Metabolic Investigations into Dairy Cow Longevity

by

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DVM (Distinct.), MPVM, DACVPM, DABVP (Dairy Practice)

A thesis submitted in fulfilment of

the requirements of the Degree of

Doctor of Philosophy

School of Life and Environmental Sciences

Faculty of Science

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STATEMENT OF ORIGINALITY

I hereby certify that the content of this thesis is my own work and has not been submitted for any other degree or purpose.

I, David Sheedy, affirm that the intellectual content of this thesis is the result of my own original work. All assistance received in the preparation of this thesis has been properly acknowledged.

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ABSTRACT

Dairy cow longevity is a multifactorial outcome determined by a combination of health, reproduction, production, management practices, and chance events. Optimising longevity can reduce the demand for replacement heifers, enable diversification into beef production, reduce lifetime emission per litre of milk produced, improve feed efficiency, and enhance the social license of the dairy industry. Increasing age and parity are associated with increased risk of adverse health events, reproductive inefficiency, culling and mortality, and prevent optimal herd longevity. Despite knowing that these poor outcomes increase with age, the underlying metabolic changes that occur with aging have received little attention. Identifying the physiological changes that occur with age and how these modulate cow health and survival may reveal opportunities to improve herd longevity and optimise production. This thesis explored physiological aspects of cow longevity through three primary investigations: 1) body tissue reserves and association with dairy housing systems of pasture-based production and confinement-based production, parity and blood metabolites; 2) association between age, housing system, reproduction and health events; and 3) lipidomic and standard metabolite panel profile's associations with cow age, housing systems, and survival. The study design enabled multiple lines of inquiry from a single set of biological samples. Thirty farms (15 pasture-based, 15 total mixed ration, confinement farms) contributed 29 randomly selected, parity stratified cows to both a dry-cow and peak-milk cow cohort, totalling approximately 1,700 samples. Liquid chromatography – mass spectrometry targeted lipidomics identified 185 lipids species in plasma, and colorimetric analysis measured 15 other metabolites in serum. Novel statistical approaches that included machine learning, cluster analysis, and variable stability selection were used to address each chapter's objectives, and to present interpretable and actionable results. The first chapter of the thesis reviewed the role of blood glycerophospholipids, specifically the subclasses of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine, in dairy cattle health and longevity and provided rationale for a lipidomic approach to investigate aging and survival. Topics discussed in Chapter 1 included the oxidative pathway theory of aging, 1-carbon metabolism, the endocannabinoid system, phosphoinositides, alternate liver phosphatidylcholine pathways to mediate hepatic lipidosis, and the bioavailability of different dietary forms of poly-unsaturated fatty acids. Chapters 2 and 3 examined the association between measures of body tissue reserves, including a novel metric that combined body condition score and body weight, with

parity, housing systems, and a standard panel of serum metabolites. Higher parity cows had greater BW but lower BCS compared to lower parity cows. Albumin was consistently associated with all metrics, suggesting protein metabolism efficiency is an important determinant of body tissue reserves. Chapter 4 explored the relationship between parity, housing systems and the important longevity-related outcomes of reproduction, mastitis, and lameness. This chapter introduced the Dairy UP database, which integrates multiple data sources that include different herd management software, pasture information, milk production and quality, and weather. Increasing parity was associated with reduced reproductive efficiency, and increased hazards of mastitis and lameness. Housing system did not differ for reproductive or lameness risk, but confinement-based farms had increased mastitis risk compared to pasture-based farms across all parity groups. Chapters 5, 6 and 7 explored lipidomic associations with 1) age and parity, 2) housing systems, and 3) survival, respectively. Glycerophospholipids containing omega-3 fatty acids had reduced plasma concentrations in older cattle compared to younger cattle, had lower concentrations in confinement-based housing systems compared to pasture-based systems, and lower concentrations were associated with increased hazards of culling. Collectively, these findings suggest omega-3 fatty acid pathways may be altered with age and could partially explain the increased risk of adverse outcomes in older cattle. We speculated that diet was the major differentiating factor between housing system lipid profiles, with the corn-silage based total mixed rations provided in confinement-based farms containing greater amounts of omega-6 fatty acids and less omega-3 fatty acids compared to pasture-based diets. This body of work used an exploratory, hypothesis-generating approach to dairy cow longevity and represents the first application of lipidomics to characterise aging and survival in dairy cattle. It identified associations between housing systems and blood metabolite profiles, and introduced novel statistical approaches to high-dimensional agricultural data. The thesis provides actionable targets for interventional studies aimed at optimising cow longevity. A logical follow-up to this thesis would be long-term, randomised controlled feeding trials of lipids identified by this thesis, particularly omega-3 fatty acids, to determine whether targeted lipid supplementation can: 1) shift the lipid profile of older cows toward a more youthful phenotype; 2) shift the lipid profile of confinement-based cows towards a more pasture-based phenotype; and 3) ultimately reduce the risk of adverse health events, reproduction failure, and removal. Developing practical strategies to improve the lipid balance and mitigate age-associated metabolic changes could substantially enhance optimisation of cow longevity and the long-term sustainability of the dairy industry.

