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Palatability of flunixin and pharmacokinetics when administered to sheep through feed

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Applying analgesics to feed is a potentially easy method of providing pain-relief to sheep and lambs that undergo painful husbandry procedures. In order for sheep to consume medicated feed it needs to be know if the medication has an adverse odour or flavour that may affect consumption. It is also important to determine if therapeutic concentrations of a non-steroidal anti-inflammatories (NSAIDs) can be achieved when administered to sheep as a feed supplement. Pelleted feed was supplemented with flunixin (4.0mg/kg liveweight) and administered to eight sheep, which they were able to consume over a 12 h period. Blood samples were taken over 48 h and plasma drug concentrations were determined using Ultra High Pressure Liquid Chromatography. The mean time required to reach maximum concentration was 6 ± 1.46 h and ranged from 1 to 12 h. Average maximum plasma concentration was 1.78 ± 0.17µg/mL and ranged from 1.61 to 2.80 µg/mL. The average half-life of flunixin was 7.95 ± 0.77 h and there was a mean retention time of 13.62 ± 1.17 h. Sheep did not show aversiveness to pellets supplemented with flunixin. When consuming medicated feed ad libitum all sheep were able to obtain inferred therapeutic concentrations of flunixin in plasma within 6 h. Provision of flunixin in the feed may provide a practical way to provide pain relief to sheep and lambs following painful husbandry procedures removing the need for multiple injections, reducing handling stress and minimising labour requirements.

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Palatability of flunixin and pharmacokinetics when administered to sheep through feed[1]

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ABSTRACT:

Applying analgesics to feed is a potentially easy method of providing pain-relief to sheep and lambs that undergo painful husbandry procedures. In order for sheep to consume medicated feed it needs to be know if the medication has an adverse odour or flavour that may affect consumption. It is also important to determine if therapeutic concentrations of a non-steroidal anti-inflammatories (NSAIDs) can be achieved when administered to sheep as a feed supplement. Pelleted feed was supplemented with flunixin (4.0mg/kg liveweight) and administered to eight sheep, which they were able to consume over a 12 h period. Blood samples were taken over 48 h and plasma drug concentrations were determined using Ultra High Pressure Liquid Chromatography. The mean time required to reach maximum concentration was 6 ± 1.46 h and ranged from 1 to 12 h. Average maximum plasma concentration was 1.78 ± 0.17μg/mL and ranged from 1.61 to 2.80 μg/mL. The average half-life of flunixin was 7.95 ± 0.77 h and there was a mean retention time of 13.62 ± 1.17 h. Sheep did not show aversiveness to pellets supplemented with flunixin. When consuming medicated feed ad libitum all sheep were able to obtain inferred therapeutic concentrations of flunixin in plasma within 6 h. Provision of flunixin in the feed may provide a practical way to provide pain relief to sheep and lambs following painful husbandry procedures removing the need for multiple injections, reducing handling stress and minimising labour requirements.

Key words: Flunixin, Pharmacokinetics, Sheep, Oral administration, Pain relief

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42

43 **INTRODUCTION**

44 Flunixin meglumine is a potent non-steroidal anti-inflammatory (NSAID) that is commonly used
45 in veterinary medicine for its anti-inflammatory, analgesic and antipyretic activity. Like other
46 NSAIDs, flunixin reduces inflammation by inhibiting cyclooxygenase and, in turn, decreasing
47 the production of prostaglandin (Cheng et al. 1998b), an important inflammatory mediator.
48 Flunixin is known to be effective at relieving pain in various domesticated species such as horses
49 (Keegan et al. 2008; Toutain et al. 1994) and cattle (Currah et al. 2009) and is currently
50 registered for use for these animals in the US, Europe and Australia (Feely et al. 2002). Although
51 flunixin has also been shown to be effective for pain relief in sheep (Paull et al. 2007; Welsh
52 1995), there are currently no registered NSAIDs in Australia for use in sheep. Pain relief can be
53 logistically difficult and costly to administer to livestock raised in extensive systems due to
54 feasibility of repeated application overtime and availability of registered drugs is limited
55 (Lizarraga & Chambers 2012). A potential practical method of providing pain-relief is through
56 oral administration, allowing farmers to either provide NSAIDs as a drench or through feed in
57 the form of granules or a liquid formulation. It is known that the rumen can decrease the
58 bioavailability of NSAIDs following oral administration (Mosher et al. 2012; Odensvik 1995),
59 consequently in previous work, the dose of NSAIDs required when administered orally in cattle
60 has been double that recommended for parenteral dosing (Coetzee et al. 2012). If flunixin was
61 added to feed, there is the potential for animals to display a neophobic reaction or reduced
62 consumption of feed if flunixin is unpalatable. Therefore the objectives of this study were 1) to

