IC ₂₀ :IC ₈₀			0.735:1
% inhibition of COX-1 (0%–100%)			
for 50% COX-2			6.2%
for 80% COX-2			26.8%
for 90% COX-2			49.9%
for 95% COX-2			71.4%
for 99% COX-2			95.1%

Note: IC_x: concentration of meloxicam producing x% inhibition

multiplying factor of 1.5 was used to convert to whole blood clearance (emu blood haematocrit averaged 33.5%). Predicted doses were calculated using Equation (8) and displayed in Table 5:

$$\frac{\text{Dose}}{\text{Dosing interval}} = \frac{\text{plasma clearance} * 1.5 * \text{target concentration}}{F} \quad (8)$$

TABLE 5 Dose extrapolation of meloxicam from PK and PD results. IC₈₀ COX-2 as determined by PD analysis. Plasma clearance used from population PK two-compartment model. Whole blood C_{max} from plasma C_{max} from NCA divided by 1.5 (plasma/blood conversion ratio). Percentage inhibitions were extrapolated from rescaled sigmoid curves. Time above IC_x estimated from concentration–time curves in Figure 5 and expressed as time for the 50% quantile of the population (5% quantile – 95% quantile). Range to >180 indicates the time upper quantile was greater than 3 hr and therefore not determinable from the adult emus' concentration–time graph

Parameters	All birds	Chicks	Juveniles	Adults
IC ₈₀ COX-2 (µM) whole blood assay	0.2	0.2	0.2	0.2
IC ₈₀ COX-2 (µg/L) whole blood target	105.1	105.1	105.1	105.1
Predicted daily dose for 80% inhibition COX-2 (µg/kg/24hr)	1,365	3,375 (6.7 times 500 µg/kg)	1,440 (2.9 times 500 µg/kg)	949 (1.9 times 500 µg/kg)
Empirical dose (µg/kg)	500	500	500	500
Corresponding plasma C _{max} (µg/L) empirical dose	4,994	1976	5,228	6,388
Corresponding blood C _{max} (µM)	9.5	3.8	9.9	12.2
% inhibition COX-1 at C _{max}	97.7	93.2	97.8	98.3
% inhibition COX-2 at C _{max}	99.5	98.7	99.5	99.6
Time above IC _x with empirical dose				
Time above IC ₅₀ COX-2 (min)	120 (60–216)	72 (48–108)	114 (72–192)	138 (84 - >180)
Time above IC ₈₀ COX-2 (min)	84 (36–144)	42 (30–60)	72 (45–105)	90 (54–144)
Time above IC ₁₀ COX-1 (min)	108 (48–96)	60 (42–96)	102 (66–165)	126 (75 - >180)
Time above IC ₂₀ COX-1 (min)	42 (108–198)	48 (33–72)	78 (48–126)	105 (66–165)

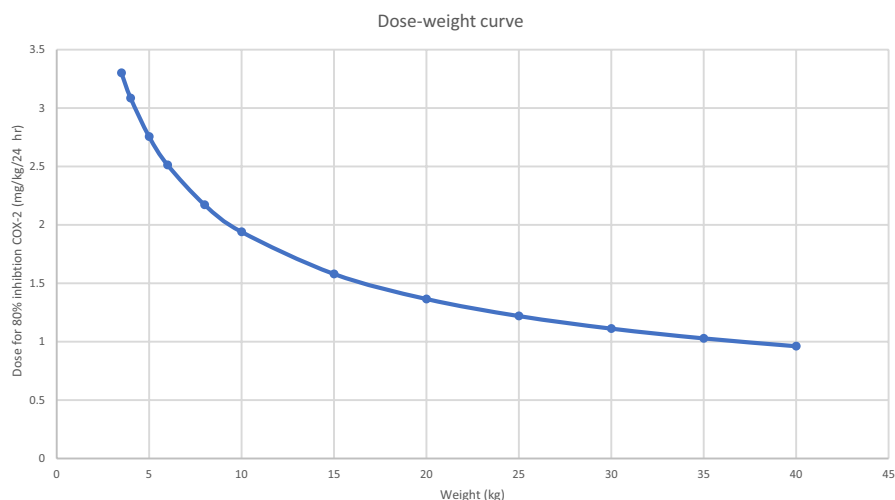
Where the target concentration is IC_{x%} COX-1 or COX-2 and F (bio-availability) = 1. The current empirical dose (0.5 mg/kg/24 h) in emus is too low as COX-2 inhibition is only above 50% for an average of 2 hr (72 min in chicks versus 138 min in adults). Predicted doses required for 80% inhibition of COX-2 are between 1.9 (adults) and 6.7 (chicks) times higher than the empirical dose. Using the relationship between weight and clearance (Figure 2), Figure 4 illustrates predicted doses for 80% inhibition of COX-2. For birds of all ages, COX-1 inhibition up to 20% for between 48 min (chicks) and 105 min (adults). No doses have been evaluated for safety of in vivo dosing in emus.

