Final report

The Welfare of Bobby Calves in the Meat Supply Chain

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Abstract

The bobby calf supply chain into the meat industry is unique to Australia and New Zealand. This project studied 'at-risk' welfare and health variables in a representative sample of bobby calves. Blood samples were analysed from 4484 bobby calves at 3 abattoirs in Victoria with significant bobby calf supply chains. Variables measured include those indicative of hydration status, energy status, muscle damage and fatigue, and colostral immunity. Results showed that most calves had adequate hydration and energy. With increasing distance transported, calves were more likely to show evidence of a negative energy balance or dehydration. The estimated effect of distance overall was small, but for calves transported more than 500 km the blood glucose concentration declined more per kilometre. The calves' farm of origin accounted for a substantial amount of the random variation between calves for total protein and plasma glucose. A total of 35% of calves showed evidence of failure of passive transfer of maternal immunity. These results highlight the importance of calf management and preparation on farm to ensure good bobby calf welfare during transport and lairage. Improving colostral management, calf nutrition and calf health on farm is likely to improve bobby calf welfare during transport and lairage.

Executive summary

Background

The bobby calf supply chain into the meat industry is unique to Australia and New Zealand. The vast majority of bobby calves in Australia are transported and/or processed in Victoria, due to the concentration of the dairy industry in the state making it viable for a number of abattoirs to process calves. Male purebred dairy calves that are born in southern NSW or in the eastern part of South Australia may be transported to Victoria for slaughter.

The bobby calf sector is the focus of animal welfare concern that ~~is~~ has the potential to be damaging to the public perception of the farming industries – both meat and dairy. This animal welfare concern for bobby calves arises from the age and vulnerability of the animals, the fact that they are difficult to handle in the conventional manner (i.e. they do not herd easily), and because each animal is of relatively low value.

The dairy industry and regulators are contributing to manage the issue through use of NLIS tag scanning to capture time in transport, and by the use of standards on maximum transport time and duration off-feed. Despite these efforts, there a paucity of published objective data on bobby calf welfare status available to validate the industry's performance or enable it to identify improvements.

This project aimed to measure relevant, 'at-risk' welfare and health variables in a substantial, representative sample of bobby calves in the supply chain, and to test for relationships with distance travelled and farm of origin. The results of the research will be used and made available for recommendation for good practice in bobby calf preparation and management in the supply chain.

Objectives

- Determination and validation of the welfare state of bobby calves at the end of the meat supply chain under commercial conditions
- Identification of some of the key on-farm and transport-related risk factors for bobby calf welfare
- Development of evidence-based management guidelines for the preparation, selection and management of bobby calves through the meat supply chain to minimise risks to their welfare
- Data-based evidence and management strategies to mimimise the animal welfare reputational risk to the meat sector through bobby calf welfare problems

All the project objectives were achieved.

Methodology

We analysed blood samples from 4,484 Australian bobby calves originating from Victoria, NSW and South Australia, after transport, fasting and lairage. Packed cell volume (PCV), plasma glucose, and serum urea, total protein, creatine kinase (CK), β-hydroxybutyrate (BHB) and γ-glutamyl transferase (GGT) were measured. Ear tag data was used to estimate the distance that the calves were transported, and to identify the farm of origin. Data were analyzed using linear mixed models, except for β-hydroxybutyrate which was analyzed using a Goodman-Kruskal gamma test due to left censoring of the data.

Results/key findings

With increasing distance transported, calves were more likely to show evidence of a negative energy balance or dehydration. The estimated effect of distance overall was small, but for calves transported more than 500 km the blood glucose concentration declined more per kilometre. The calves' farm of origin accounted for a substantial amount of the random variation between calves for total protein and plasma glucose. A total of 35% of calves showed evidence of failure of passive transfer of maternal immunity.

Benefits to industry

These results highlight the importance of calf management and preparation on farm to ensure good bobby calf welfare during transport and lairage. Improving colostral management, calf nutrition and calf health on farm is likely to improve bobby calf welfare during transport and lairage.

Future research and recommendations

Improving colostral management, calf nutrition and calf health on farm is likely to improve bobby calf welfare during transport and lairage. Policy/extension efforts should focus on these areas. Additionally, one variable for which we were unable to collect data is calf mortality (through death or necessary euthanasia) on arrival or while in lairage. New Zealand collects and publishes this statistic, and has tracked a decline in bobby calf mortality in the supply chain from 0.68% in 2008 to 0.04% in 2019. We should also collect and track this measure.

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1. Background

The bobby calf supply chain into the meat industry is unique to Australia and New Zealand. The vast majority of bobby calves in Australia are transported and/or processed in Victoria, due to the concentration of the dairy industry in the state making it viable for a number of abattoirs to process calves. Male purebred dairy calves that are born in southern NSW or in the eastern part of South Australia may also be transported to Victoria for slaughter.

The bobby calf sector is the focus of animal welfare concern that has the potential to be damaging to the public perception of the farming industries – both meat and dairy. Because the calves are in the meat supply chain once they leave the farm and are visibly on livestock transport vehicles and then are held in lairage and killed at abattoirs, the reputational risk surrounding incidences of apparent poor welfare is relevant to the meat sector, as well as dairy.

This animal welfare concern for bobby calves arises from the age and vulnerability of the animals, the fact that they are difficult to handle in the conventional manner (i.e. they do not herd easily), and because each animal is of relatively low value.

The contentiousness of the bobby calf issue may be reduced in the future by developments in sexed semen technology, such as reduced semen cost and increased efficacy, or by improved economic gains for veal calf rearing. Having said this, it is relevant for both calf welfare and for the reputational risk for the farming sector to aim to address animal welfare of bobby calves. The dairy industry and regulators are contributing to manage the issue through use of NLIS tag scanning to capture time in transport, by the use of standards on maximum transport time and duration off-feed, and by information capture at abattoirs. Despite these efforts, there remain several R&D knowledge gaps, a lack of independent publishable information, and a paucity of published objective data on bobby calf welfare status available to validate the industry's performance or enable it to identify improvements.

In New Zealand and Australia, non-replacement dairy calves, or bobby calves, can legally be transported from farms to abattoirs for slaughter from the age of 4 or 5 days, respectively (Animal Health Australia, 2012, New Zealand Government, 2018). This process involves a number of risks to the welfare of calves, including fasting, transport stress, handling, social stress, injury and disease. While transport combined with fasting is recognized as a stressful experience for any age of cattle (Warriss et al., 1995, Grandin, 1997, Knowles, 1999), neonatal calves may be at an especially high risk of welfare compromise. This is due to a number of reasons. Firstly, young calves have low body fat reserves (Bell, 1979), which puts them at a higher risk of energy depletion during the fasting that accompanies transport and lairage. Secondly, young calves are reliant on good colostrum management for immunity from infectious disease (Cuttance et al., 2018). Transfer of colostral immunity is often variable in practice, with 33-42% of dairy calves in Australia and New Zealand estimated to have failure of passive transfer of immunity (Vogels et al., 2013, Cuttance et al., 2017b, Abuelo et al., 2019). Finally, young calves have not learned herding and following behaviour, and this can make them more difficult to handle (Jongman and Butler, 2013), particularly at loading and unloading. This difficulty may increase calves' risk of poor handling in relation to transport. These factors mean that bobby calves are at a higher risk of poor welfare outcomes during transport and fasting than adult cattle.

There have been a number of studies that have assessed the impact of transport and fasting on young calf welfare in an experimental setting. Most of these studies used selected serum biochemistry and haematology values as part of the welfare assessment (e.g. Todd et al., 2000,

Stafford et al., 2001, Fisher et al., 2014). Stafford et al. (2001) found that blood variable analysis results were in agreement with physical examination welfare assessments at abattoirs. This supports the use of selected blood variables as a welfare assessment tool for young calves.

Previous research in an experimental setting has shown that during transport and fasting, bobby calves' plasma glucose decreases, and BHB increases, indicating a negative energy balance (Todd et al., 2000, Fisher et al., 2014). Hypoglycaemia can result if fasting is prolonged (24-30 hours), which is likely to compromise calf welfare. While New Zealand regulations stipulate a maximum 24 hours fasting time for bobby calves (New Zealand Government, 2018), no such legal requirement exists in Australia, though industry guidelines suggest a maximum of 30 hours of fasting (Animal Health Australia, 2015). There is currently no published data about the prevalence of hypoglycaemia in transported Australian bobby calves, or how long they are typically fasted for. Prolonged fasting also has the potential to impact calf hydration, as neonatal calves' diet is exclusively milk.

It is also unknown how prevalent failure of passive transfer is in bobby calves. Previous research has indicated that farms may give heifer calves preferential treatment when it comes to colostrum management, with some farms reporting that bull calves (which usually become bobby calves) do not receive any colostrum after separation from the dam (Vogels et al., 2013). Lack of good colostral management has the potential to be a major calf health and welfare issue, with the risk of morbidity and mortality being markedly higher in calves with failure of passive transfer (Tyler et al., 1999, Cuttance et al., 2018). Although bobby calves may be slaughtered before some of the health problems become apparent in retained calves that receive insufficient colostrum, a failure of passive transfer nonetheless leaves bobby calves vulnerable to infection and sepsis during their life on farm and in transit and lairage.

The majority of the published research studies on bobby calf welfare, besides mortality prevalence studies, have been conducted under experimental conditions. Results from these studies have suggested that the majority of well managed young calves that are transported up to 12 hours, and fasted up to 24 hours, have blood results comparable to published reference ranges for fasted young calves (Stafford et al., 2001, Fisher et al., 2014, Roadknight et al., in press – see Appendix 1). However, none of the available studies has assessed Australian bobby calf welfare in the commercial system. It is therefore unknown whether these experimental studies are representative of bobby calf welfare in the commercial Australian dairy industry, or whether industry standards of calf management align with best practice experimental management.

Our study aimed to address this knowledge deficit, and to identify optimal areas of bobby calf management to target for calf welfare improvement. We hypothesized that bobby calves in the commercial system that were transported over longer distances would be more likely to have blood variables consistent with poor welfare, such as high packed cell volume (PCV) and urea indicating dehydration, low glucose and high BHB indicating depleted energy, and high creatine kinase (CK) indicating muscle fatigue or damage. We also expected that the calves' farm of origin would impact total protein and glucose, due to differences in farm colostral management, calf nutrition and calf management. This will provide objective data on industry performance, as well as forming the basis of updated advice to farmers on areas of calf preparation on which to focus for ensuring optimal calf welfare. Together, these outcomes can contribute to a greater resilience of the industry in response to current concerns around calf welfare.