test the palatability of flunixin and 2) determine the pharmacokinetics of flunixin in sheep when feed containing flunixin was offered ad libitum. We hypothesised was that all sheep would achieve therapeutic concentrations of flunixin in plasma when consuming feed supplemented with flunixin.

MATERIALS AND METHODS

Experimental animals

Nine, 2-year-old, maiden Merino ewes with an average liveweight of 38.8 ± 0.9 kg were used in this study. The sheep were housed in individual pens in a covered shed which was open North facing and were in close proximity to allow visual and social interaction with other experimental animals. Animals were fed a complete pelleted ration (Ridley Agriproducts, Australia; 17% crude protein dry matter; 9.04 MJ/kg dry matter) ad libitum (approx. 800-1000g) and given 100 g of oaten chaff daily and provided water ad-libitum. The experiment was undertaken at CSIRO's FD McMaster Laboratory, Armidale, New South Wales (NSW). The protocol and conduct of the experiment was approved by The CSIRO Armidale Animal Ethics Committee under the NSW Animal Research Act, 1985 (ARA 14/01).

Palatability test

One week prior to the experiment commencing, animals were acclimatised to eating from two troughs and daily food intake was recorded. The palatability test was run for 2 days; in the morning sheep were offered feed in two troughs, one containing 2 kg of the standard animal house pelleted ration and one with 2 kg of the same standard animal house pellet supplemented with 20 mL (200 mg) of liquid flunixin (Flunixin Oral solution, 15mg/mL, Pia Pharma Pty Ltd, Gladesville, NSW, Australia). The amount of flunixin added per kg of feed was equivalent to an

approximate single dose for the live weight of the ewes (i.e. eating 1 kg of feed with flunixin would give them 1 dose). The feed was prepared each morning by mixing the liquid flunixin into the pellets by hand; even incorporation of the liquid was noted by the change in colour of the pellets. Both troughs were placed into the pen at the same time and the locations of the troughs were swapped for the second day of testing.

Pharmacokinetic protocol

After the palatability test, the ewes were kept in a paddock for a 2-week flush-out period. They were then returned to the same individual pens that they were in for the palatability test, 1 week prior to the commencement of the pharmacokinetic experiment. The sheep were again fed the complete pelleted ration ad libitum and 100 g of oaten chaff once a day. The day prior to supplementation of feed with flunixin, sheep were weighed and had the wool clipped from their necks. To allow for intensive blood sampling, catheters were inserted aseptically in the jugular vein using a 12 G catheter needle to puncture the vein. A piece of catheter tubing was then threaded through the needle, the line flushed with heparinised saline and then liquid withdrawn till blood was flowing visibly to ensure the catheter was inserted correctly. Catheters were then re-flushed with heparinised saline. The catheter needle was removed and the line was sealed with a three-way tap adaptor containing a leur lock syringe port. The line was secured to the animal at the exit point with Elastoplast tape, the remaining catheter tubing was then encased in 7.5 cm wide Elastoplast bandage which was gently wrapped around the sheep's neck.