4 | DISCUSSION

This study determined the PK parameters of meloxicam in emus (CL = 0.54 L/kg/h, V = 0.095 L/kg, for a typical 20kg bird) and demonstrated significant species differences to comparable studies (Table 6). The clearance was similar to ostriches, another larger ratite species. Clearance was high compared with other bird species except for red-tailed hawks. While volume of distribution in emus is not as high as in ostriches, it is similarly higher than in mammalian species (not showed in Table 6). The large variability in PK between avian species is one of the main issues with analgesic therapy in birds as cross-species allometric scaling is not reliable and therefore PK/PD analysis is required in every species (Baert & De Backer, 2003).

The emus in this study were under general anaesthesia, and concurrent drugs may have an effect on PK parameters. The alpha 2-adrenergic agonist xylazine used in this study's anaesthetic protocol may have altered hepatic blood flow. The presence

FIGURE 4 PK/PD informed dosage regimen (mg/kg/24 hr) and adjustment with increasing weight to achieve an average plasma concentration corresponding to 80% inhibition of COX-2 [Colour figure can be viewed at wileyonlinelibrary.com]



of an α_2 -adrenergic agonist can reduce clearance by 50% (Bennett et al., 2017; Gozalo-Marcilla, et al., 2019; Gozalo-Marcilla et al., 2019). This may be a confounding factor causing the increased clearance in chicks, as two out of the three received half the xylazine dose (1.5 mg/kg versus 3 mg/kg) of the rest of the study population. While the chick receiving the 3 mg/kg xylazine dose had the lowest clearance of all chicks (1.22 L/kg/h), its clearance however remained much higher than one of the juvenile and adult emus, suggesting that xylazine dose was not a major influencing factor in this case.

Pelligand et al. (2016) and Karademir et al. (2016) demonstrated, for two NSAIDs (robenacoxib in cats and meloxicam dogs, respectively), an effect of anaesthesia on volume of distribution but no significant effect on clearance. The elimination half-life of meloxicam in dogs (12.7 h) extended well beyond the duration of the anaesthesia (29 min surgery) in Karademir et al. (2016). Hence, robenacoxib in cats is a better comparator to meloxicam in birds because (a) most of the drug was eliminated in the perioperative period and a lesser proportion during recovery and (b) protein binding is equally high (unknown for meloxicam in birds but 97% in dogs and cats, and > 98% for robenacoxib in cats).

From well-stirred venous equilibration model (Benet & Hoener, 2002):

$$CL_{hepatic} = \frac{Q_{hepatic} \cdot f_u \cdot Cl_{int}}{Q_{hepatic} + f_u \cdot Cl_{int}}$$

Because the renal contribution to the systemic clearance of robenacoxib is negligible in cats (2% excreted as unchanged), and assuming this is the same in birds, we can also write the following:

$$CL = \frac{Q_{hepatic} \cdot f_u \cdot Cl_{int}}{Q_{hepatic} + f_u \cdot Cl_{int}}$$

Considering the cardiac output of 37.5 kg Emu at rest 67.9 mL/kg/min (Grubb et al., 1983), the extraction ratio (CL/ cardiac output) of meloxicam in emus was $E_{\text{meloxicam}} = 6.45 / 67.9 = 0.095$ (present adult Emus clearance 0.387 L/kg/h). This is between a low clearance

($E < 0.05$) and medium clearance ($E = 0.15$), as per Toutain and Bousquet-Melou (2004).