ACKNOWLEDGMENTS

The acknowledgement page is both a wonderful opportunity for me to express sincere thanks and appreciation to the many, many people who have guided, aided, and cajoled me through the completion of my thesis. It is also simultaneously a wholly inadequate vehicle to address the depth of gratitude I owe you all.

Firstly, I wish to thank my supervisory team of Ian Lean, Helen Golder, and Sergio "Yani" Garcia. Ian – I cannot express how profoundly lucky I feel to have landed under your mentorship. Your borderline pathological compulsion to constantly remind me to "return to your hypothesis" is now so thoroughly ingrained in me that I am sure to be on sound footing for any future endeavours. However, the value of your academic rigour and advice simply pales into statistical insignificance compared to the value of your camaraderie and friendship. Thank you.

Helen – Thank you for your guiding hand throughout the project, your fine-toothed-comb edits, the shared tears over the database, for being the disembodied voice that would answer questions I yelled through my wall, and for your friendship and support. I fear dire repercussions if I also failed to give specific thanks to both you and Ian for your invaluable assistance during fieldwork, especially when I was otherwise incapacitated through various injuries or the coming of Kai.

Yani – Thank you for your constant support, for always providing a solid sounding board, helping me navigate the intricacies of the University system, and for the much-needed feedback when manuscripts began to wander and lose focus.

I also extend my greatest thanks to everyone at the *Scibus* office, including Jo Brown, Yatza Grbic-Clinch, and Neil Moss. You have all made me and my little crew feel welcomed and at home. Thank you to Andrew Lean for taking on the huge burden of work with the Dairy UP database. Without your efforts, I would not have had a spare brain-cell left to function.

Thank you to the team at DairyBio/AgriBio who lent me their 'omics' expertise and laboratory know-how, without which I would have been completely lost. With special thanks to Priyanka Reddy for your encouragement and mentorship, Zhiqian Liu for introducing me to the wonderful world of lipidomics, and Simone Rochfort for guiding the project and making it all possible.

I am also grateful to the Veterinary Medicine Teaching and Research Centre team in Tulare, California, with a particularly special thanks to Deniece Williams and Sharif Aly. Thank you for your continued friendship, for opening the door to my journey into research and statistical rigour, and (to Deniece) for quite literally opening the door to your home.

Thank you to the 30 farms directly involved in this thesis, and for your ongoing support of the Dairy UP project. Ultimately, these research efforts are intended to benefit the entire dairy industry, but they would not be possible without your time and commitment, for which we are all truly grateful. I also thank the broader Dairy UP team, including co-researchers, committee members, and funders. It has been wonderful to collaborate with such dedicated people, and I hope we can continue this work going forward. I truly believe the results of this thesis will provide an important foundation for optimising the longevity of dairy cattle.

Finally, I wish to thank my family: Mum and Dad, Catherine, Elizabeth, Paul, Grant, and Alex. You are the most special and dear people to me. Kai-kun, thank you for the happiness you have brought into our lives and for teaching me the true value of sleep – I'll be sure to repay that favour in the future! And above all, I cannot thank enough my beautiful wife, Miki, the true unsung hero of this PhD journey. いつも支えてくれて、本当にありがとう。

PREFACE

All chapters of this thesis have been written in publication style. Accordingly, the General Introduction and Thesis Outline provide the overarching background, scope, and justification for the body of work. Chapters 1, 2, 3, 5, 6 and 7 have been published in top international peer-reviewed journals, as indicated on their cover-pages, and are reproduced in this thesis in their respective published journal formats. Chapters 1 and 7 were *in-press* at the time of submission and presented in the formatting style of this thesis. Chapter 4 is intended to be submitted for peer-review publication with minor modifications. David Sheedy was listed as the first author on all chapters except for chapter 2, where he was listed as a joint first author, and as the corresponding author. The Australian English language is used throughout, except in the published peer-reviewed chapters that have been published in United States of American English. Assistance given by others is indicated in the *Author Attribution Statements* section.