2. Objectives

- Determination and validation of the welfare state of bobby calves at the end of the meat supply chain under commercial conditions
- Identification of some of the key on-farm and transport-related risk factors for bobby calf welfare
- Development of evidence-based management guidelines for the preparation, selection and management of bobby calves through the meat supply chain to minimise risks to their welfare
- Data-based evidence and management strategies to mimimise the animal welfare reputational risk to the meat sector through bobby calf welfare problems

All the project objectives were achieved.

3. Methodology

3.1 Animals

Blood samples were analysed from 4,484 bobby calves at 3 commercial abattoirs in Victoria, Australia. Calves were both male (3,776 calves, 84%) and female (481, 11%), with 227 calves (5%) where sex was not recorded. Calves originated from Victoria (3,722 calves, 83%), New South Wales (613 calves, 14%), and South Australia (13 calves, 0.3%), with 136 calves (3%) where the state of origin was not identified. Calf breeds were assessed based on phenotype as Holstein-Friesian (n = 2079, 46%), Jersey (n = 903, 20%) or other breed/crossbreed (n = 1,369, 31%), with 133 calves (3%) where breed was not recorded.

3.2 Animal handling and management

Calves were processed as per normal abattoir practices, by abattoir staff. Calves were stunned using electrical head-only stunning, followed by exsanguination via thoracic sticking and cervical cutting. During exsanguination, National Livestock Identification System RFID ear tags were scanned and external numbers were read aloud into a voice recorder. This allowed each calf and the farm that they originated from to be uniquely identified.

3.3 Sample collection, handling and processing

Blood samples were collected on 45 days between August 2017 and April 2018, corresponding to the spring 2017 and the autumn 2018 calving seasons. Samples from 4,540 calves from 1,117 farms were collected, with 4,484 of these samples being used for analysis of at least one blood variable. Samples from 56 calves were discarded prior to analysis, and not all blood variables were measured for all calves. This was due to technical reasons such as clotting of the sodium fluoride/potassium oxalate tube preventing measurement of PCV, failure to collect blood in all tubes for all calves, insufficient volume of sample, and contamination of some tubes (e.g. with hair or other foreign material).

Blood samples were collected during exsanguination, as described by Roadknight et al. (2020). In brief, blood samples were collected soon after stunning, thoracic stick, and cervical cutting. Tubes (a

Vacuette® (Greiner Bio-One, USA) 8 mL serum separator clot activator tube, and a Vacuette® 2 mL sodium fluoride/potassium oxalate tube) were uncapped and blood was collected mid-stream directly from the thoracic stick site or from the major cervical vessels. Samples were placed immediately into a container cooled by icepacks, where they remained while being transported to the laboratory.

Serum was separated by centrifuging samples at 2500g for 5 minutes. Serum was then transferred to plain tubes and frozen on the same day as sampling at -20°C until analysis. PCVs were assessed using the microhaematocrit method on the day of sampling, with blood from the sodium fluoride/potassium oxalate tube. Microhaematocrit tubes were centrifuged at 9838 g for 5 minutes. After PCV measurement, plasma was separated and handled in the same manner as serum. Serum was thawed and analyzed for CK, urea, γ-glutamyl transferase (GGT), total protein and BHB, and plasma was thawed and analyzed for glucose. This blood panel gave indications of calves' energy status (glucose and BHB), hydration (PCV, urea), colostral immunity (GGT, total protein) and muscle fatigue or damage (CK). Serum and plasma analysis was carried out using a Cobas Integra® 400 Plus biochemistry analyzer (Roche, Switzerland) within 3 weeks of being frozen.

3.4 Distance and duration of transport and lairage calculations

The distance that calves were transported was estimated using the parish (local area) of origin information from National Livestock Identification System calf ear tags, and Google Maps to approximate the distance from the parish to the abattoir. The duration of transport and lairage was estimated using the ear tag scanning records for 3,744 calves. Calves were scanned when loaded on to the truck at the farm, and at slaughter, and these scans were time stamped and compared to calculate the total time spent in transport and lairage. Durations of more than 72 hours (75 calves) were not included in the analysis, as we considered that these very long durations were likely due to scanning or data entry error. Some calves (n = 1,741) went to a sale yard prior to being transported to the abattoir. However, because the locations of these sale yards were not known, this was not factored into the transport distance estimate.

3.5 Statistical analyses

Data were analysed using R statistical software, version 4.0.0 (R Core Team, 2020). Linear mixed models were run using the 'lme4' and 'lmerTest' packages (Bates et al., 2015, Kuznetsova et al., 2017), with blood variables as the dependent variables, distance transported, calf breed and calf sex as fixed effects, and farm identification number as a random effect. Individual fixed effects models were fitted for all explanatory variables, and were considered for the final model if $P < 0.1$. Fixed effects and interaction terms were removed from the final model if they were not significant ($P > 0.05$). Model residuals were inspected for approximately normal distribution and equal variance in q-q plots, histograms and residuals versus predicted values scatterplots. Urea, GGT and CK were natural log transformed when used in the models, to better satisfy the model assumptions. Results for model estimates and confidence intervals for log transformed variables were back-transformed and expressed as percentage change in the blood variable of interest.

For BHB, results for 999 calves were left censored, due to being less than the limit of detection for the analyser (0.1 mmol/L). For this variable, maximum likelihood estimation and bootstrapping was used to calculate the mean and standard deviation, using the R packages 'MASS', 'boot' and 'fitdistrplus' (Venables and Ripley, 2002, Delignette-Muller and Dutang, 2015, Canty and Ripley, 2019, Davison and Hinkley, 1997). The log normal distribution was chosen as the best fit for the data, based on visual inspection of plotted predicted versus actual values. Values for the mean and

standard deviation generated in the log normal scale were then transformed to the linear scale. Linear mixed models were not used to analyse BHB due to left censoring. Instead, a Goodman-Kruskal gamma test was run, using BHB categories of less than 0.60 mmol/L (normal), and more than 0.59 mmol/L (high), from the fasted calf reference range for BHB (Roadknight et al., in press, see Appendix 1). Distance transported categories used for the Goodman-Kruskal gamma test were 0-199 km, 200-399 km, 400-599 km, 600-799 km, and more than 799 km.

4. Results

The means and medians for all blood variables measured were within the published reference intervals for fasted dairy calves aged 5-12 days (Roadknight et al., in press, see Appendix 1) – see Table 1 for descriptive statistics. In addition, most individual calves' blood results were within the reference intervals (Table 2). However, a proportion of calves had values that fell outside of the reference interval. These included 36% of calves that had CK greater than the upper reference limit, with 1% of calves returning a CK result of more than 2000 U/L, and 35% of calves that had total protein concentrations consistent with failure of passive transfer.

The distance that calves were transported had significant effects on all blood variables except for urea (Tables 3-8) and BHB. There was no significant association between the distance transported category and the BHB category ($\gamma = 0.05$, 95% CI (-0.17, 0.27), $P = 0.64$). Visual inspection of glucose data suggested that, for calves being transported more than approximately 500 km, each km travelled was associated with a greater decline in glucose compared to calves transported shorter distances (Figure 1). This was confirmed when a linear mixed model was used to analyze only those calves being transported more than 500 km – glucose declined by an estimated 0.41 mmol/100 km for these calves (95% CI (-0.5, -0.3), $P < 0.001$), an 8-fold increase on calves travelling all distances (-0.05 mmol/100 km, Table 3).

No significant effects of duration of transport and lairage were evident for any of the blood variables (Tables 3-8). Few calves (6.5%) had recorded duration of transport and lairage times of more than 24 hours; the highest recorded duration of transport and lairage was 48 hours.
Estimates from the final models indicated that a substantial percentage of the variation in plasma glucose and total protein between calves was due to the farm of origin of the calves. Farm of origin accounted for 20% of the random variability in glucose, and 12% of the random variability in total protein.

Table 1. Descriptive statistics for blood variables, distance transported, and duration of transport and lairage for bobby calves at abattoirs.

Variable	Units	Mean	Median	Min	Max	SD	n calves
PCV	L/L	0.30	0.29	0.11	0.52	0.06	1723
Urea	mmol/L	5.0	4.2	0.6	47.8	3.4	4348
Glucose	mmol/L	5.0	5.0	0.8	13.3	1.0	4345
BHB	mmol/L	0.20[1]	0.16	<0.1	2.18	0.15[1]	4346
Total protein	g/L	59	58	9	109	13	4348
GGT	U/L	397	237	3	5928	503	4347
Creatine kinase	U/L	355	257	7	15,779	424	4348
Distance transported	km	245	180	8	1006	213	4219
Duration of transport + lairage	hours	20.0	19.1	2.3	47.8	6.1	3743

[1]Mean and standard deviation for BHB calculated using Maximum Likelihood Estimation and bootstrapping with a log normal distribution, then back-transformed.

Abbreviations: PCV, packed cell volume; GGT, gamma-glutamyl transferase; Min, minimum; Max, maximum; SD, standard deviation.

Table 2. Percentage of calves at abattoirs that had PCV and biochemistry results outside the normal fasted calf reference interval.

Variable	Units	Reference interval[1]	No. calves with results <lower reference limit (%)	No. calves with results >upper reference limit (%)
PCV	L/L	0.23-0.45	208 (12.1%)	7 (0.4%)
Urea	mmol/L	1.6-7.7	78 (1.8%)	472 (11%)
Glucose	mmol/L	2.8-6.9	60 (1.4%)	100 (2.3%)
BHB	mmol/L	<0.60	N/A	91 (2.1%)
Total protein	g/L	45-82	656 (15.1%)	192 (4.4%)
GGT	U/L	32-1037	555 (12.8%)	367 (8.4%)
Creatine kinase	U/L	46-326	4 (0.1%)	1548 (35.6%)

[1]From Roadknight et al. (in press, see Appendix 1)

Abbreviations: PCV, packed cell volume; GGT, gamma-glutamyl transferase; N/A, not applicable

Table 3. Glucose - mixed model results for individual explanatory variables. Results for the final model for glucose are presented below the dashed line.