For drugs exhibiting low extraction ($Q_{hepatic} \gg f_u \cdot Cl_{int}$), the hepatic clearance can be approximated by the product of the unbound fraction and the intrinsic clearance as follows:

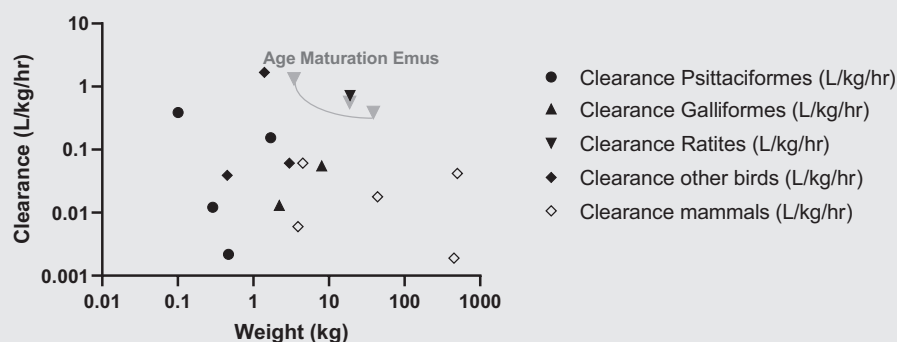
$$CL \propto f_u \times Cl_{int}$$

Hepatic NSAID elimination is therefore perfusion-independent and only dependent on the (a) free fraction of NSAID within the plasma and (b) the intrinsic enzyme activities of the liver (Anderson & Holford, 2007). For the first (a), enzyme activities will change with emu maturation, but not because of anaesthesia provided that normothermia is maintained. For the second (b), anaesthesia may change the free fraction. However in these circumstances, and as described by Benet and Hoener (Benet & Hoener, 2002), the free steady state concentration which is pharmacodynamically active has no dependency upon the free fraction. Consequently, potential changes in protein binding due to anaesthesia would have little impact on the effect of meloxicam for postoperative pain relief medication in emu.

Chicks had significantly higher clearance and volume of distribution per unit weight than juvenile and adult emus (Table 6), suggesting that scaling doses solely on body weight could result in incorrect dosing in young emus. This concept is seen in paediatric human medicine where doses are scaled with an allometric weight exponent and a maturation function accounting for age (Germovsek et al., 2017) due to physiological changes affecting drug distribution, metabolism and excretion. Changes in body composition with age, in particular decreasing extracellular water content, result in higher volumes of distribution of hydrophilic drugs such as meloxicam in infants. Plasma protein concentrations are lower in neonates, resulting in less protein binding in plasma and greater tissue penetration, further increasing the initial volume of distribution. This is likely to have transient effect on meloxicam free concentration due to its high degree of plasma protein binding (>99.5%) (Turck et al., 1996), which is a limitation of the current study.

TABLE 6 Comparison of pharmacokinetics of meloxicam in difference species, after intravenous (IV) administration. CL = clearance