On the day of the study, sheep were offered 800 g of feed containing a dose of flunixin (at a rate of 4.0 mg/kg live weight) adjusted for each animal's body weight. Flunixin was added to feed as described for the palatability test. The first sheep was presented with the medicated feed at 0700

h and the remaining sheep were given their medicated feed at 2 min intervals thereafter. Blood samples (10mL) were collected before the medicated feed was offered (0 h) and at 5, 10, 15, 20, 30, 45 min and 1, 2, 4, 6, 8, 12, 24, 36, 48 h after each sheep was observed consuming the medicated feed. Prior to taking each blood sample, a small volume of blood was withdrawn from the catheter and discarded to ensure fresh blood was collected for each sample. Blood samples were centrifuged (2000 × g) and separated plasma collected and frozen at -20^{o}C immediately after collection. Residual feed remaining in the feed bin was weighed at each blood sampling time point until 24 h post-initial ingestion.

Plasma flunixin concentration determination

Plasma samples were transported frozen to Pia Pharma Pty Ltd, Gladesville, NSW for flunixin concentration determination using an Ultra High Liquid Chromatography Tandem Mass Spectrometry (UHPLC-MSMS).

Each plasma sample was thawed to room temperature on the day of analysis. For determination, a 250 µL aliquot of each plasma sample was dispensed into a 2mL polypropylene centrifuge tube .Internal standard, flunixin-d3 internal standard (50µL of 2.0 µg/mL flunixin-d3) was added and the sample mixed gently prior to addition of 350 µL acetonitrile. The sample was vortexed (1 min) and centrifuged (13000 rpm/5 min) to remove any sediment. Water (0.5 mL) was added to the extract and the mixture then filtered through a 0.45 µm filter prior to determination. An aliquot of sample extract (5µL) was injected into an Eksigent® Ekspert™ ultraLC 100-XL Liquid Chromatograph fitted with a Supelco Ascentis® Express 50x2.1 mm, 2.7 µm analytical column maintained at 40 °C. A gradient elution program, based on a combination of 0.1 % formic acid and acetonitrile as mobile phase constituents operating at 0.4 mL min-1, resolved

129 flunixin and flunixin-d3 (retention time of 2.5 min) from matrix interferences and endogenous
130 sample components. The identity of peaks was predicted using an AB Sciex API 3200 triple-
131 quadrupole mass spectrometer was interfaced with the liquid chromatograph. The detector was
132 configured with a proprietary turbo V source for desolvation and operated in negative
133 electrospray ionisation (-ve ESI) mode (-4500 V), desolvation temperature 550 °C, for optimum
134 analyte selectivity and sensitivity. The transitions for flunixin and flunixin-d3 were
135 295.1→191.0, 298.2→254.0 respectively.
136 Matrix matched calibration standard solutions of flunixin were prepared at incremental
137 concentrations between 10 and 4000 ng/mL in plasma from animals prior to treatment. The
138 calibration curve was prepared by plotting the nominal flunixin concentration (x axis) against the
139 determined peak area ratio of flunixin and flunxin-d3 for each calibrator. A correlation co-
140 efficient (r) greater than 0.99 was required for the calibration curve to be used for quantitative
141 purposes. Analyte concentrations were calculated using the peak area ratio of flunixin detected in
142 each sample relative to the corresponding flunixin-d3 internal standard, and the regression
143 equation of the calibration curve.

144 Method accuracy and precision were monitored with the inclusion of fortified quality control
145 samples. Four plasma samples containing flunixin concentrations of 13.1, 328.5, 1314.1, 3942.3
146 ng/mL (n=3) were prepared on the day of the analysis. The mean percentage of accuracy was
147 90.8% at LLOQ and 102.9 – 111.6 % at all other concentrations. The Coefficient of variation at
148 LLOQ was 2.9%, and 1.3-3.1% at other concentrations. Quality control data were acceptable.
149 ***Statistics***

150 Palatability data was analysed with R (RStudio, Boston, Massachusetts) using nlme (Pinheiro et
151 al. 2015) to perform a linear mixed effects model. Fixed effects included in the analysis model

included feed type (flunixin present or absent), day (1 or 2), and location of medicated feed trough (left or right) and the interaction of feed type by day. Sheep number was fitted as a random effect. One ewe was excluded from data analysis as she did not consume any of the feed containing flunixin on either day. Data were tested for normality using the Shapiro-Wilk test. $P < 0.05$ was considered statistically significant.