Explanatory variable	Estimate of effect (mmol/L glucose)	95% CI	P-value	No. calves	No. farms
Transport distance	-0.05/100 km	(-0.07, -0.03)	<0.001	4068	1044
Duration of transport + lairage	-0.05/10 hours	(-0.11, 0.002)	0.057	3606	946
Breed – Friesian	reference	-	-	4215	1096
Breed – Jersey	-0.24	(-0.32, -0.15)	<0.001	4215	1096
Breed – other/crossbreed	-0.02	(-0.09, 0.04)	0.496	4215	1096
Sex – male	Reference	-	-	4122	1088
Sex – female	0.03	(-0.06, 0.12)	0.534	4122	1088
Transport distance	-0.05/100 km	(-0.07, -0.03)	<0.001	3950	1039
Breed – Friesian	reference	-	-	3950	1039
Breed – Jersey	-0.24	(-0.32, -0.15)	<0.001	3950	1039
Breed – other/crossbreed	-0.03	(-0.10, 0.04)	0.372	3950	1039

Table 4. Packed cell volume - mixed model results for individual explanatory variables. Results for the final model for PCV are presented below the dashed line.

Explanatory variable	Estimate of effect (L/L PCV)	95% CI	P-value	No. calves	No. farms
Transport distance	0.002/100 km	(0.0002, 0.004)	0.029	1636	614
Duration of transport + lairage	-0.007/10 hours	(-0.016, 0.002)	0.133	1494	565
Breed – Friesian	reference	-	-	1658	647
Breed – Jersey	-0.012	(-0.020, -0.003)	0.009	1658	647
Breed – other/crossbreed	0.006	(-0.001. 0.012)	0.090	1658	647
Sex – male	reference	-	-	1637	637
Sex – female	0.004	(-0.006, 0.014)	0.413	1637	637
Transport distance	0.002/100 km	(0.0003, 0.004)	0.020	1573	612
Breed – Friesian	reference	-	-	1573	612
Breed – Jersey	-0.011	(-0.0200, -0.0026)	0.011	1573	612
Breed – other/crossbreed	0.006	(-0.0009, 0.0128)	0.089	1573	612

Table 5. Urea (log transformed) - mixed model results for individual explanatory variables. Results for the final model for urea are presented below the dashed line. Estimates and confidence intervals have been back-transformed, so are expressed as percentage change in urea.

Explanatory variable	Estimate of effect (% change in urea)	95% CI	P-value	No. calves	No. farms
Transport distance	-0.1/100 km	(-1.1, 0.9)	0.824	4066	1039
Duration of transport + lairage	-0.7/10 hours	(-3.6, 2.3)	0.660	3597	941
Breed – Friesian	reference	-	-	4227	1088
Breed – Jersey	6.3	(1.8, 11.1)	0.005	4227	1088
Breed –other/crossbreed	0.6	(-3.0, 4.2)	0.758	4227	1088
Sex – male	reference	-	-	4138	1081
Sex – female	0.2	(-4.6, 5.4)	0.923	4138	1081
Breed – Friesian	reference	-	-	4227	1088
Breed – Jersey	6.3	(1.8, 11.1)	0.005	4227	1088
Breed –other/crossbreed	0.6	(-3.0, 4.2)	0.758	4227	1088

Table 6. Total protein - mixed model results for individual explanatory variables. Results for the final model for total protein are presented below the dashed line.

Explanatory variable	Estimate of effect (g/L total protein)	95% CI	P-value	No. calves	No. farms
Transport distance	0.4/100 km	(0.12, 0.63)	0.004	4066	1039
Duration of transport + lairage	0.13/10 hours	(-0.66, 0.92)	0.742	3597	941
Breed – Friesian	reference	-	-	4227	1088
Breed – Jersey	5.4	(4.3, 6.5)	<0.001	4227	1088
Breed –other/crossbreed	5.2	(4.3, 6.1)	<0.001	4227	1088
Sex – male	reference	-	-	4138	1081
Sex – female	1.8	(0.5, 3.1)	0.007	4138	1081
Transport distance	0.4/100 km	(0.20, 0.)	<0.001	3954	1034
Breed – Friesian	reference	-	-	3954	1034
Breed – Jersey	5.5	(4.4, 6.7)	<0.001	3954	1034
Breed –other/crossbreed	5.3	(4.4, 6.3)	<0.001	3954	1034

Table 7. γ-glutamyl transferase (GGT, log transformed) - mixed model results for individual explanatory variables. Results for the final model for GGT are presented below the dashed line. Estimates and confidence intervals have been back-transformed, so are expressed as percentage change in GGT.

Explanatory variable	Estimate of effect (% change in GGT)	95% CI	P-value	No. calves	No. farms
Transport distance	-5.3/100 km	(-7.6, -2.9)	<0.001	4065	1039
Duration of transport + lairage	-1.4/10 hours	(-9.0, 6.9)	0.732	3596	941
Breed – Friesian	reference	-	-	4226	1088
Breed – Jersey	83.6	(64.1, 105.5)	<0.001	4226	1088
Breed –other/crossbreed	71.5	(56.2, 88.3)	<0.001	4226	1088
Sex – male	reference	-	-	4226	1088
Sex – female	18.9	(4.2, 35.8)	0.010	4138	1081
Transport distance	-4.7/100 km	(-7.0, -2.3)	<0.001	3953	1034
Breed – Friesian	reference	-	-	3953	1034
Breed – Jersey	84.5	(64.4, 107.2)	<0.001	3953	1034
Breed –other/crossbreed	71.9	(56.0, 89.3)	<0.001	3953	1034

Table 8. Creatine kinase (CK, log transformed) - mixed model results for individual explanatory variables. Results for the final model for CK are presented below the dashed line. Estimates and confidence intervals have been back-transformed, so are expressed as percentage change in CK. Because there was a significant interaction between distance and sex, we present estimates for the effect of distance for both males and females in the final model.

Explanatory variable	Estimate of effect (% change in CK)	95% CI	P-value	No. calves	No. farms
Transport distance	-2.8/100 km	(-4.0, -1.5)	<0.001	4066	1039
Duration of transport + lairage	1.9/10 hours	(-2.0, 6.0)	0.346	3597	941
Breed – Friesian	reference	-	-	4227	1088
Breed – Jersey	1.8	(-3.7, 7.7)	0.531	4227	1088
Breed –other/crossbreed	-2.0	(-6.3, 2.6)	0.396	4227	1088
Sex – male	reference	-	-	4138	1081
Sex – female	14.5	(7.5, 22.0)	<0.001	4138	1081
Transport distance					
Sex – male	-2.4/100 km	(-3.8, -1.1)	<0.001	3868	1026
Sex – female	-7.3/100 km	(-10.4, -3.9)	<0.001	3868	1026

Figure 1. Relationship of bobby calf plasma glucose to estimated distance transported from farm to abattoir. Red line is the linear regression line, blue line is a loess line, which is a non-parametric, locally estimated scatterplot smoothing regression line.