Species/Order	Route (weight)	CL (L/kg/hr)	Author
Birds			
Emu (chicks)	<i>Dromaius novaehollandiae</i> Casuariiformes, IV (3.45 kg)	1.32	Present study
Emu (juveniles)	Ratites IV (18.75 kg)	0.559	
Emu (adults)	IV (38.75 kg)	0.387	
Pigeon	<i>Columba livia domestica</i> Columbiformes IV (0.45 kg)	0.039	Baert and De Backer (2003)
Duck	<i>Anas platyrhynchos</i> Anseriformes IV (3.0 kg)	0.061	
Turkey	<i>Meleagris gallopavo</i> Galliformes IV (8.0 kg)	0.055	
Ostrich	<i>Struthio camelus</i> Struthioniformes, Ratites IV (19 kg)	0.72	Lacasse et al. (2013)
Chicken	<i>Gallus gallus</i> Galliformes IV (2.2 kg)	0.013	
Red-tailed hawk	<i>Buteo jamaicensis</i> Accipitriformes IV (1.4 kg)	1.675	
Great horned owl	<i>Bubo virginianus</i> Strigiformes IV (1.7 kg)	0.154	Montesinos et al. (2017)
African grey parrot	<i>Psittacus erithacus</i> Psittaciformes IV (0.47 kg)	0.00218	
Hispaniolan Amazon parrots	<i>Amazona ventralis</i> Psittaciformes IV (0.29 kg)	0.0122	
Cockatiels	<i>Nymphicus hollandicus</i> Psittaciformes IV (0.101 kg)	0.388	Dhondt et al. (2017)
Mammals			
Camel	<i>Camelus bactrianus</i> IV (450 kg)	0.0019	Wasfi et al. (2012)
Piglet	<i>Sus scrofa</i> IV (4.5 kg)	0.061	Fosse et al. (2008)
Cat	<i>Felis catus</i> IV (3.9 kg)	0.006	Giraudel, et al. (2005)
Goat	<i>Capra aegagrus hircus</i> IV (43.9 kg)	0.0179	Ingvast-Larsson et al. (2011)
Horses	<i>Equus caballus</i> IV (500 kg)	0.042	Lees et al. (1991)

Meloxicam clearance (all species)

NB: the CL for the 3 emu ages are represented in grey inverted triangles, with an arrow indicating the direction of maturation with ageing

Meloxicam is metabolized to inactive metabolites by cytochrome P450 isozymes and oxidation, which are then excreted in faeces and urine (Grude et al., 2010). Hepatic metabolism is affected by the increased relative size of the liver in neonates resulting in increases hepatic blood flow relative to body weight and therefore clearance (Strolin Benedetti et al., 2005). Maturation of enzyme expression and activity also affects clearance to a variable degree (Koukouritaki et al., 2004). Renal clearance is increased with increased relative size of the kidneys in neonates; however, as less than < 0.25% of meloxicam is excreted as its active compound, this will not have significantly affected clearance in chicks (Batchelor & Marriott, 2015).

Selectivity of NSAIDs for COX-1 and COX-2 varies between species and assays used (Schmid et al., 2010). The ratio IC_{50} COX-1:COX-2 in emus was 9.1:1, similar to that in dogs (value of 7.3:1 from (King et al., 2010)), indicating meloxicam is COX-2 preferential in this species. Whole blood assays most closely represent in vivo physiological conditions and are most useful at predicting clinical relevance. IC_{20} COX-1: IC_{80} COX-2 indicates selectivity relating to clinical use, essentially a ratio of therapeutic efficacy to side effects. This was 0.735:1 in the emus in this study, higher than that in dogs (0.46:1) and cats (0.25:1) (King et al., 2010; Schmid et al., 2010). Two limitations of the COX-2 assay were that that only one curve was

produced, and the best signal obtained was from a TxB_2 assay. In mammalian assays, PGE_2 conventionally indicates COX-2 activity; however, no PGE_2 was detected in the COX-2 assay in this study. This may be due to PGE_2 playing a less significant role in avian inflammation than in mammals, as indicated by Golemboski et al. (1990) and Fraifeld et al. (1995), and therefore, a different assay may be required (using TxB_2 for example). While both COX-1 and COX-2 genes are encoded within the emus genome, their roles in the inflammatory response remain uncertain (Altschul et al., 1997) and there is a lack of information on inflammation and biomarkers in avian species.

In a urate synovitis model (Anhut et al., 1979), the PGD_2 response was higher in chickens synovial fluid compared with PGE_2 , but the PGE_2 response was still measurable (and more substantial than the TxB_2 one). In our COX-1 assay, PGE_2 was also produced and measured (though not in quantities as high as TxB_2), but not in the COX-2 assay. In the validation of our ex vivo assay, PGE_2 could however be measured in the absence of aspirin (either with or without LPS, corresponding to a COX-1 contribution), but could not be measured on addition of aspirin. It is possible that the LPS stimulation did not activate COX-2 in all birds at the doses used (100 $\mu\text{g}/\text{ml}$) or with this specific LPS (*Escherichia coli* serotype O26:B6). From de Boever et al., (2010), in vivo intravenous administration of LPS in chicken (1.5×10^6 units/kg or 2.5 mg/kg *E. coli* O127 B8) produced a blood PGE_2 response that peaked at 1.5 h and lasted a couple of hours. This response could be significantly suppressed by ketoprofen, but this does not rule out an in vivo effect of LPS on COX-1 predominantly compared with COX-2.