FINANCIAL SUPPORT

This research was supported by the Postgraduate Research Scholarship in Dairy Science from Dairy UP project (https://dairyup.com.au), a joint project between the University of Sydney (Camperdown, NSW, Australia), Scibus (Camden, NSW, Australia), the New South Wales Department of Primary Industry and Regional Development (Orange, NSW, Australia), and Dairy Australia (Southbank, VIC, Australia); funded through the Australian and New South Wales Government's Bushfire Local Economic Recovery Fund (Orange, NSW, Australia) with co-contributions by Australian Fresh Milk Holding Ltd. (Orange NSW, Australia), Dairy Australia (Southbank, VIC, Australia), DairyNSW (Shellharbour, NSW, Australia), eastAUSmilk (North Quay, QLD, Australia), Hunter Local Land Services (Taree, NSW, Australia), Leppington Pastoral Co. (Bringelly, NSW, Australia), Norco Dairy Co-Op (South Lismore, NSW, Australia), NSW Farmers (St Leonards, NSW, Australia), the New South Wales Department of Primary Industries and Regional Development (Orange, NSW, Australia), Scibus (Camden, NSW, Australia), South East Local Land Services (Bega, NSW, Australia), and the University of Sydney's Dairy Research Foundation (Brownlow Hill, NSW, Australia). This research was also supported by an Australian Government Research Training Program fee offset scholarship.

AUTHOR ATTRIBUTION STATEMENTS

I, David Sheedy, confirm that the following authorship attribution statements provided in this thesis are accurate and reflect the contributions of all listed authors.

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CHAPTER 1: Glycerophospholipids in dairy cow health and longevity: A review

I, David Sheedy, was the primary author of Chapter 1 of this thesis, which has been published in the Journal of Dairy Research (*In-press*). Conceptualization was performed by myself; funding acquisition by H.Golder, S.Garcia and I.Lean; investigation by myself; methodology by myself; project administration and resource management H.Golder, S.Garcia, I.Lean, and myself; supervision by H.Golder, S.Garcia and I.Lean; visualisation by myself; original draft by myself; and final reviews and edits were performed by all co-authors.

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CHAPTER 2: Holstein dairy cows lose body condition score and gain body weight with increasing parity in both pasture-based and total mixed ration herds

Chapter 2 of this thesis has been published in the Journal of Dairy Science Communications, Volume 3, Issue 6, Page 431 – 435, 2022. I, David Sheedy, shared primary authorship with Adjunct Professor I.Lean and am the corresponding author of this publication. Conceptualization was performed by I. Lean, and H.Golder; data curation by I.Lean, S.LeBlanc, T.Duffield, J.Santos, H.Golder, and myself; formal analysis by I.Lean, and myself; funding acquisition by I.Lean, S.LeBlanc, T.Duffield, J.Santos, and H.Golder; investigation by I.Lean, H.Golder, and myself; methodology by I.Lean, and myself; software by myself; project administration and resource management by I.Lean, and H.Golder; supervision by I.Lean, and H.Golder; validation by I.Lean, H.Golder ,and myself; visualisation by I.Lean, H.Golder, and myself; original draft by myself; and final reviews and edits were performed by all co-authors.

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CHAPTER 3: Associations among body condition score, body weight, and serum biochemistry in dairy cows

I, David Sheedy, was the primary author of Chapter 3, which has been published in the Journal of Dairy Science, Volume 108, Issue 4, Page 4131 – 4148, 2025. Conceptualization was performed by I.Lean, and myself; data curation by myself; formal analysis by I.Lean, D.Vincent, and myself; funding acquisition by H.Golder, S.Garcia, J.Pryce, and I.Lean; investigation by H.Golder, P.Reddy, J.Hemsworth, D.Vincent, I.Lean, and myself; methodology by P.Reddy, J.Hemsworth, D.Vincent, and myself; software by J.Hemsworth, D.Vincent, and myself; project administration and resource management by H.Golder, S.Garcia, S.Rochfort, J.Pryce, I.Lean, and myself; supervision by H.Golder, S.Garcia, P.Reddy, S.Rochfort, and I.Lean; validation by P.Reddy, J.Hemsworth, D.Vincent, and I.Lean; visualisation by H.Golder, I,Lean, and myself; original draft by myself; and final reviews and edits were performed by all co-authors.

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CHAPTER 4: Reproduction, mastitis, and lameness in confinement and pasture-based systems: Associations with parity

I, David Sheedy, was the primary author of Chapter 4 of this thesis. Conceptualization was performed by I.Lean, and myself; data curation by H.Golder, A.Lean, and myself; formal analysis by I.Lean, and myself; funding acquisition by H.Golder, S.Garcia, and I.Lean; investigation by I.Lean, and myself; methodology by myself; software by myself; project administration and resource management by H.Golder, S.Garcia, I.Lean, and myself; supervision by H.Golder, S.Garcia, and I.Lean; validation by I.Lean; visualisation by H.Golder, I,Lean, and myself; original draft by myself; and final reviews and edits were performed by all co-authors.