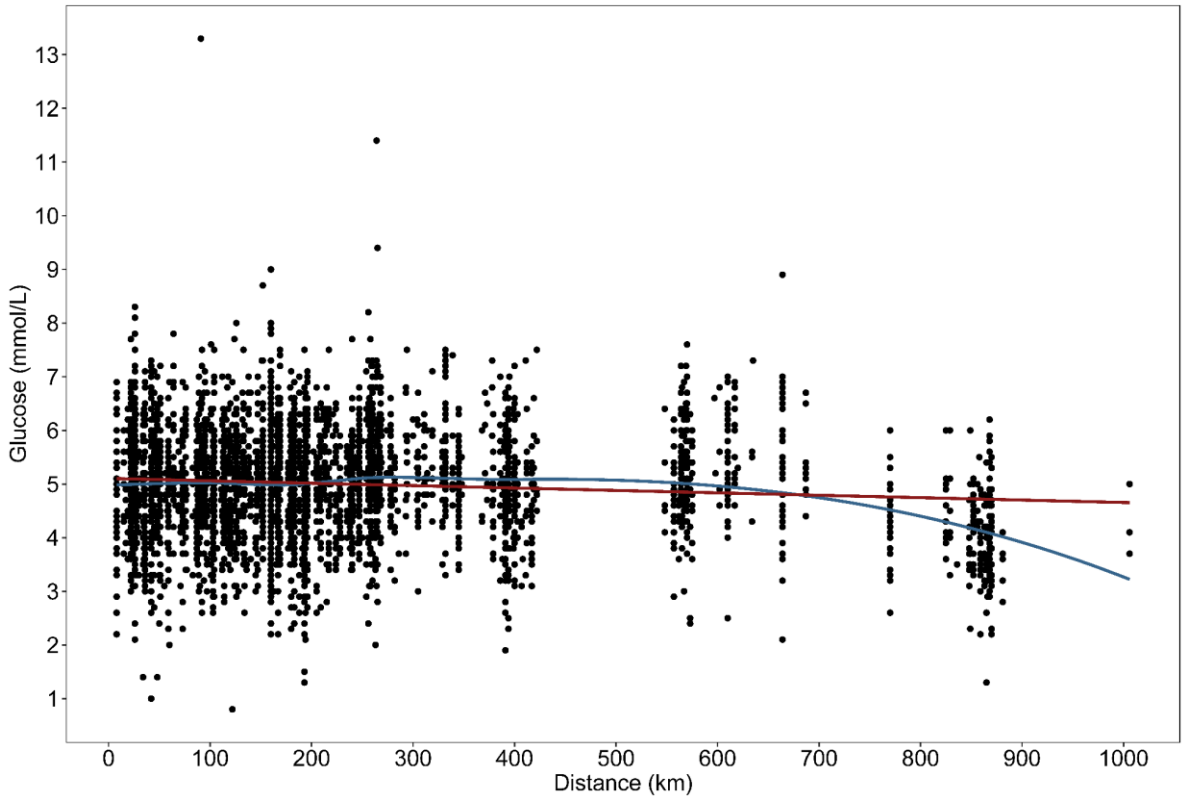
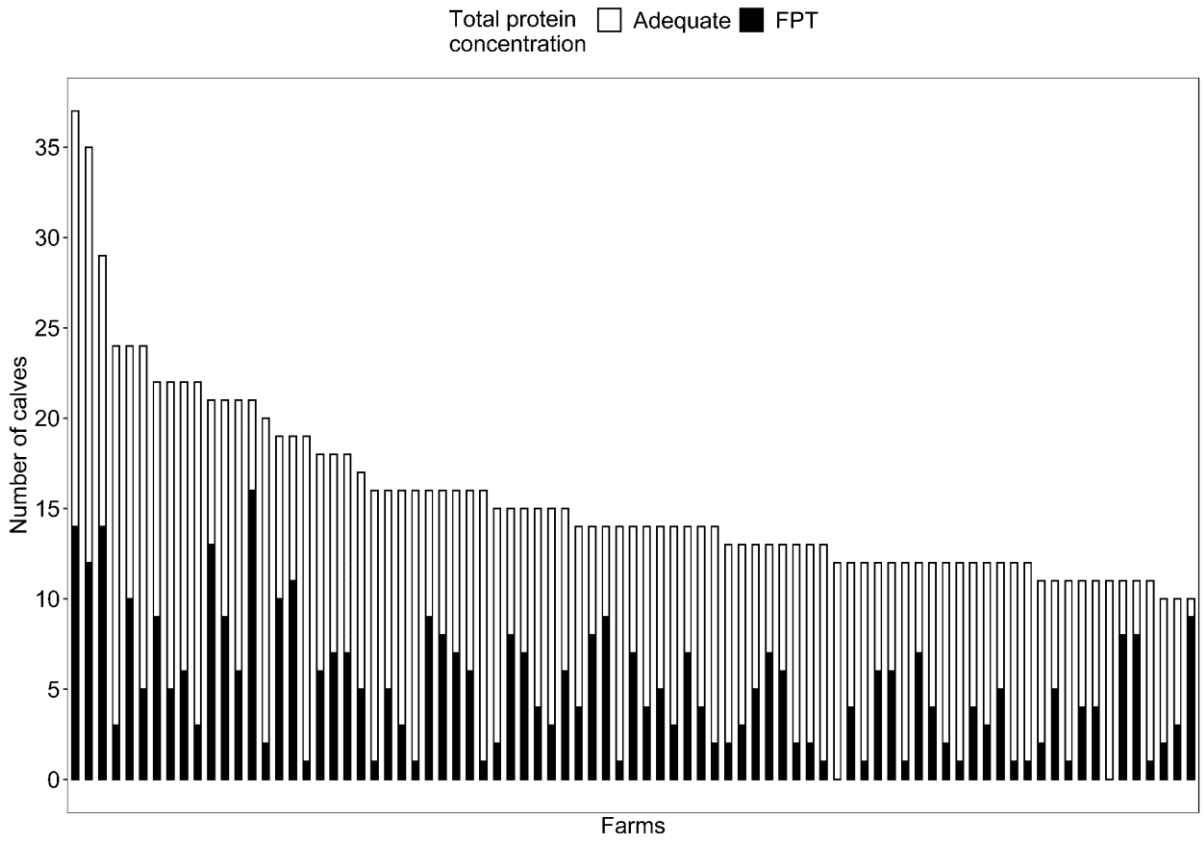


Figure 2. Numbers of bobby calves with failure of passive transfer (FPT, total protein 52 g/L or less (Cuttance et al., 2017a), filled) compared to adequate passive transfer (total protein >52 g/L, unfilled), for farms with more than 10 and less than 40 calves in the dataset.



5. Discussion

Our results show that, while most bobby calves sampled had blood results within the established young calf reference intervals for fasted calves, a notable proportion of the calves had results outside of this range. This indicates the potential for physiological and welfare compromise for some calves in the supply chain. The main area of concern was failure of passive transfer. Over a third of the calves sampled showed total protein concentrations of 52 g/L or less, which is indicative of failure of passive transfer of immunity (Cuttance et al., 2017a, Cuttance et al., 2017b). As this rate of failure of passive transfer is similar to previously reported rates in both male and female Australian dairy calves (Vogels et al., 2013, Abuelo et al., 2019), this is likely to be a dairy industry-wide problem, rather than being specific to bobby calves.

While GGT is a less reliable measure of passive transfer than total protein (Cuttance et al., 2017a), especially when the age of the calf is not known, the GGT results were in broad agreement with the total protein results. When a cut-off of 210 U/L GGT was used to assess passive transfer, which is recommended for calves 5-8 days old (Cuttance et al., 2017a), 46% of the calves had GGT activity indicative of failure of passive transfer. This may be an overestimate; because GGT decreases rapidly with age (Parish et al., 1997), calves older than 8 days may have lower GGT activity due to normal decline, rather than failure of passive transfer. The exact age of calves in this study is unknown, but is estimated based on visual assessment and anecdotal reporting to be 5-10 days of age. Conversely, dehydration could mean that the proportion of calves with failure of passive transfer is underestimated using total protein, as dehydration can lead to increases in total protein concentration (George and Zabolotzky, 2011). There was some evidence of dehydration in approximately 11% of calves, which had urea concentrations higher than the upper reference limit (Table 2) (Roadknight et al., in press, see Appendix 1). Increases in urea are not expected in neonatal calves until the third day of fasting (Dalton, 1967); therefore factors other than fasting time may be contributing to the increase in urea concentration. These could include diarrhea or other illness, restricted milk feeding, or lack of water availability on the farm. Previous research has reported that Australian dairy farmers start providing access to water on average at 5 days calf age (Phipps, 2016), despite industry recommendations to provide water from the day of birth (Dairy Australia, 2017). Therefore, it is possible that some bobby calves did not have access to water prior to being transported, and this may have contributed to dehydration causing high urea concentrations.

PCV results, though, did not show evidence of dehydration in many calves, with only 0.4% of calves having PCV above the upper reference limit. However, PCV results showed that 12% of calves had results consistent with mild to moderate anaemia (PCV less than 0.23 L/L, Table 2) (Roadknight et al., in press, see Appendix 1). Therefore, the effects of dehydration could be masked due to this tendency towards anaemia. Anaemia has been previously reported in young dairy calves (Benesi et al., 2019), though there is some disagreement about what the lower reference limit for PCV should be; values from 0.17-0.25 L/L have been proposed (Lumsden et al., 1980, Panousis et al., 2018, Benesi et al., 2019). We used the reference interval reported by Roadknight et al. (in press, see Appendix 1) because this study was based on Australian calves that were likely to have the most similar genetics, environmental, and management conditions to the bobby calves. The reason for the anaemia in our sampled bobby calves is unknown; previous research has shown correlations between anaemia and diarrhea (Prodanović et al., 2019), and iron deficiency has been theorized to be a potential aetiology of anaemia in other studies (Hibbs et al., 1963, Benesi et al., 2019). Therefore, the tendency towards anaemia is suggestive of underlying pathology or deficiency, either of which could impact calf welfare.

Most bobby calves had energy profiles consistent with reference intervals from non-transported calves fasted for 14-25 hours (Roadknight et al., in press, see Appendix 1). Very few calves (1%)

showed evidence of hypoglycaemia (plasma glucose less than 2.8 mmol/L), and 2% had high BHB (more than 0.59 mmol/L) indicating metabolism of fat as an energy source (Roadknight et al., in press, see Appendix 1). It should be noted that calves fasted for 14-25 hours may not reflect an ideal welfare state, although it does reflect standard calf management practice on Australian dairy farms, where feeding generally occurs once or twice daily. Therefore, the low proportions of calves with hypoglycaemia and high BHB does not suggest that calves weren't hungry, or low in energy reserves. Rather, it suggests that they had similar glucose and BHB concentrations to fasted, non-transported calves managed on a restricted feeding schedule. Most bobby calves sampled in this study appeared to have been fasted for 24 hours or less, based on comparisons of glucose concentrations with calves fasted for a known time period (Fisher et al., 2014, Roadknight et al., in press, see Appendix 1).

Thirty-six percent of bobby calves had CK results greater than the upper reference limit of 326 U/L (Roadknight et al., in press, see Appendix 1). This was not unexpected, as CK is known to increase with transport, even under experimental conditions (Todd et al., 2000, Fisher et al., 2014). An increase in CK can indicate muscle activity (e.g. bracing against truck movements), or muscle damage, such as bruising from knocks or falls. CK is a sensitive indicator of muscle fatigue or damage, but has a short half-life of approximately 4 hours in cattle (Parkinson et al., 2019). Therefore, the timing of sample collection in relation to muscle damage will affect CK activity results. In this study, samples were taken after overnight lairage, so CK activity may have decreased with this rest period. However, the sampling techniques used may have increased CK activity, as blood collection at exsanguination in calves leads to, on average, 102% higher CK activity (Roadknight et al., 2020). Given that calves likely rested for a period equivalent to several CK half-lives between transport and sampling, it is possible that the effects of the rest after transport and the sampling technique cancelled each other out to some degree.

One percent of calves had moderately high CK activity (more than 2000 U/L) and 2 calves (0.05%) had markedly high CK (more than 5000U/L) (Hall and Bender, 2011). High CK activity suggests that calves may be bruised or have muscle fatigue related to transport or handling. Previous research in New Zealand reported that 50% of bobby calves at abattoirs showed evidence of stifle bruising at an abattoir (McCausland et al., 1977). Further research is needed to identify the highest risk points in the supply chain for muscle damage and fatigue, as it is unclear at what point in the supply chain muscle damage is most likely to occur.

The GGT results may also have been affected by the exsanguination sampling technique, though likely to a lesser extent than the CK results. Such GGT measurements have been reported to decrease on average by 8% when collected at exsanguination (Roadknight et al., 2020). However, this is unlikely to affect the results for the general trends for GGT and its relation to risk factors such as distance transported and farm of origin. Blood collection at exsanguination may have also had some minor effects on plasma glucose and total protein measurements, but is unlikely to have impacted PCV, urea and BHB results (Roadknight et al., 2020).