NSAIDs have variable safety in avian species with diclofenac associated with significantly high mortality in vultures due to carcass consumption (Swan et al., 2006). High dosing with NSAIDs is associated with intestinal lesions, degenerative bone changes and renal impairment (Palocz et al., 2016). The results obtained indicate the current empirical dose of 500 $\mu\text{g}/\text{kg}/24$ hr will inhibit 50% COX-2 for 2 hr or less and therefore is too low. The predicted therapeutic dose required for 80% COX-2 inhibition for 24 hr was 3.375 $\mu\text{g}/\text{kg}$ in chicks, down to 949.3 $\mu\text{g}/\text{kg}$ in adult emus. While meloxicam is the safest NSAID in birds, raising the dose to the level predicted in this study may result in adverse side effects (Cuthbert et al., 2007). A dose of 1 mg/kg has been recommended for African Grey and Hispaniolan Amazon parrots and may be more appropriate for adult emus (Hawkins et al., 2018). Therapeutic analgesia in emus could be achieved through a single higher IV dose, repeated IV administration, a longer-acting subcutaneous formulation or using a multi-modal approach including opioids and nonopioids. The safety and practicality of this should be considered in particularly with younger emus, due to their higher dose requirements.

5 | CONCLUSION

Meloxicam is a widely available analgesic with safety demonstrated in many avian species; however, doses used are often empirical due

to lack of data in individual species. This may lead to inadequate analgesia due to the high degree of variability of PK and PD in different veterinary species. This study demonstrates that emus clear meloxicam more rapidly, have a higher volume of distribution than many mammalian species and display similar PK parameters to ostriches. This difference in drug exposure (AUC) was lower in younger emus than in adults, which may be due to weight and developmental stage or due the anaesthetic protocol used. Our PD analysis using the first whole blood assay generated in birds, further indicates the empirical dose used was too low in this species for once daily dosing and requires revision.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

AUTHOR CONTRIBUTION

The authors contributed to this study as follows: LP contributed to conceptualization, project administration, overall supervision, data modelling and resources (task shared with LA); DC, LL and LP were involved in vivo studies; SdB and SC were involved in measurement of meloxicam and biomarker concentrations using HPLC-UV and LC-MS/MS at their analytical laboratory facilities, LA was involved in data interpretation, pharmacokinetic modelling and wrote the manuscript together with DC and LP. All authors have read and approved the final manuscript. The author also thanks Prof. John Hutchinson and Prof. Russel Main for sharing their experiences and biomechanical study design.

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APPENDIX 1

Analytical equipment sources

Equipment	Source
HPLC-UV system: Model P1000XR pump with vacuum degassing AS3000 autosampler UV6000LP diode array detector set at 355nm Hypersil GOLD column & pre-column (dp: 5 µm, 100 × 3 mm i.d.)	ThermoFischer Scientific, Breda, The Netherlands Thermo Fisher Scientific, Merelbeke, Belgium
LC-MS/MS system: Xevo TQ-S triple quadrupole mass spectrometer Hypersil Gold column (50 × 2.1 mm i.d., dp: 1.9 µm) Acquity UPLC® BEH C18 column (50 × 2.1 mm i.d., dp: 1.7 µm)	Waters, Zellik, Belgium Thermo Fisher Scientific, Merelbeke, Belgium Waters, Zellik, Belgium