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CHAPTER 5: A large, multi-site lipidomic investigation of parity and aging in dairy cows

I, David Sheedy, was the primary author of Chapter 5, which has been published in the Journal of Dairy Science, Volume 108, Issue 3, Page 2897 – 2913, 2025. Conceptualization was performed by H.Golder, I.Lean, and myself; data curation by myself; formal analysis by I.Lean, and myself; funding acquisition by H.Golder, S.Garcia, J.Pryce, and I.Lean; investigation by H.Golder, P.Reddy, Z.Liu, I.Lean, and myself; methodology by Z.Liu, P.Reddy, I.Lean, and myself; software by Z.Liu, P.Reddy, and myself; project administration and resource management by H.Golder, S.Garcia, S.Rochfort, J.Pryce, I.Lean, and myself; supervision by H.Golder, S.Garcia, Z.Liu, P.Reddy, S.Rochfort, and I.Lean; validation by Z.Liu, P.Reddy, and I.Lean; visualisation by H.Golder, I,Lean, and myself; original draft by myself; and final reviews and edits were performed by all co-authors.

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CHAPTER 6: Confinement and pasture-based dairy herds differ in plasma lipid profiles

I, David Sheedy, was the primary author of Chapter 6, which has been published in the Journal of Dairy Science, Volume 108, Issue 11, Page 12421-12437, 2025. Conceptualization was performed by H.Golder, I.Lean, and myself; data curation by myself; formal analysis by I.Lean, and myself; funding acquisition by H.Golder, S.Garcia, J.Pryce, and I.Lean; investigation by H.Golder, P.Reddy, Z.Liu, J.Hemsworth, D.Vincent, I.Lean, and myself; methodology by Z.Liu, P.Reddy, J.Hemsworth, D.Vincent, I.Lean, and myself; software by Z.Liu, P.Reddy, J.Hemsworth, D.Vincent, and myself; project administration and resource management by H.Golder, S.Garcia, S.Rochfort, J.Pryce, I.Lean, and myself; supervision by H.Golder, S.Garcia, Z.Liu, P.Reddy, S.Rochfort, and I.Lean; validation by Z.Liu, P.Reddy, J.Hemsworth, D.Vincent, and I.Lean; visualisation by H.Golder, I,Lean, and myself; original draft by myself; and final reviews and edits were performed by all co-authors.

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CHAPTER 7: A large, multi-site investigation into the lipidomics of survival in dairy cows

I, David Sheedy, was the primary author of Chapter 7 of this thesis, which has been published in the Journal of Dairy Science, Volume 109, Issue 3, Page 2969-2988. Conceptualization was performed by H.Golder, I.Lean, and myself; data curation by myself; formal analysis by I.Lean, and myself; funding acquisition by H.Golder, S.Garcia, J.Pryce, and I.Lean; investigation by H.Golder, P.Reddy, Z.Liu, J.Hemsworth, D.Vincent, I.Lean, and myself; methodology by Z.Liu, P.Reddy, J.Hemsworth, D.Vincent, I.Lean, and myself; software by Z.Liu, P.Reddy, J.Hemsworth, D.Vincent, and myself; project administration and resource management by H.Golder, S.Garcia, S.Rochfort, J.Pryce, I.Lean, and myself; supervision by H.Golder, S.Garcia, Z.Liu, P.Reddy, S.Rochfort, and I.Lean; validation by Z.Liu, P.Reddy, J.Hemsworth, D.Vincent, and I.Lean; visualisation by H.Golder, I,Lean, and myself; original draft by myself; and final reviews and edits were performed by all co-authors.

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ARTIFICIAL INTELLIGENCE

During the preparation of this thesis I, David Sheedy, used ChatGPT 4o (https://chat.openai.com), to assist in debugging R-language errors (https://www.R-project.org), minor edits to grammar, and for one image creation that has been appropriately acknowledged and cited (Chapter 1). The author confirms that where text was modified by generative AI, the content was thoroughly reviewed for possible errors, inaccuracies, and bias. Generative AI was not used to perform any statistical analysis, interpretate results and no confidential information or data was shared. The author takes full responsibility for the submitted thesis and ensures the work is their own and has used generative AI in accordance with University guidelines and policies.

LIST OF PUBLICATIONS FROM THESIS

Peer-reviewed publications

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Sheedy, D.B., H.M. Golder, S.C. Garcia, Z. Liu, P. Moate, P. Reddy, S.J. Rochfort, J.E. Pryce, and I.J. Lean. 2025. A large multisite lipidomic investigation of parity and aging in dairy cows. J. Dairy Sci. 108:2897–2913. doi:10.3168/jds.2024-25578.