The effect of distance travelled on most blood variables was in the direction expected. For example, PCV and total protein increased with longer distances of transport, indicating a trend towards dehydration with longer distances. Similarly, glucose decreased with longer transport durations, indicating longer fasting times or greater energy utilization with long transport distances. However, CK decreased with increasing distance transported, which was unexpected, as CK usually increases with transport (Grigor et al., 2001, Marcato et al., 2020), and more so with longer transport durations (Fisher et al., 2014). We theorize that this may be due to loading being the most likely process to increase CK levels, due to knocks, falls or resistance to handling. If this was the case,

calves transported for longer distances may also have a longer time to recover normal CK levels after muscle insult.

Another finding that was unexpected was that GGT decreased with distance transported. We hypothesize that this may be due to a calf age factor. GGT generally decreases quite rapidly in the first days to weeks of calves' lives (Braun et al., 1982). It is possible that calves travelling from greater distances may be on average older, as calf transporters may pick up calves from further away less frequently. Total protein concentrations, another measure of colostral immunity, are consistent with this explanation, as they have a positive correlation with distance, suggesting that the relationship between GGT and distance is less likely to be due to passive immunity factors. Urea and BHB were not significantly affected by distance transported. For urea, previous nutrition and access to water may be more important than the distance that calves travelled. For BHB, some calves with low body fat reserves may not show a marked increase in this variable with a negative energy balance. Additionally, using the Goodman-Kruskal gamma test for statistical analysis for BHB may have masked small distance effects, as some detail was lost dividing the data into categories, in contrast to the linear mixed models used for other variables which allowed a more detailed understanding of the effect of distance on the variables.

Although distance transported had significant effects on all blood variables except urea and BHB, most of the effects estimated by the models were very small (Tables 3-8). For example, glucose was estimated to decrease by 0.05 mmol/L per 100 km, and PCV to increase by 0.002 L/L. When a subset of calves that were transported more than 500 km were analysed, it was found that glucose declined more with each km of transport for these calves, compared with calves overall (Figure 1). For calves transported for more than 500km, glucose decreased 8 times faster per km transported compared with calves overall. This suggests that the welfare impact of transport may be greater per km if calves have already been transported over a long distance.

The farm of origin, when included in the final model for glucose, accounted for 20% of the random variation in glucose and 12% of the random variation in total protein between calves. Estimates of the effect of farm suggested that the farm of origin had a relatively large impact on plasma glucose and serum total protein. For instance, when analysing a subset of calves from farms where more than 5 calves were included in the dataset, the estimated difference in plasma glucose between the 2.5% and the 97.5% quantile was 1.1 mmol/L, but the estimated effect of distance transported for the same group of calves was only -0.05 mmol/L per 100 km. Similarly, for total protein, there was a difference of 12.4 g/L between these quantiles for farms, but the effect of distance transported for the same cohort was estimated to be only 0.5 g/L per 100 km. Figure 2 shows an example of the between-farm variation in the proportion of calves with failure of passive transfer (total protein 52 g/L or less (Cuttance et al., 2017a)). Plasma glucose could also reflect the health status of calves from particular farms, with unwell calves being likely to have lower plasma glucose due to inappetence. These results highlight the importance of on-farm calf colostral management and nutrition for ensuring calf welfare during transportation, sales and lairage.

The reasons why the duration of transport and lairage had no significant effects on most blood variables could be because of inaccuracies in scanning data (e.g., if calves were scanned at unloading instead of loading), impacts of stop overs such as at sale yards, where some calves may have been fed, and the fact that lairage and transport duration could only be assessed together. For instance, it is possible that transport but not lairage had an impact on calf blood variables, or that they had opposing effects, making interpretation of this variable difficult. Although 6.5% of calves had transport and lairage times of more than 24 hours recorded, this may not reflect the calves' time off feed, as calves may be fed at sale yards. Anecdotally, it has been reported that calves from New South Wales may be fed at the sale yards before travelling to Victoria. Also, calves may be fed on

farms up to 6 hours prior to loading, if Australian government guidelines are adhered to (Animal Health Australia, 2012).

One limitation of this study is that calves that were suffering very poor welfare may not have been sampled. Because sampling occurred at slaughter, only calves that were considered fit enough for transport, lairage and slaughter, as assessed by farmers, transporters and abattoir staff, would survive until the end of the commercial supply chain; others may have died or been euthanized prior to this point. We suggest that future research on bobby calf welfare be directed towards quantifying calf morbidity and mortality rates between birth and slaughter.

6. Conclusion

6.1 Key findings

Calves transported very long distances were impacted more per km of distance transported than calves overall, as shown by more rapid declines in plasma glucose for calves travelling more than 500 km. However, the estimated effects of the distance that calves were transported were small, even for calves travelling more than 500 km, and the prevalence of hypoglycaemia was low. As expected, longer transport distances were associated with higher PCVs and lower plasma glucose. Unexpectedly, CK decreased with distance transported; this may be related to the short half-life of CK in cattle, and the timing of the highest muscle insult risk. Only a small proportion of calves had CK results suggestive of welfare compromise due to muscle fatigue or damage, which could be related to handling or injuries/fatigue during transport.

The farm of origin had a relatively large effect on calf welfare at abattoirs, with substantial proportions of the variability between calves for glucose and total protein being due to farm factors. We theorize that this variability was most likely to be due to differences in feeding practices, calf health, and colostral management. Over a third of bobby calves sampled showed evidence of failure of passive transfer, while 11% showed evidence of dehydration, and 12% were anaemic; again, these factors are likely to be related to on-farm calf management, such as nutritional management and disease prevention. Overall, these results suggest that on farm calf management and preparation, especially colostral management, nutrition, and appropriate calf selection, are likely to be the best targets for improving Australian bobby calf welfare in the current commercial system. Further research in this area should aim to quantify morbidity and mortality data in the commercial non-replacement calf supply chain, from birth to slaughter.

6.2 Benefits to industry

This study has provided a clear snapshot of the Australian bobby calf industry and the welfare of the calves in the supply chain. The research shows that while the majority of calves sampled were physiologically uncompromised, future attention on emphasising to farmers the importance of calf preparation and selection, aiming to transport these young animals less than 500 km if possible, and capturing and tracking mortality data are of future benefit to the industry and calf welfare.

7. Future research and recommendations

One of the goals of this project was to identify evidence-based management guidelines for the preparation, selection and management of bobby calves through the meat supply chain to minimise risks to their welfare. As outlined above, our results primarily indicate that on farm calf management and preparation, especially colostral management, nutrition, and appropriate calf selection, are likely to be the best targets for improving Australian bobby calf welfare in the current commercial system. This therefore does not result in new recommendations, other than us recommending that the current welfare advice be reiterated to farmers preparing bobby calves to enter the meat supply chain. Accordingly, while the bobby calf meat supply chain continues to exist, key recommendations for the welfare of bobby calves should include:

- Overall management:
    - Calves destined to be consigned as bobby calves need to be given the same standard of care as other calves on the farm
- Provision of colostrum:
    - Bobby calves need to be fed be fed 2 x 2-litre feeds of good (known) quality colostrum within the first 12 hours of life, with a further feed of 2 litres of good quality colostrum in the next 12 hours being beneficial
- Nutritional management:
    - Bobby calves need to have been fed on the farm within 6 hours of pick up for transport, with a record indicating when feeding occurred
- Selection for transport:
    - Bobby calves need to be actively selected for transport and must be fit for the intended journey

The significant contribution of farm of origin to the variation seen in our results (greater for example than the effect of distance transported) indicates that not all sources of bobby calves are achieving the desired level of preparation and selection.

Furthermore, our results support a recommendation that, if possible, bobby calves be transported no more than 500 km on a single journey to slaughter.

Finally, given that: 1) our results relate to calves that were well enough to walk up to the stun box; and 2) we were not able to easily obtain and collate validated mortality data; it is our recommendation that the Australian bobby calf sector compile and track data on bobby calf mortality based on a combination of calves dead on arrival and calves euthanased after arrival at the slaughter plant. New Zealand has compiled (and reported) on this overall mortality figure for a number of years, and it has enabled tracking of significantly improved performance over time. Linked with feedback to farms of origin (encouraging better performance in calf management and selection), such mortality data recording should enhance both calf welfare and the industry itself in the future.

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9. Appendix 1 – Paper (in press) on calf blood reference ranges

Roadknight, N., N. Courtman, P. Mansell, E. Jongman, Z. Loh, and A. Fisher. In press. Biochemistry, hematology and electrolyte reference intervals for dairy calves aged 5-12 days. Vet. Clin. Pathol.

Biochemistry and hematology reference intervals for neonatal dairy calves aged 5-12 days

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Abstract

Background

Comprehensive hematology and biochemistry reference intervals are currently lacking in the literature for young dairy calves based on sample sizes more than 120. Young dairy calves are at a relatively high risk of poor health and welfare outcomes. They have a high risk of morbidity and mortality in the first 2 weeks of life, and many are transported and fasted during this time. For example, non-replacement calves in Australia and New Zealand are usually 5-12 days old when transported to abattoirs, meaning that calves of this age group are potentially at risk of both health and welfare compromise. Given these factors, sound, comprehensive, age-specific biochemical and hematological reference intervals are needed for both veterinary clinical practice, and to inform research on calf health and welfare.

Objectives

The aim of this study was to generate age-specific hematology and biochemistry reference intervals for dairy calves aged 5-12 days.

Methods

We collected blood samples from 141 fasted, healthy dairy calves on 10 Australian farms. Reference Value Advisor software was used to calculate non-parametric reference intervals for multiple biochemistry and hematology variables.

Results

Reference intervals for a panel of hematology and biochemistry variables in dairy calves aged 5-12 days old were derived.

Conclusions

These reference intervals will be useful for clinical veterinary practice, as well as for research on dairy calf health and welfare.

Keywords

blood, heifer, non-replacement calf, Reference Value Advisor, plasma, serum

Introduction

Young dairy calves are at a relatively high risk of health and welfare challenges due to inadequate quantity, quality and timing of colostrum ingestion, poor hygiene of calf housing, restricted milk feeding and low body fat reserves. High rates of morbidity and mortality in calves can occur during the first 2 weeks of life[1,2]. Consequently, young calves may be the focus of individual and herd health investigations by veterinarians. Additionally, non-replacement calves that are sold for slaughter, and replacement heifers that are transferred to calf rearing facilities, experience the added stressors of fasting and transport. Numerous studies have investigated the welfare impact of transporting and fasting young dairy calves. These studies often include the use of hematology and biochemistry measurements[3-5]. It is important to establish reference intervals that are age-specific because many biochemistry and hematology variables vary with age in cattle[6-10].