Pharmacokinetic analysis

Pharmacokinetic modelling of flunixin in plasma was performed using an open source pharmacokinetic program (PK Solver, China Pharmaceutical University, Nanjing, Jiangsu, China) (Zang et al. 2010). Using non-compartmental analysis, the maximum flunixin concentration (Cmax) in plasma, the time required to reach Cmax (Tmax), mean residence time (MRT) and elimination half-life (t1/2) were determined. The area under the concentration vs. time curve (AUC0–t) was calculated using the linear trapezoidal rule. All parameters were calculated as an overall average as well as for each individual animal.

RESULTS

Palatability

Location of the different feeds (left or right trough) had no effect on the amount of each feed (flunixin treated versus untreated) that was consumed. Although overall there was no feed type effect (P=0.10), a trend was observed for the day by feed type interaction (P = 0.08), with animals consuming on average 551 ± 218 g more of the untreated feed than feed containing flunixin (P=0.02) on day 1. On day 2, sheep ate significantly less untreated feed compared to their consumption on day 1 (P=0.03, -490 ± 218g). However, on day 2 there were no differences observed in the consumption of untreated feed and feed containing flunixin (Figure 1).

Pharmacokinetics

Pharmacokinetic parameters for plasma flunixin in individual animals and the group average are shown in Table 1. The plasma concentration time curve of flunixin averaged across all sheep plasma is shown in Figure 2. All sheep started to eat within a few minutes of being offered feed. There was large variability between sheep in the amount of feed that was consumed at each time-point (Figure 3). This led to a large variability in the Tmax, which ranged from 1 to 12 h. The Cmax average was 1.8 ± 0.2 µg/mL and the flunixin meglumine plasma $t_{1/2}$ was 7.95 ± 0.77 h.

It took between 8 and 12 h for all sheep to consume the total 800 g of feed. Most of the sheep spread consumption of the feed throughout the day except for ewe 466 who ate 350 g of feed in the first 5 min and ewe 627 who consumed 332.5 g in the last 4 h of the first day. Flunixin was absorbed rapidly, all sheep had detectable plasma concentrations (>20 ng/mL) at 10 min after initial consumption of supplemented feed with the exception of one animal (ewe 627), who only ate 21.5 g of feed in the first 10 min.

DISCUSSION

When consuming feed ad-libitum, the majority of sheep (7 out of 8) achieved plasma flunixin concentrations above 1.0 µg/mL within 2 h of consuming medicated feed, with maximum concentrations (between 1.33 and 2.80 µg/mL) being reached on average by 6 h. Concentrations observed in this current study were somewhat less to those reported in our previous study (Marini et al. 2015) where flunixin concentration in plasma reached between 2.6 - 4.1 µg/mL 2 h after a single oral dose (4mg/kg) in sheep. Reports of therapeutic concentrations of flunixin in farm animals are limited, however, Toutain et al. (1994) reported therapeutic effects in horses when plasma concentrations reached 0.2-0.9 µg/mL. We can infer therefore that in the current

196 study, that therapeutic concentrations of plasma flunixin were observed following consumption
197 of medicated feed.

198 Although displaying an initial preference for untreated pelleted feed over flunixin-treated feed on
199 the first day, there were no overall feed preference effects observed. The initial preference of
200 untreated pelleted feed may have been due to the novelty of the odour or flavour of flunixin.
201 Odour and flavour help sheep distinguish food types and they are more likely to eat novel food
202 types that contain familiar flavours (Hinch et al. 2004; Launchbaugh et al. 1997). Sheep are
203 known to initially avoid new feed types taking several days before they start to consume a feed
204 to which they have not been previously exposed (Chapple et al. 1987). Having added flunixin to
205 a feed with which the ewes were familiar may have reduced neophobia. With the exception of
206 one ewe who did not consume any feed containing flunixin over the two days, the consumption
207 of treated and untreated feeds was similar on the second day of testing.