Sheedy, D.B., H.M. Golder, S.C. Garcia, P. Reddy, J.E. Hemsworth, D.E. Vincent, S.J. Rochfort, J.E. Pryce, and I.J. Lean. 2025. Associations among body condition score, body weight, and serum biochemistry in dairy cows. J. Dairy Sci. 108:4131-4148 doi:10.3168/jds.2024-25425.

Sheedy, D.B., H.M. Golder, S.C. Garcia, Z. Liu, P. Reddy, S.J. Rochfort, J.E. Hemsworth, D.E. Vincent, J.E. Pryce, and I.J. Lean. 2025. Confinement and pasture-based dairy herds differ in plasma lipid profiles. Journal of Dairy Science. doi.org/10.3168/jds.2025-26782.

Sheedy, D.B., H.M. Golder, S.C. Garcia, Z. Liu, P. Reddy, S.J. Rochfort, J.E. Hemsworth, D.E. Vincent, J.E. Pryce, and I.J. Lean. 2026. A large, multisite investigation into the lipidomics of survival in dairy cows. J. Dairy Sci. 109:2969-2988. doi.org/10.3168/jds.2025-27687.

Sheedy, D.B., H.M. Golder, S.C. Garcia, and I.J. Lean. Accepted 2026. Glycerophospholipids in dairy cow health and longevity: A review. J. Dairy Res. (*Accepted, In-Press*)

Peer-reviewed conference abstracts

Sheedy, D.B., H.M. Golder, S.C. Garcia, Z. Liu, P. Reddy, S.J. Rochfort, J.E. Hemsworth, D.E. Vincent, J.E. Pryce, and I.J. Lean. 2025. Jun. 22. Variable stabilization in adaptive lasso cox frailty models to explore lipidomic associations with survival of dairy cows. Animal Science Modellers Special Interest Group. Louisville, Kentucky, USA.

Sheedy, D.B., H.M. Golder, S.C. Garcia, Z. Liu, P. Reddy, P. Moate, S.J. Rochfort, J.E. Pryce, and I.J. Lean. 2025. Jun. 22-25. Do cows in total mixed ration systems have old lipid

profiles compared with pasture-based cows? J. Dairy Sci. 108 (Suppl.1): 1445. Louisville, Kentucky, USA

Sheedy, D.B., H.M. Golder, S.C. Garcia, P. Reddy, J.E. Hemsworth, D.E. Vincent, S.J. Rochfort, J.E. Pryce, and I.J. Lean. 2024. Jun. 16-19. Associations among body condition score, body weight, and serum biochemistry in dairy cows. J. Dairy Sci. 107 (Suppl.1): 2306. West Palm Beach, Florida, USA. (Poster*)

Sheedy, D.B., H.M. Golder, S.C. Garcia, P. Reddy, J.E. Hemsworth, D.E. Vincent, S.J. Rochfort, J.E. Pryce, and I.J. Lean. 2023. Nov. 11-15. Associations among body condition score, body weight, and serum biochemistry in dairy cows. 17th International Symposium on Veterinary Epidemiology and Economics, Sydney, New South Wales, Australia. (Poster*)

Sheedy, D.B., H.M. Golder, S.C. Garcia, and I.J. Lean. 2023. Nov 7-8. Culling reasons on Australian dairy farms. Dairy Research Foundation Symposium. Camden, New South Wales, Australia.

Non peer-reviewed conference presentations

Sheedy, D.B., H.M. Golder, S.C. Garcia, Z. Liu, P. Reddy, S.J. Rochfort, J.E. Hemsworth, D.E. Vincent, J.E. Pryce, and I.J. Lean. 2025. Nov 26. Life and death in pasture and TMR farms - the fat?. Dairy Research Foundation Symposium, Wollongong, New South Wales, Australia.

Sheedy, D.B., H.M. Golder, S.C. Garcia, Z. Liu, P. Reddy, S.J. Rochfort, J.E. Hemsworth, D.E. Vincent, J.E. Pryce, and I.J. Lean. 2024. Nov 11. Metabolic investigation into dairy cow longevity. School of Life and Environmental Sciences Higher Degree Research Showcase, Sydney, New South Wales, Australia.

Sheedy, D.B., H.M. Golder, S.C. Garcia, and I.J. Lean. 2024. Feb 27-28. Survival and longevity. Raising the roof. Hunter Valley, New South Wales, Australia

Sheedy, D.B., H.M. Golder, S.C. Garcia, and I.J. Lean. 2023. Nov 6. Insights into exit reasons between intensively housed and pasture-based dairy systems. School of Life and Environmental Sciences Higher Degree Research Showcase, Sydney, New South Wales, Australia. (Poster*)

Sheedy, D.B., H.M. Golder, S.C. Garcia, and I.J. Lean. 2022. Nov 11. A metabolomic investigation into Australian dairy cow longevity. School of Life and Environmental Sciences Higher Degree Research Showcase, Sydney, New South Wales, Australia.