Despite the numerous studies on the changes in cattle hematology and biochemistry variables with age, there have been few published studies on hematology and biochemistry reference intervals for young calves[10-13]. Of the studies that have been published, one had a relatively low sample size (41 for the relevant age group) and did not specify whether calves had been fasted or not[11], another reported hematology values only[12] or select biochemistry values only with minimal (4 hour) fasting[10], while another had a smaller than ideal sample size (53)[13].

Additionally, young calf reference intervals for beta-hydroxybutyrate (BHB) have not, to our knowledge, been previously reported. Although BHB is not commonly used diagnostically in calves, it can be a useful research tool, as it indicates a negative energy balance and metabolism of fat (where sufficient body fat is available)[14]. For this reason, BHB has been used in a number of studies on young calf welfare, particularly in relation to fasting and transport[3-5,15].

This study aims to address these knowledge deficits by establishing reference intervals for fasted, 5-12 day old dairy calves using a sample size appropriate for non-parametric statistical methods[16-18]. This allows for a more comprehensive and specific reference interval for use both in clinical veterinary work and for research on young calf health and welfare. We expected that the reference intervals for most variables would be reasonably well aligned with previously reported intervals for young calves, with some discrepancies due to factors such as differences in sample sizes, fasting periods prior to sample collection, genetics, environment, management factors, analytical methods and location. We anticipated that the upper reference limit for BHB in calves would be lower than that for adult cattle because BHB has been shown to increase with age[9].

Materials and methods

Ethics

This research was approved by the University of Melbourne Faculty of Veterinary and Agricultural Sciences Animal Ethics Committee (animal ethics identification number 1814448.2). Research was carried out in compliance with the *Prevention of Cruelty to Animals Act 1986* and the *Australian Code for the Care and Use of Animals for Scientific Purposes 2013.* Farmers/farm managers gave written, informed consent for animals to take part in the research.

Inclusion and exclusion criteria

Farm selection

Participating farms in Northern and Western Victoria, Australia were recruited through local veterinarians, or through contacts provided by other farmers in the area. Farms were not randomly selected, but based on a convenience sample of farms which had calves available at the required time, and whose contact details were provided. Ten farms were visited to sample calves, 9 of which

were commercial dairy farms, and one of which was a dairy research farm. Some farms were visited on multiple days.

Northern Victorian farms (n = 7) were in an inland irrigated area, with mean monthly temperatures ranging from a low of 3°C (38° F) to a high of 32° C (90° F)[19]. Western Victorian farms (n = 3) were in a coastal area, with mean monthly temperatures ranging from a low of 6° C (43° F) to a high of 25° C (77° F)[19].

Calf selection

Blood was collected from 141 calves, in August 2018 and January/February 2019. We aimed to achieve a sample size of 120 calves minimum, in line with recommendations for non-parametric analytical methods[16]. We collected samples from 141 calves to ensure this, while allowing for the fact that some samples may need to be excluded due to post analytical factors. Criteria for inclusion in the study were that calves: were of dairy breed(s); aged 5-12 days old at the time of sampling, fasted from milk for at least 12 hours; were assessed as clinically healthy on physical examination, and had not been given any medication. All calves present on the farms at the time of the visit that fulfilled the inclusion criteria were sampled. Both female (n=128) and male (n= 12) calves were sampled (sex was not recorded for one calf). It was not possible to get even numbers of samples from males and females, as most dairy farmers in Australia do not keep male calves for much longer than 5 days, which is the legal minimum age for calves to travel to sale yards or abattoirs[20]. Calves that were found to have abnormal heart sounds, severe ocular discharge, current diarrhea or pyrexia (rectal temperature >103.1°F (39.5°C)) were excluded from the study – see Figure 1 for details on excluded calves.

Background information about the calves was collected verbally in a standardized questionnaire format from farm owners/managers, and recorded in written form by the researcher. Prior to sample collection, calves were examined either by a veterinarian or by a final year veterinary student under veterinary supervision. Physical examinations included cardiac and pulmonary

auscultation, rectal temperature, mucous membrane color, condition of umbilicus, presence of a suckle reflex, calf demeanor, ocular or nasal discharge, and presence/absence of diarrhea.

Direct, a priori sampling was used.[16] Samples were collected prior to morning feed, between approximately 6.30 and 11.30 am. Apart from a slightly delayed morning feed on the day of sampling, calves were fed, housed and cared for as per normal farm practice. Calves had previously been fed a milk or milk replacer diet once (n = 41) or twice (n = 100) daily, with total daily volume fed ranging between 3.5 and 6 liters. Based on farmer reported feeding times, calves had been fasted for approximately 14-15 hours (n = 89), 18-19 hours (n = 11) or 24-25 hours (n = 41) at the time of sampling. All calves were kept under shelter in bedded pens; most calves (n = 107) were housed in group pens within larger 3-sided sheds, while the remainder of the calves (n = 34) were housed in individual pens under a roof with solid half height walls on 3 sides.

Sample collection and handling

Samples were collected from calves that were manually restrained in either sternal recumbency or in a standing position. Blood was collected by jugular venipuncture using a 20-gauge, 1-inch needle. Blood was collected into a Vacuette® (Greiner Bio-One, USA) 2 mL sodium citrate tube, a Vacuette® 4 mL serum separator tube, a Vacuette® 2 mL lithium heparin tube and a 1 mL K3 EDTA tube, after preparing the venepuncture site with a gauze swab soaked in 70% alcohol. Not all tubes were collected/used for every calf, due to animal ethics reasons (e.g. calf was resisting restraint and sampling was terminated early), or for sample quality reasons (e.g. hemolysis). All tubes were rotated gently at least 6 times, before being placed in a container cooled by ice packs. Serum separator tubes were centrifuged on farm at 3461 g for a minimum of 5 minutes on a Hettich® EBA 20 (Sigma-Aldrich, USA) centrifuge within 4 hours of sample collection (most samples within 1-3 hours of collection). Blood smears were prepared on farm. Samples were then transported chilled directly to a veterinary clinical pathology laboratory approximately 3-4 hours' drive away and refrigerated on arrival. All samples were analyzed within 10 hours of collection.

Citrate tubes noted to be under-filled or over-filled by more than 3mm (n = 11) were excluded from the fibrinogen (modified Clauss method) analysis. Total protein (refractometer) results were excluded if plasma was more than slightly hemolyzed (n = 4). Biochemistry results including electrolytes were also excluded if serum was noted to be more than slightly hemolyzed (n = 12). Hematology and fibrinogen results were excluded if there was evidence of inflammation, based on the presence of bands (more than 0.1 x 10^9/L (adult reference interval for the laboratory)), toxic change in neutrophils (basophilic or foamy cytoplasm, Döhle bodies), or Millar fibrinogen concentration greater than 7.5g/L (adult reference interval for the laboratory, n = 3). Platelet results of samples with platelet clumping noted on the blood smear were excluded (n = 10). Six EDTA tubes were clotted; for these samples, hematology was analyzed on blood from citrate tubes (dilution corrected, n = 5) or a lithium heparin tube (n = 1). Figure 1 summarizes excluded samples.

Laboratory analytical methods

Blood smears were stained with Wright's Giemsa using Siemens Hematek (Siemens, Germany). All blood smears were examined and manual differential counts were performed. Hematology was analyzed on a Sysmex XT-2000i analyzer (Sysmex, Japan), fibrinogen on the Stago Compact Max (Stago, France), and manually by the Millar method for a subset of samples (n = 50)[21]. Serum biochemistry was analyzed using a Cobas Integra® 400 Plus biochemistry analyzer (Roche, Switzerland). Electrolytes were measured on an IDEXX VetLyte Na+K+Cl- analyzer (IDEXX, USA). A subset of samples were also analyzed for total protein using a hand held refractometer (REF312ATCbp, Bacto Laboratories, Australia) (n = 54). See Tables 1 and 2 for details on the reagents used for analysis. The biochemistry assays in this study are commonly used in commercial laboratories and have undergone a general validation in the laboratory.

Statistical analysis

The number of calves enrolled in this study was based on guidelines from the American Society for Veterinary Clinical Pathology[18]. Reference intervals were calculated using Reference Value Advisor Software[22]. Non-parametric methods were used for all variables with more than 120 samples, therefore, normality and distribution tests were not required for these variables[18], though histograms for all variables were visually inspected for evidence of any unusual/unexpected distributions or data points. Outliers were retained unless there were pre-analytical factors or post-analytic factors that could account for the results. This resulted in one calf being excluded with a high outlier CK result as it was known to have had increased handling compared to other calves. For variables with less than 120 samples, data distribution was assessed using a combination of visual assessment using histograms and Q-Q plots, the Anderson-Darling test for normality and the symmetry test for the Robust method, both before and after Box-Cox transformation. For total protein (refractometer, n=54), data were assessed as non-Gaussian and non-symmetrical before and after transformation using the above methods. Therefore, the non-parametric method was used for this variable despite the smaller sample size. For Millar fibrinogen (N=50) and plateletcrit (N = 105), the data were assessed visually as non-Gaussian and non-symmetrical before and after transformation, and so the non-parametric RI was also used for these variables. Platelet distribution width, platelet large cell ratio and mean platelet volume (all n = 105), were also assessed by non-parametric methods as the sample sizes were large enough, though their data were assessed visually as approaching Gaussian distribution.

The effects of sex on blood variables were not analyzed, due to the lack of male calves in the sample (only 9% of calves were male). Similarly, the effects of breed on the blood variables were not analyzed, as approximately half the calves were Holstein-Friesian, with the other half almost exclusively being Holstein-Friesian crossbreds, making comparative analysis of limited use.