208 In sheep, the pharmacokinetics of flunixin has been investigated following intramuscular and
209 intravenous administration (Cheng et al. 1998a; Welsh et al. 1993). When administered
210 intravenously, flunixin meglumine's elimination half-life has been reported to be 2.48 h (Cheng
211 et al. 1998a) and 3.83 h (Welsh et al. 1993). The elimination half-life observed in the current
212 study (following oral administration) was longer (7.95 ± 0.77 h). It was similarly observed for
213 the mean retention time of flunixin following intravenous versus oral administration, with MRT
214 in plasma being 3.20 ± 0.18 h (Cheng et al. 1998a) compared with 13.59 ± 1.17 h in the current
215 study. However, Cheng et al. (1998a) reported a longer MRT when flunixin concentrations were
216 measured from exudate and transudate obtained from an acute inflammation model (12.98±1.01
217 h and 15.35±0.64 h respectively). The AUC observed in the current study (37.62 ± 1.69

μg/mL*h) was similar to that reported by Cheng et al. (1998a) (30.61+3.41 μg/mL*h). It is probable that our higher AUC was due to the higher dose rate used in our study.

In sheep, the pharmacokinetics of flunixin following intramuscular and intravenous administration has been reported previously (Cheng et al. 1998a; Welsh et al. 1993). When administered intravenously, half-life has been reported to be 2.48 h (Cheng et al. 1998a) and 3.83 h (Welsh et al. 1993). The elimination half-life observed in the current study (following oral administration) was (7.95 ± 0.77 h). Similarly mean retention time of flunixin following intravenous administration was shorter (3.20 ± 0.18 h) (Cheng et al. 1998a) than was observed in the current study (13.59 ± 1.17 h). When flunixin is administered intramuscularly and intravenously it is given as one complete dose, which allows rapid absorption and elimination to occur. The longer half-life and mean retention time observed in this study would be due to animals having access to consume their dose of flunixin over a period time, rather than in one go. The AUC observed in the current study (37.62 ± 1.69 μg/mL*h) was similar to that reported by Cheng et al. (1998a) (30.61+3.41 μg/mL*h). It is probable that our higher AUC was due to the higher dose rate used in our study.

The pharmacokinetics of orally administered flunixin has been studied in goats (Königsson et al. 2003), horses (Pellegrini-Masini et al. 2004; Welsh et al. 1992) and cattle (Odensvik 1995). Following oral administration in the absence of feed in these species, flunixin is absorbed rapidly and concentrations can still be detected up to 30 h after administration (Königsson et al. 2003; Odensvik 1995). Horses that had ad libitum access to hay following the oral administration of flunixin had a slower absorption of flunixin and a lower Cmax (Welsh et al. 1992). Although concentrations of flunixin in plasma were maintained for longer when animals had access to food compared with when they were fasted (Welsh et al. 1992). The AUC was not significantly

different between fasted and non-fasted animals with suggesting that the overall concentration of flunixin absorbed is not affected by fasting. In the current study, flunixin was found to be absorbed rapidly when consumed with feed, with detectable levels present within 10 min in sheep that consumed more than 22 g within that period. Flunixin concentrations remained detectable, but were below therapeutic concentrations, 36-40 h after the last medicated food was consumed. Currently there is no toxicity data for flunixin in sheep, however the healthy sheep used in this study did not show any visible side effects as a result of consuming medicated feed.

Previous work in cattle by Odensvik (1995; 1998) showed that oral administration of flunixin as a granule inhibited the production of prostaglandin $PGF_2\alpha$ by up to 60%, which was as effective as the standard therapeutic dose of flunixin (2.2 mg/kg) used parenterally. Although the authors did not directly measure the effectiveness of oral flunixin at reducing inflammation, they concluded that an anti-inflammatory effect was likely due to reduced production of $PGF_2\alpha$ which acts as a pro-inflammatory following injury (Ricciotti & FitzGerald 2011). Although further studies are required it is expected that oral administration of flunixin could provide effective pain-relief in sheep.