*Posters are available to view in the supplementary documents: https://doi.org/10.6084/m9.figshare.29456534.

ABRREVIATIONS

The following abbreviated terms have been used throughout the thesis and are defined at first used in each chapter. Abbreviations that are used exclusively in Tables or Figures are not listed here and are defined within their respective Tables or Figures.

%	percentage
±	plus-minus
µL	microlitre(s)
µm	micrometre(s)
µmol	micromole(s)
100DICR	100 day in-calf rate
2-AG	2-arachidonoylglycerol
A:G	albumin:globulin ratio
AA	amino acids
AEA	n-arachidonoylethanolamine
AIC	Akaike information criterion
AKT	protein kinase b
ALA	alpha-linolenic acid
ARA	arachidonic acid
ANOVA	analysis of variance
ASCA	ANOVA simultaneous component analysis
AT	adipose tissue
AU	Australia
AUC	area under the receiver operator curve
BCG	body classification group
BCS	body condition score
BHB or BHBA	β-hydroxybutyrate acid
BW	body weight
CAN	Canada
CDP	cytidine diphosphate
CI	confidence interval
CONFINE	confinement-based dairy system, according to lactating herd housing
CP	crude protein
CVal	cross validation
d	day(s)
DBC	days before calving
DBE	double bond equivalent
DGLA	dihomo-γ-linolenic acid
DHA	docosahexaenoic acid
DIM	days in milk
dL	decilitre(s)
DMI	dry matter intake
DPA	docosapentaenoic acid
E.coli	*Escherichia coli*
ECS	endocannabinoid system
EPA	eicosapentaenoic acid
EPO	external parameter orthogonalization

FA	fatty acyls or fatty acids
FDR	false discovery rate
g	gram(s)
g	gravitational force equivalent
GA	genetic algorithm
GEA	Gesellschaft für Entstaubungsanlagen
h	hour(s)
HCULL	hazards of culling
HETE	8,11,13-eicosatrienoic acid
HLAME	hazards of lameness
HMAST	hazards of mastitis
HMORT	hazards of mortality
HPREG	hazards of pregnancy
HR	hazard ratio
kg	kilogram(s)
L	litre(s)
LA	linoleic acid
LASSO	least absolute shrinkage and selection operator
LC-MS	liquid chromatography mass spectrometry
LC-PUFA	long-chain polyunsaturated fatty acids
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
m/z	mass-to-charge ratio
MCP	minimax concave penalty
mg	milligram(s)
min	minute(s)
mL	millilitre(s)
MLR	multiple linear regression
mm	millimetre(s)
m*M*	millimole(s) per litre
mmol	millimole(s)
MP	metabolisable protein
MUN	milk urea nitrogen
n	number
NEFA	non-esterified fatty acids
NSW	New South Wales
NZ	New Zealand
^{o}C	degrees Celsius
OEA	oleoylethanolamide
O-PLS	orthogonal partial least squares
O-PLS-DA	orthogonal partial least squares discriminant analysis
P	probability
PAST or PB	pasture-based dairy system, according to lactating herd
PC	phosphatidylcholine
PCA	principal component analysis
PC-O or PC(O)	ether-linked phosphatidylcholine
PE	phosphatidylethanolamine
PEMT	phosphatidylethanolamine N-methyltransferase
PE-O or PE(O)	ether-linked phosphatidylethanolamine
PI	phosphatidylinositol

PI3K	phosphoinositide-3-kinase
PIP	phosphatidylinositol phosphates
PL	glycerophospholipid or phospholipid
PS	phosphatidylserine
PUFA	poly-unsaturated fatty acids
QC	quality control
RF	random forest
RMSE	root mean square error
RMSECV	root mean square error of cross-validation
RMSEP	root mean square error of prediction
ROC	receiver operator curve
RPC	rumen-protected choline
s	second(s)
SCAD	smoothly clipped absolute deviation
SD	standard deviation
SE	standard error
SM	sphingomyelin
spp.	species
SQL	structured query language
SR	selectivity ratio
SVM	support vector machines
TG	triacylglycerol
TMR	total mixed ration
VIC	Victoria
VLDL	very low-density lipoproteins
w/w	weight per weight
wk	week(s)
yr	year(s)

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CHAPTER 1: Glycerophospholipids in dairy cow health and longevity: A review

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

Dairy cow longevity is determined by a combination of health, reproduction, milk production, management practices, and stochastic events. While definitions of longevity vary, this thesis defines longevity as the length of productive life from first calving to culling or death (Compton et al., 2017; Schuster et al., 2020). Extending cow longevity offers several potential benefits, including a reduced demand for replacement heifers and the opportunity to diversify revenue streams through beef production (Clasen et al., 2019; Hüneke et al., 2025); the improved feed efficiency in older cows (Hurley et al., 2018); reduced lifetime emissions per litre of milk produced, driven by improved lifetime feed efficiency and longer productive lifespans (Browne et al., 2014; Lehmann et al., 2019; De Vries and Marcondes, 2020); and enhancing the social licensing of the dairy industry (Wolf and Tonsor, 2017; De Vries, 2020). Any negative impact to herd genetic improvement from extended longevity is minimal, with strategic genomic selection and sexed-semen use capable of completely mitigating the impact (De Vries, 2017, 2020; Clasen et al., 2019).