For BHB, results for 51 calves were left censored due to being less than the limit of detection for the analyzer (0.1 mmol/L). Reference Value Advisor was not used for this variable and instead, non-parametric bootstrap reference intervals, confidence intervals and median were calculated

using the MASS and boot packages in R statistical software[23]. The accelerated bias-corrected percentile limits method was utilized, using 10,000 bootstrap samples.[24, 25] Maximum likelihood estimation was used to calculate the mean and standard deviation for BHB, using the R package fitdistrplus. The log normal distribution was chosen as the best fit for the data, based on visual inspection of plotted predicted versus actual values, as well as the Akaike information criterion and the Bayesian information criterion. Values for the mean and standard deviation generated in the log normal scale were then transformed to the linear scale using the R package tsiMisc.

Results

Calves' breeds were reported by owners/managers as Holstein-Friesian (n =63), Holstein-Friesian/Jersey cross (n = 42), Holstein-Friesian/Jersey/Australian Red cross (n = 34), Jersey (n = 1), and dairy cross breed (n = 1).

Reference intervals for hematology, biochemistry and electrolyte variables for dairy calves are presented in Tables 3 and 4, including 90% confidence intervals for the upper and lower limits and select descriptive statistics.

Discussion

The reference intervals presented in this paper will be useful both as a diagnostic tool for veterinarians treating sick dairy calves, and as a research tool. Dairy calves are often fasted and transported at a young age, either prior to slaughter or for transfer to a rearing facility. In countries such as Australia and New Zealand, non-replacement calves are fasted and transported to abattoirs as young as 5 days old,[20] but this is likely to range from between 5-12 days old due to the pick-up schedules of calf transporters. Previous research on the effect of transport or fasting on calf welfare has utilized hematology and biochemistry, either comparing these values over time[3-5] or between

different groups of calves[15,26]. The reference intervals presented here for 5-12 day old calves are a useful reference for future studies in this area.

The reference intervals in this study are in broad agreement with previous studies[11-13]. Relatively minor differences between studies are seen for all analytes, probably reflecting differences in sample sizes, genetics, environmental factors, management factors, and/or analytical methods[16]. The upper reference limit for BHB was, as expected, lower than that reported for adult cattle (0.59 mmol/L for calves compared to 1.00 mmol/L for adult cattle)[27]. We believe that our study represents the most comprehensive source of young calf biochemistry and hematology reference intervals, and the only young calf reference interval for BHB.

Some potential limitations of this study relate to calf management on the farms. While the reference intervals presented in this paper are likely to reflect expected values in healthy calves on typical Australian dairy farms, this does not necessarily mean that calves had been managed to the best standards possible. For example, the reference interval reported in this paper for total protein is 44-82 g/L, but values for total protein below 50-52 g/L in young calves are regarded as being indicative of failure of passive transfer of immunity[28,29]. In the current study, 7% of included calves had total protein results that indicated failure of passive transfer (<52 g/L), which means that these calves are at a higher risk of morbidity and mortality[30,31]. However this rate of failure of passive transfer is still much lower than previously reported in Victoria[28], and the first author (NR) considered the participating dairy farms to be representative of conditions that would be found on average Australian dairy farms.

Despite being assessed as reflecting normal industry standards, a relatively large number of calves (35 calves, or 15% of available calves) were not sampled due to evidence of illness on physical examination (Figure 1). Of these, most were excluded due to severe ocular discharge (12 calves) or diarrhea (11 calves); other reasons including pyrexia, cardiac murmurs, or prior illness. The rates of illness in the calves, and the proportion of calves sampled that had failure of passive transfer, indicate that, although these calves were considered to be managed in a way that was broadly

consistent with standard industry practices, this may not reflect the gold standard of calf management, and, therefore, calf health.

Another limitation of the study relates to calf feeding practices on farms. While individual animal intakes were not recorded, calves were fed an average of 4.3L per day each, with a range of 3.5-6L. The lower to middle end of this range represents approximately 10% of body weight per day. There is now a large body of scientific evidence that supports the feeding of higher volumes (generally around 15-20% of body weight) for improved calf growth, health, and future milk production (reviewed in Khan et al 2011 and Kertz et al 2017)[32-34]. In addition, 3 of the 10 farms participating in this study fed calves once a day, despite Dairy Australia guidelines recommending that calves less than 2 weeks old are fed a minimum of twice daily.[34] These factors mean that some of the reference intervals reported here may be different if calves were fed more often and with greater volumes, in line with current recommendations. Variables that are likely to be most affected by feeding regimes and fasting times are plasma glucose and serum BHB, with a higher glucose reference interval and a lower BHB upper limit expected with increased feed volume and frequency of feeding.

Another point to note is that the assays that we used in this study had not been specifically validated for cattle, as they form a minority of our laboratory caseload, and species validation of these assays was beyond the scope of this study. This would be a worthwhile future study.

Conclusion

Young dairy calves are particularly vulnerable to poor health and welfare outcomes; high morbidity and mortality rates have been reported for the first 2 weeks of life, and calves often undergo stressful management practices such as transport and fasting. This has made young calves a focus of research on calf health and welfare, which often utilizes biochemistry and hematology measurements. Our results support the hypothesis that our reference intervals would be similar to those that have been published previously, with some small differences likely due to variations in

sample size, genetics, animal management and location, and methodology. Our results also support our hypothesis that the upper reference limit of BHB for calves would be lower than that for adult cattle. The comprehensive biochemistry and hematology reference intervals presented here for fasted calves aged 5-12 days old can be utilized both for clinical veterinary practice and for research purposes.

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Table 1. Reagents used for hematology analysis

Reagent	Use
Cellpack	Red blood cell, platelet and hemoglobin diluent; rinsing of instrument; hydrodynamic focusing (Sysmex, Japan)
Stromatolyser-4DL	Differential lysing reagent (Sysmex, Japan)
Stromatolyser-4DS	Differential stain (Sysmex, Japan)
Stromatolyser-FB	Diluent for white blood cell count and differential lysing agent (Sysmex, Japan)
Sulfolyser	Sodium lauryl sulphate, a non-cyanide hemoglobin lysing agent (Sysmex, Japan)

Ret-Search (II) Diluent	Dilutes sample for reticulocyte analysis (Sysmex, Japan)
Ret-Search (II) Dye	Stains reticulocytes and platelets for analysis (Sysmex, Japan)

Table 2. Reagents used for biochemistry analysis

Variable	Reagent
Albumin (g/L)	Albumin Gen.2, (Roche, Switzerland)
ALP (U/L)	ALP IFCC Gen.2, (Roche, Switzerland)
AST (U/L)	Aspartate Aminotransferase Liquid Reagent, (Roche, Switzerland)
BHB (mmol/L)	D-3-Hydroxybutyrate, (kinetic enzymatic assay) (Randox Laboratories, UK)
Calcium (mmol/L)	Calcium Gen.2, (Roche, Switzerland)
Cholesterol (mmol/L)	Cholesterol Gen.2, (Roche, Switzerland)
Creatine kinase (U/L)	Creatine Kinase Gen.2 Liquid Reagent, (Roche, Switzerland)
Creatinine (µmol/L)	Creatinine Jaffé Gen.2 Compensated Method for Serum and Plasma, (Roche, Switzerland)
GGT (U/L)	γ-Glutamyltransferase ver.2 (enzymatic colorimetric assay); (Roche, Switzerland)
GLDH (U/L)	GLDH Gen.3 Utility Cassette Set, (Roche, Switzerland)
Globulin (g/L)	Calculated (Protein g/L – Albumin g/L)
Glucose (mmol/L)	Glucose HK Gen.3 (enzymatic reference method with hexokinase); (Roche, Switzerland)
Magnesium (mmol/L)	Magnesium, (Roche, Switzerland)
Phosphorous (mmol/L)	Phosphate (inorganic) ver.2, (Roche, Switzerland)
Total protein (g/L)	Total Protein Gen.2 (colorimetric biuret); (Roche, Switzerland)
Triglycerides (mmol/L)	Triglycerides, (Roche, Switzerland)
Urea (mmol/L)	Urea (kinetic test with urease and glutamate dehydrogenase); (Roche, Switzerland)
Sodium (mmol/L)	Ion selective electrode, (VetLyte Idexx USA)
Potassium (mmol/L)	Ion selective electrode, (VetLyte Idexx USA)

Chloride (mmol/L)	Ion selective electrode, (VetLyte Idexx USA)
Total protein (g/L)	Refractometer (Bacto Laboratories, Australia)
Millar fib (g/L)	Millar heat precipitation
Modified Clauss fib (g/L)	STA®Liquid Fib with Owen-Koller Buffer

Abbreviations: ALP, alkaline phosphatase; AST, aspartate aminotransferase; BHB, beta-hydroxybutyrate, GGT, gamma-glutamyl transferase; GLDH, glutamate dehydrogenase; fib, fibrinogen.

Table 3. Hematology reference values for 5-12 day old Australian dairy calves

Variable	Units	Mean	Median	SD	Min	Max	LRL (90%CI)	URL (90%CI)	n
WBC	x 10^9/L	9.6	9.3	2.9	4.0	21.2	4.8 (4.0-5.2)	16.3 (14.2-21.2)	135
RBC	x 10^{12}/L	9.0	9.1	1.2	5.2	12.3	6.2 (5.2-7.3)	11.9 (10.9-12.3)	135
Hemoglobin	g/L	114	116	17	64	151	73 (64-84)	148 (139-151)	135
Hematocrit	L/L	0.35	0.35	0.05	0.19	0.47	0.23 (0.19-0.26)	0.45 (0.42-0.47)	135
MCV	fL	38.4	38.3	2.7	26.5	44.5	33.1 (26.5-34.5)	44.2 (42.8, 44.5)	135

MCH	pg	13	13	1	9	15	11 (9-12)	14 (14-15)	135
MCHC	g/L	328	329	9	297	348	309 (297-312)	346 (343-348)	135
Reticulocytes	x 10^9/L	10	6	13	0	85	-	51 (32, 85)	135
RCDW-SD	fL	36.1	35.9	3.3	26.8	60.2	31.2 (26.8-32.4)	41.6 (40.2-60.2)	135
RCDW-CV	%	29.4	29.3	2.8	23.9	49.7	25.5 (23.9-25.9)	34.1 (32.9-49.7)	135
Platelets	x 10^9/L	752	769	238	161	1313	238 (161-308)	1213 (1159-1313)	124
PDW	fL	7.9	7.9	0.7	6.3	10.4	6.7 (6.3-7.0)	9.6 (9.0-10.4)	105
MPV	fL	6.5	6.5	0.3	5.7	7.4	6.0 (5.7-6.0)	7.3 (7.0-7.4)	105
PLCR	%	4.9	4.6	1.9	1.5	10.7	1.7 (1.5-2.1)	10.0 (7.8-10.7)	105
Plateletcrit	L/L	0.005	0.005	0.001	0.001	0.008	0.002 (0.001-0.002)	0.008 (0.007-0.008)	105