In conclusion, results of this study demonstrates that when flunixin is administered orally through feed to sheep, it is absorbed rapidly into the bloodstream and despite variability in consumption rates of pellets, all sheep reached inferred therapeutics concentrations of flunixin within 6 h. Further studies are required to investigate potential binding of flunixin to various feed components and potential impacts such binding may have on toxicity if binding resulted in slower absorption into the body. Flunixin also appears to have neither odour nor flavour that inhibits consumption by sheep. Supplementation of feed with flunixin may provide a practical way to provide pain relief to sheep and lambs prior to and after painful husbandry procedures

eliminating the need for multiple injections, reducing handling stress and minimising labour requirements.

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344 **Table 1.** Pharmacokinetic parameters of flunixin following oral administration through feed at the rate of 4mg/kg for eight sheep.
345 (t1/2) elimination half-life, (Cmax) the maximum flunixin concentration in plasma, (Tmax) the time required to reach Cmax, (AUC0–
346 t) area under the concentration vs. time curve and (MRT) mean residence time.

347

	Sheep ID								
Parameter	305	466	580	612	621	627	648	732	Mean ± SEM
$t_{1/2}$, h	4.59	5.39	8.23	6.29	7.31	4.85	11.04	5.19	7.95 ± 0.77
Tmax, h	8	1	6	6	2	12	12	4	6 ± 1.46
Cmax, µg/mL	2.39	1.61	2.18	1.89	2.16	1.33	1.63	2.80	1.78 ± 0.17
AUC0-t, µg/mL*h	29.96	38.00	38.21	40.99	42.78	31.84	42.75	36.05	37.68 ± 1.69
MRT, h	9.36	14.34	13.36	13.43	12.98	15.80	19.48	9.32	13.59 ± 1.17

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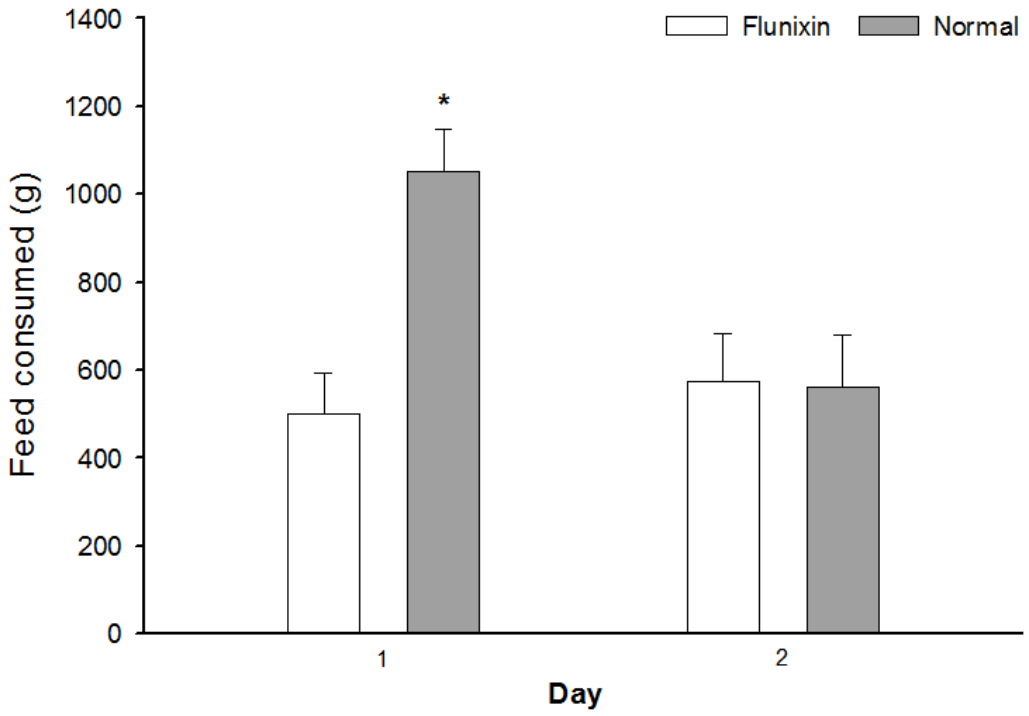