Despite these potential benefits, increasing cow longevity is currently challenged by the strong associations between increased age or parity and increased risk of adverse health events (Jamali et al., 2018; Oehm et al., 2019; Lean et al., 2023b), poor reproductive performance (Moss, 2001; Bonneville-Hébert et al., 2011; Lean et al., 2023a), and ultimately, a greater likelihood of involuntary culling and mortality (Stevenson and Lean, 1998; Miller et al., 2008; Pinedo et al., 2010; Lean et al., 2023a). As a result, producers require a sufficiently high replacement rate to replace not only cows that were involuntarily culled or died, but to also lower the number of older cows in their herds to reduce the herd-level risk of future undesirable outcomes. Although replacing older cows with young stock may reduce the herd-level risk of adverse events, this strategy may be sub-optimal if it leads to premature culling of cows that could have remained productive and profitable, and prevents producers from realising the potential benefits of increased cow longevity (De Vries, 2020; De Vries and Marcondes, 2020; Hüneke et al., 2025).

If the risk of adverse health and reproductive events in older cows could be reduced, producers would have the option to shift herd management strategies towards a lower replacement rate and extend longevity. However, while substantial collective research efforts have focused on individual diseases and reproductive challenges, there have been no similar effects into investigating the broader biological changes that occur with the aging process in

dairy cattle. This lack of research interest is somewhat surprising given the consistent associations between parity and incidence of diseases and reproductive failure. Understanding the biological underpinnings of aging in cows may reveal novel targets for intervention that allow cows to maintain their health and productivity as they age.

A compelling platform for investigating the biology of dairy cow aging is lipidomics, the large-scale study of lipids. The important role of lipid metabolism in cattle health and reproduction has long been established (Yamdagni and Schultz, 1970; Reid, 1983; Bauchart, 1993; Ospina et al., 2010). The continued advances in laboratory analytical technology now allow precise quantification of very-low abundance, polar and non-polar lipids, including their stereochemistry (Liu and Rochfort, 2023), facilitating the identification of clinically relevant bioactive lipids. With lipidomics now an accessible research tool, there have been an increase in cow lipid metabolism studies, reflected by recent reviews on topics of sphingomyelin and ceramide metabolism (McFadden and Rico, 2019), omega fatty acids (Sordillo, 2016; Moallem, 2018; Fabjanowska et al., 2023) and the endocannabinoid system (Myers et al., 2021; Zachut et al., 2025). Lipidomics is also being used to study healthy aging and age-related diseases in humans (Gonzalez-Covarrubias, 2013; Almeida et al., 2021). To date, translation from exploratory or associative lipidomic studies to interventional studies (Kra et al, 2022; Myers et al, 2019; Tate et al, 2024) and on-farm application is limited (Zhao et al, 2025). Improving the generation of actionable outcomes from lipidomic analyses will depend on adoption of standardised lipid reporting (Liebisch et al. 2020), larger and more diverse study cohorts, and hypothesis-driven intervention trials informed from prior exploratory results. Given these developments, lipidomics was considered an appropriate analytical platform to investigate the biological changes associated with cattle aging for this thesis. The lipid subclass of glycerophospholipids was the specific topic for the literature review (Chapter 1), and lipidomics data was used in the final three chapters of the thesis (Chapter 5 – 7).

While lipidomics may provide novel insights into cow aging, more accessible physical metrics, including body condition score (**BCS**) and body weight (**BW**), remain important for assessing the health of dairy cows. During the transition from late-pregnancy to peak-milk production, cows mobilise their stored energy and protein reserves to meet the homeorhetic demands of milk production (Bauman and Bruce Currie, 1980; Baumgard et al., 2017). However, excessive mobilisation has been associated with increased disease incidence, reproductive inefficiencies, poor cow welfare, and is observed more often in high parity cows (Berry et al., 2006; Roche et al., 2009; Truman et al., 2022). Although BCS and BW are the

most commonly used metrics to assess body tissue reserves, their correlation is considered only low to moderate (Berry et al., 2006; Roche et al., 2007), and is influenced by differences in visceral adipose tissue (Drackley et al., 2014), stage of lactation or pregnancy, water intake (Moe et al., 1971), gut fill, and parity (Berry et al., 2006; Truman et al., 2022). To address this limitation, Chapters 2 and 3 introduce a novel combined BCS – BW classification scheme. Chapter 2 explores how BCS, BW, and the new body composition metric are associated with parity and dairy production housing system, while Chapter 3 explores the associations between body-tissue metrics and a standard metabolite panel.