Neutrophils	x 10^9/L	4.6	4.1	2.9	0.6	20.7	0.9 (0.6-1.3)	13.0 (9.2-20.7)	135
Lymphocytes	x 10^9/L	4.3	4.4	1.9	0.1	10.3	0.1 (0.1-0.1)	8.4 (7.1-10.3)	135
Monocytes	x 10^9/L	0.6	0.5	0.5	0.0	2.3	-	1.7 (1.4-2.3)	135
Eosinophils	x 10^9/L	0.1	0.0	0.1	0.0	0.5	-	0.4 (0.2-0.5)	135
Basophils	x 10^9/L	0.1	0.1	0.1	0.0	0.4	-	0.3 (0.2-0.4)	135

Reference intervals determined with Reference Value Advisor macro for Excel[22], using the nonparametric method. Abbreviations: SD, standard deviation; Min, minimum; Max, maximum; LRL, lower reference limit; URL, upper reference limit; CI, confidence interval; WBC, white blood cells; RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RCDW-SD, red cell distribution width standard deviation; RCDW-CV, red cell distribution width coefficient of variation; PDW, platelet distribution width; MPV, mean platelet volume; PLCR, platelet larger cell ratio.

Table 4. Biochemistry, electrolyte and fibrinogen reference values for 5-12 day old Australian dairy calves

Variable	**Units**	**Mean**	**Median**	**SD**	**Min**	**Max**	**LRL (90%CI)**	**URL (90%CI)**	**n**
Albumin	g/L	32	32	2	27	37	27 (27-28)	36 (36-37)	129

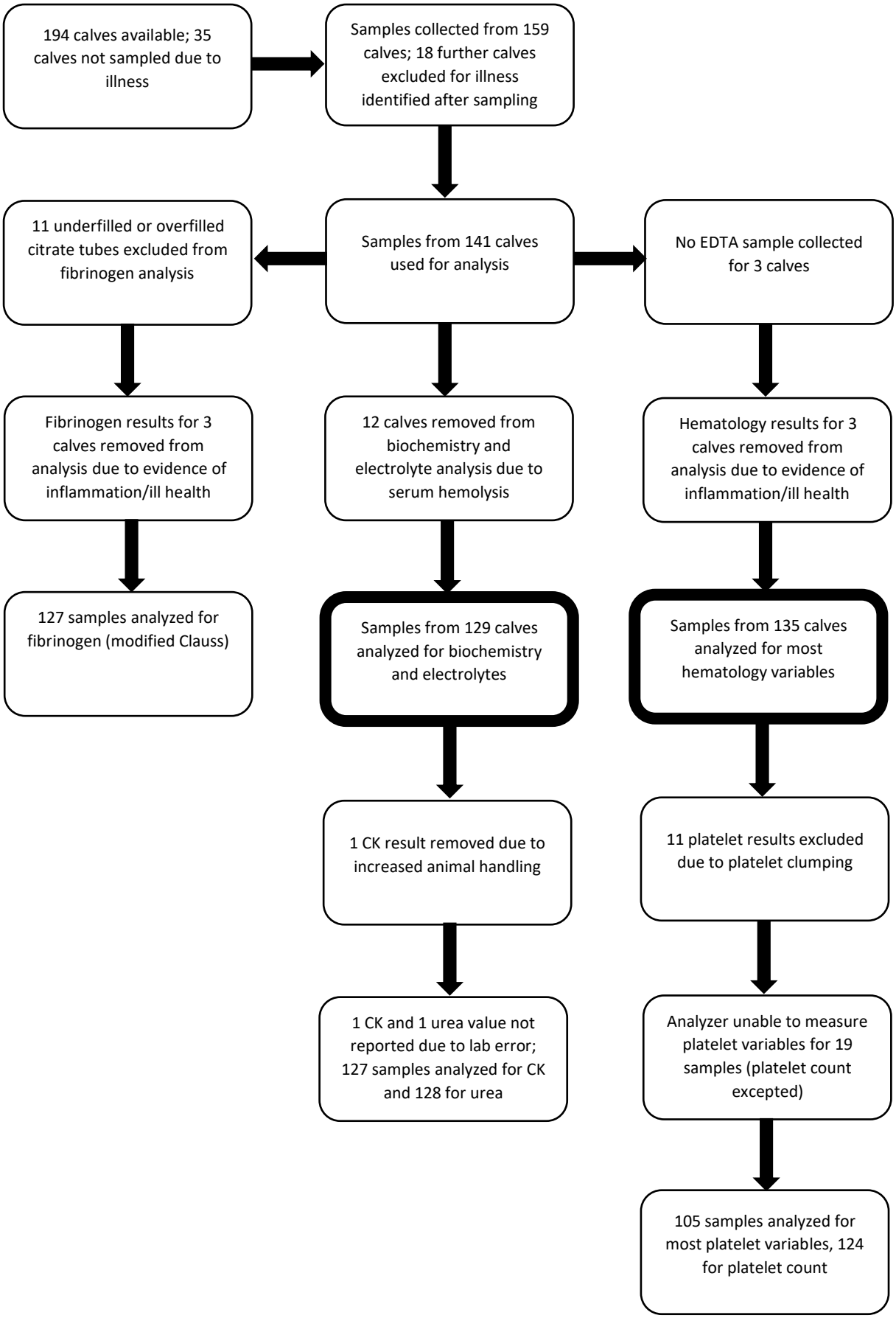
Globulin	g/L	31	31	10	11	54	14 (11-16)	53 (50-54)	129
Total protein	g/L	62	63	9	44	84	45 (44-48)	82 (80-84)	129
Total protein (refractometer)	g/L	67	65	8	50	78	50 (50-52)	78 (73-78)	54
ALP	U/L	293	313	105	123	738	152 (123-166)	578 (468-738)	129
GGT	U/L	215	297	269	26	1379	32 (26-47)	1037 (823-1379)	129
GLDH	U/L	13	17	14	5	111	5 (5- 6)	60 (36-111)	129
AST	U/L	35	35	6	24	58	25 (24-28)	51 (49-58)	129
Creatine kinase	U/L	92	117	70	34	432	46 (34-50)	326 (250-432)	127
Glucose	mmol/L	5.6	5.4	1.0	2.3	7.1	2.8 (2.3-3.3)	6.9 (6.8, 7.1)	129
BHB	mmol/L	0.11	0.16	0.14	<0.1	0.94	-	0.59 (0.44, 0.80)	129
Cholesterol	mmol/L	2.0	2.0	0.7	0.5	4.2	0.9 (0.5-1.0)	3.6 (3.3-4.2)	129

Triglycerides	mmol/L	0.3	0.3	0.2	0.1	1.2	0.1 (0.1-0.1)	1.0 (0.8-1.2)	129
Creatinine	µmol/L	75	74	14	45	118	50 (45-52)	109 (101-118)	129
Urea	mmol/L	4.1	4.2	1.3	1.1	8.4	1.6 (1.1-2.2)	7.7 (6.3-8.4)	128
Calcium	mmol/L	2.77	2.74	0.18	2.39	3.55	2.47 (2.39-2.53)	3.15 (3.07 3.55)	129
Magnesium	mmol/L	0.9	0.9	0.1	0.8	1.2	0.8 (0.8-0.8)	1.1 (1.1-1.2)	129
Phosphorous	mmol/L	2.8	2.7	0.3	2.3	3.3	2.3 (2.3-2.3)	3.2 (3.2-3.3)	129
Sodium	mmol/L	143	143	4	124	152	130 (124-134)	148 (147-152)	129
Potassium	mmol/L	5.76	5.70	0.48	4.20	7.20	4.75 (4.20-5.00)	6.75 (6.60-7.20)	129
Chloride	mmol/L	102	103	3	95	108	96 (95-98)	106 (106-108)	129
Millar fibrinogen	g/L	4.8	4.7	0.9	3.0	6.6	3.1 (3.0-3.6)	6.5 (6.1-6.6)	50

Modified Clauss fibrinogen	g/L	4.00	4.01	0.90	2.00	6.52	2.32 (2.00-2.57)	5.70 (5.34-6.52)	127

Reference intervals determined with Reference Value Advisor macro for Excel[22], using the nonparametric method, except for BHB, calculated using a bootstrap bias corrected and accelerated method. Abbreviations: SD, standard deviation; Min, minimum; Max, maximum; LRL, lower reference limit; URL, upper reference limit; CI, confidence interval; ALP, alkaline phosphatase; AST, aspartate aminotransferase; BHB, beta-hydroxybutyrate, GGT, gamma-glutamyl transferase; GLDH, glutamate dehydrogenase.

Figure 1. Exclusion flowchart for biochemistry and hematology samples. CK = creatine kinase.


194 calves available; 35 calves not sampled due to illness
Samples collected from 159 calves; 18 further calves excluded for illness identified after sampling
11 underfilled or overfilled citrate tubes excluded from fibrinogen analysis
Samples from 141 calves used for analysis
No EDTA sample collected for 3 calves
Fibrinogen results for 3 calves removed from analysis due to evidence of inflammation/ill health
12 calves removed from biochemistry and electrolyte analysis due to serum hemolysis
Hematology results for 3 calves removed from analysis due to evidence of inflammation/ill health
127 samples analyzed for fibrinogen (modified Clauss)
Samples from 129 calves analyzed for biochemistry and electrolytes
Samples from 135 calves analyzed for most hematology variables
1 CK result removed due to increased animal handling
11 platelet results excluded due to platelet clumping
1 CK and 1 urea value not reported due to lab error; 127 samples analyzed for CK and 128 for urea
Analyzer unable to measure platelet variables for 19 samples (platelet count excepted)
105 samples analyzed for most platelet variables, 124 for platelet count