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354 **Figure 1:** Average daily feed intake with standard error bars, of pelleted feed containing

355 flunixin and untreated pelleted feed over a period of two days, * indicates a $P<0.05$

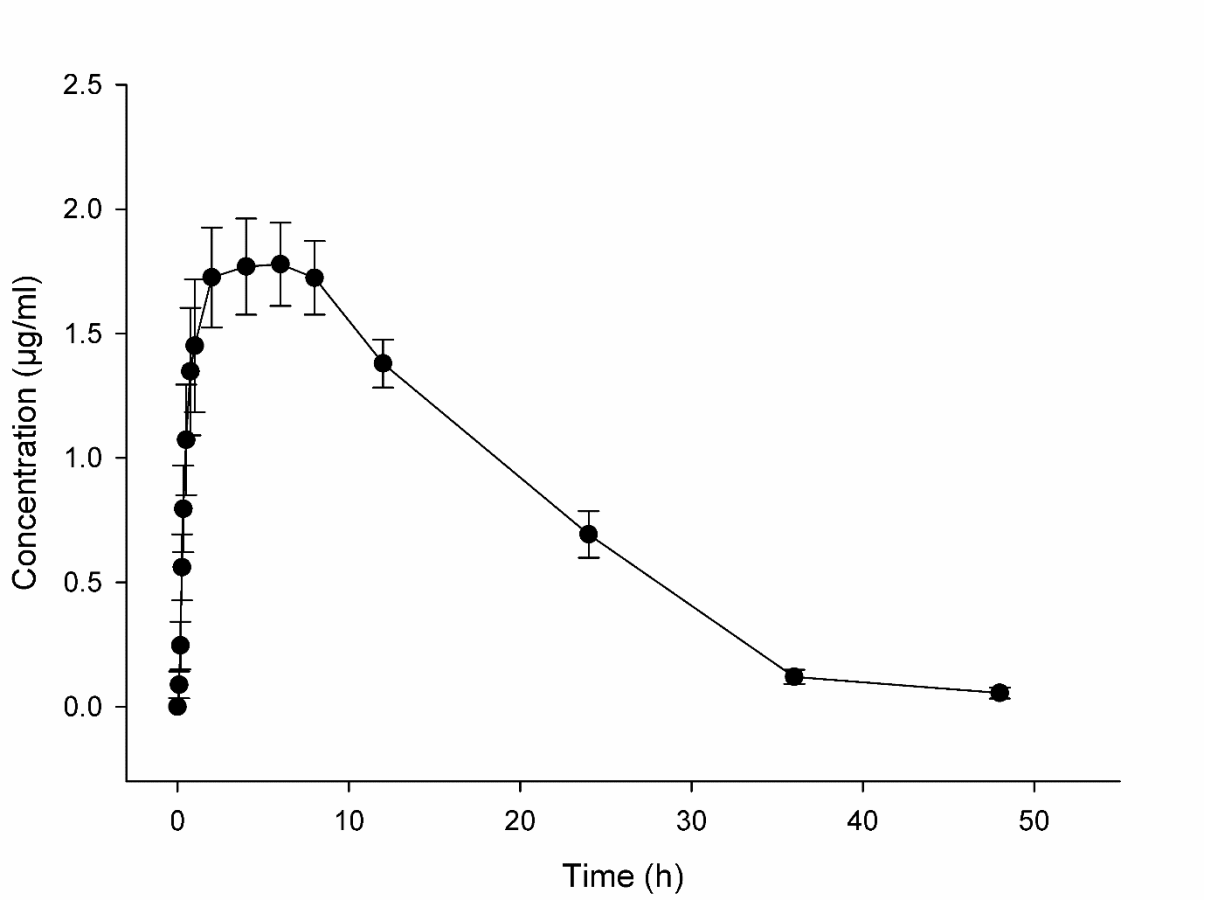

Figure 2: Concentration time curve with error bars of the average concentration of flunixin in sheep plasma over a 48 h period.