APPENDIX 2

MRM transitions and MS/MS parameters for the prostanoids under investigation

Analyte	Precursor ion (m/z) ^a	Product ions (m/z)	CE ^b (eV)	Cone (V)	Retention time (min)
6-keto-PGF _{1α}	369.10	163.00 ^c	25	25	1.87
		245.10	25	25	
(6-keto-PGF _{1α} -d4)	373.10	167.00	25	25	1.86
		249.10 ^c	25	25	
6,15-diketo-13,14-dihydro-PGF _{1α}	369.10	223.00 ^c	22	25	2.67
		267.00	22	25	
TXB ₂	369.10	168.90 ^c	15	25	2.54
		195.00	15	25	
(TXB ₂ -d4)	373.00	173.00 ^c	16	25	2.53
		199.00	13	25	
PGF _{2α}	353.14	111.02	28	40	3.03
		193.01 ^c	25	40	
(PGF _{2α} -d4)	357.10	197.00 ^c	19	25	3.00
		313.10	25	25	
PGE ₂	351.16	189.04	25	25	3.40
		271.08 ^c	15	25	
(PGE ₂ -d4)	355.16	275.05	15	25	3.37
		319.22 ^c	10	25	
11-dehydro-TXB ₂	367.10	161.10	17	25	3.61
		305.10 ^c	15	25	
(11-dehydro-TXB ₂ -d4)	371.10	165.10	17	25	3.59
		309.10 ^c	15	25	
15-keto-PGF _{2α}	351.16	219.03	18	25	3.75
		289.16 ^c	20	25	
PGD ₂	351.16	189.04	25	25	3.90
		271.08 ^c	15	25	
(PGD ₂ -d4)	355.16	275.05	15	25	3.88
		319.22 ^c	10	25	
15-keto-PGE ₂	349.11	112.97 ^c	20	25	4.19
		287.08	15	25	
13,14-dihydro-15-keto-PGF _{2α} (PGFM)	353.14	112.97	28	40	4.90
		183.05 ^c	25	40	

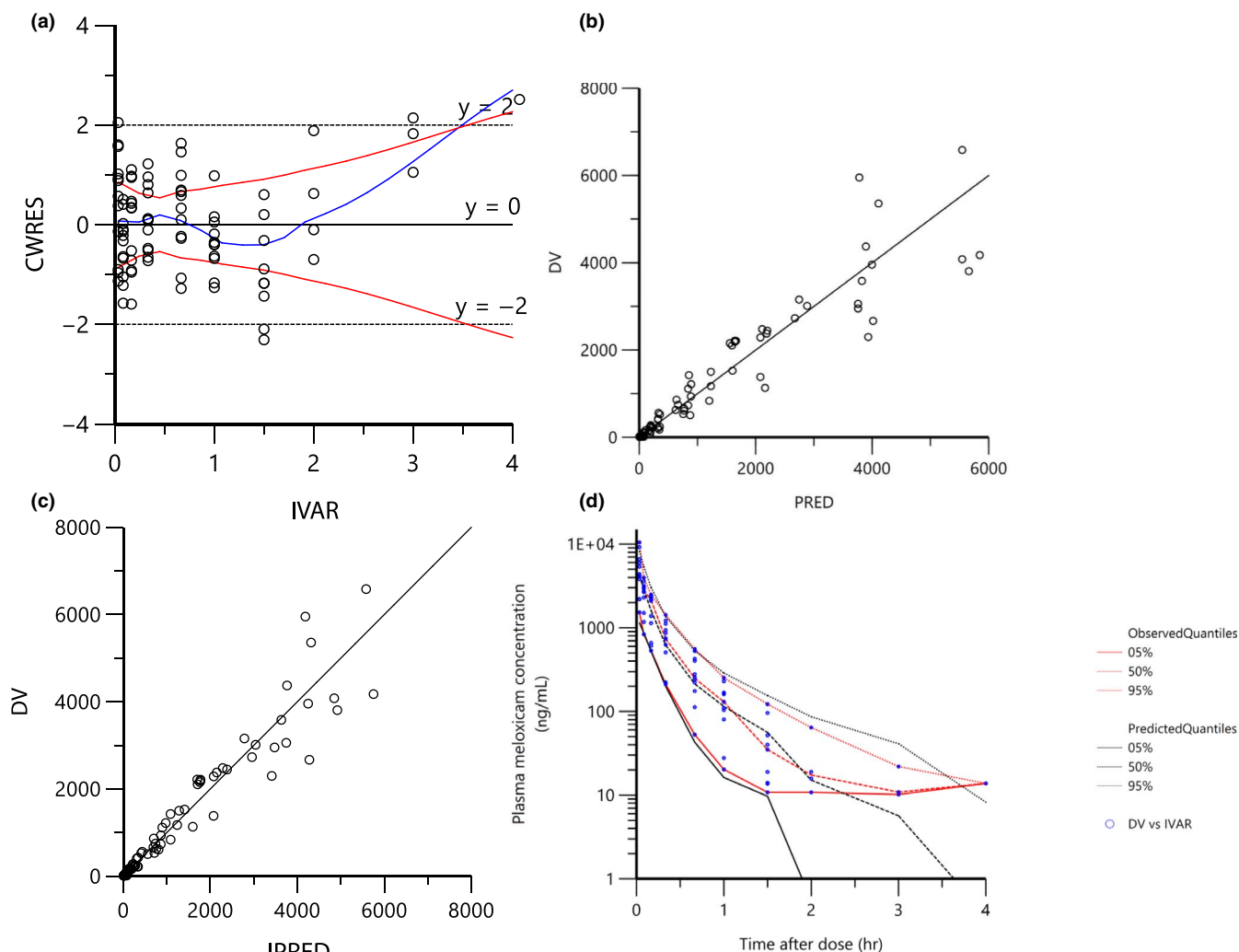
Analyte	Precursor ion (m/z) ^a	Product ions (m/z)	CE ^b (eV)	Cone (V)	Retention time (min)
13,14-dihydro-15-keto-PGE ₂ (PGEM)	351.12	108.88	25	25	5.12
		113.10 ^c	25	25	
PGJ ₂	333.12	189.00 ^c	16	25	7.55
		271.10	14	25	
PGB ₂	333.12	174.98	20	25	7.95
		315.10 ^c	17	25	
(PGB ₂ -d4)	337.20	239.00	21	30	7.88
		319.20 ^c	19	30	