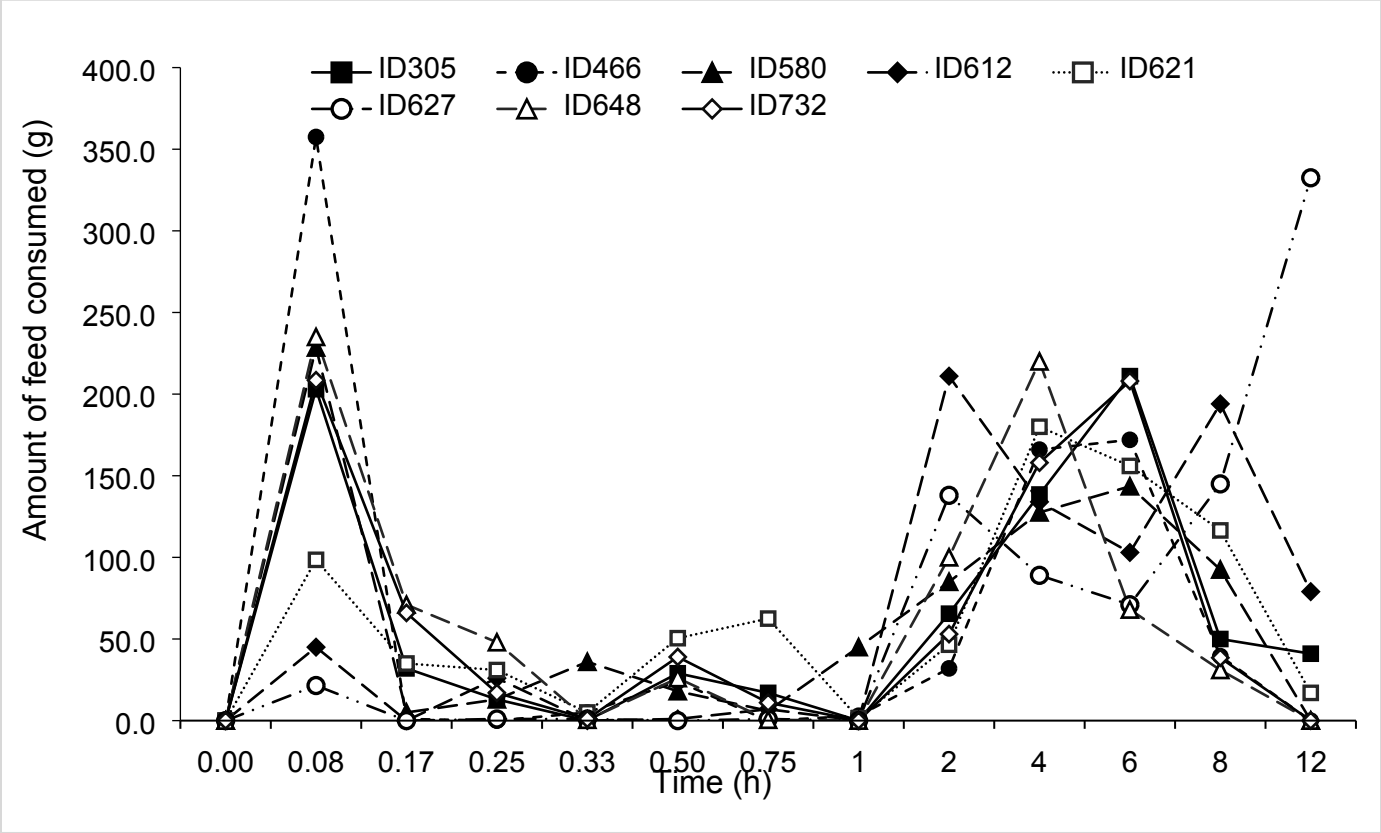

Figure 3: Amount of feed consumed by each sheep at each time point