^c ion used for quantification. For the COX-1 assay, PGF_{2α} and to some extent PGE₂ were also measured and suppressed by meloxicam. In one Emu, PGFM, BC-PGE₂, 6-keto-PGF_{1α}, 11-dehydro-TXB₂, PGB₂ and Δ-12-PGJ₂ were measurable. For COX-2, TxB₂ was measured in 3 birds (1 curve exploitable), PGF_{2α} was measurable in 2 birds. For both assays, PGEM was positively correlated with meloxicam concentration ($R^2 = 0.9360$) and initially shared the same m/z ratio until the setup was refined. None of the other prostaglandins or metabolites ever reached quantifiable levels.^a m/z = mass to charge ratio. ^b CE = collision energy.

APPENDIX 3

Diagnostic plots of PK analysis of meloxicam in Emu of different age groups after administration of 0.5 mg/kg intravenously

Panel A: Conditional weighted residuals (CWRES) versus IVAR (time), panel B: population DV (concentration) versus population prediction (PRED), panel C: population DV versus individual predictions (IPRED), panel D: population predictive check of observed and predicted quantiles



APPENDIX 4

Diagnostic plots of PD analysis of COX-1 inhibition of TXB2 production (n = 4 birds)

Panel A: Conditional weighted residuals CWRES versus population prediction (PRED) for COX-1, panel B: popDV versus population predictions (PRED) for COX-1, panel C: popDV versus individual predictions (IPRED) for COX-1, panel D: population visual predictive check (VPC), observed and predicted quantiles for COX-1 inhibition

Equations describing PD model:

$$I = I_0 + \frac{I_{\max} * C^{\gamma}}{(IC_{50}^{\gamma} + C^{\gamma})}$$

where I = percentage inhibition of COX-1 activity compared to control, I_0 = baseline inhibition, $I_{\max}$ = maximal possible inhibition, IC_{50} = concentration of meloxicam producing 50% inhibition of COX-1 activity, C = total concentration of meloxicam and γ (gamma) = slope of the concentration-effect curve.

$$IC_{50} = tvIC_{50} * \exp(\eta IC_{50})$$

$$\gamma = tv\gamma * \exp(\eta\gamma)$$

$$I_0 = tvI_0 I_{\max} = tvI_{\max}$$