Another important consideration for cow longevity is the structural differences of the farming systems in which cows are managed. There are two broad dairy production housing systems: extensive, pasture-based systems and intensive, confinement-based systems. The Australian dairy industry is currently undergoing a rapid transition from milk being produced almost exclusively from pasture-based farms to confinement-based systems now contributing approximately 20% of the national milk production, with expectations that 35-40% of milk will be produced from cows in confinement farms by 2030 (Rennie, 2024). This transition is being driven largely by motivations to expand business enterprises, an improved ability to mitigate extreme climatic events, and the decreasing availability of irrigation water (Rogers et al., 2022). As the transition from pasture to confinement systems is recent and ongoing, there was a unique research opportunity to evaluate longevity-associated performance outcomes and biological differences between the systems of dairying in a contemporary setting. Chapter 4 specifically investigates the risk profiles for reproductive outcomes and the adverse health events of mastitis and lameness in the context of the dairy production housing systems and cow parity. Chapter 6 investigates the biological differences between cows from the two housing systems of dairy production.

The overall aim of this thesis was to investigate the metabolic aspects of aging and survival in dairy cattle. This was achieved in three primary investigations: 1) body tissue reserves and their association with housing systems, parity and blood metabolites; 2) association between age, housing system, reproduction and health events; and 3) lipidomic and metabolite profile associations with cow age, housing systems, and survival. We hope that results from this thesis will provide fertile grounds for further investigation that may ‘de-risk’ old cattle, and allow producers the option to increase the longevity of their herds. In doing so, producers and the dairy industry could benefit from to increased production, increased feed efficiency, reduced emissions, and enhanced social licensing.

THESIS OUTLINE

This thesis has been written in publication style, with each chapter either submitted or intended for submission to peer-reviewed journals. Each chapter is a stand-alone manuscript, including its own abstract, introduction, materials and methods, results, discussion, and conclusion, as appropriate.

This thesis begins with a literature review on potential associations between glycerophospholipids and cow longevity (Chapter 1); followed by two chapters on a novel scoring system that combined BCS and BW and its associations with parity, housing system of dairy production (Chapter 2), and a standard metabolite panel (Chapter 3); a descriptive analysis of reproductive and health outcome differences between dairy production housing systems and parity (Chapter 4); and three chapters on the associations between plasma lipids and age or parity (Chapter 5), housing system of dairy production (Chapter 6), and survival (Chapter 7). The thesis ends with a general discussion and a conclusion.

Chapter 1 is a literature review of blood glycerophospholipids and their association with dairy cattle health, reproduction, and longevity. The review first introduces the glycerophospholipid class, their general structure and relevance to cattle health. The review then focuses specifically on the glycerophospholipid subclasses of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine. Topics discussed over the course of the review include aging, hepatic lipidosis, phosphoinositides, and the endocannabinoid system. As this review was intended for publication, it includes references from chapters within this thesis.

Chapter 2 introduces a novel body scoring system that incorporates both BCS and BW into a single metric. This was a retrospective study that utilised raw data from 16 different studies that were performed across different housing systems and nations. The association between BCS, BW, and the novel body composition metric was explored in relation to parity and housing system. Increased parity was associated with increased BW and decreased BCS.

Chapter 3 expands on the scoring system introduced in Chapter 2 and investigates the association between the BCS, BW, the body-composition metric, and a standard metabolite blood panel. Albumin was consistently positively associated with high BCS, high BW, and high milk production. This was the first chapter to introduce the sampling frame that is also used in Chapters 5 through to 7. In brief, the sampling frame was 15 pasture-based farms and 15

confinement-based farms, with two cohorts of cows sampled from each farm (a dry cow and peak-milk cow cohort) and stratified on parity groups. There were approximately 1,700 blood samples taken across the two cohorts.

Chapter 4 explores the association between parity, housing systems, and outcomes of importance to dairy production; reproduction, mastitis, and lameness. This chapter utilises a database composed of herd management software records that was created specifically for the Dairy UP project (https://dairyup.com.au) and contained approximately 100,000 lactation records for analysis. Weibull parametric survival models were used to investigate the hazards of pregnancy, mastitis, and lameness. The effect of increasing parity was consistently associated with reduced hazards of pregnancy, and increased hazards of mastitis and lameness. In contrast, the effect of dairy housing system had limited to no effect on the hazards of pregnancy or lameness. However, the hazards of mastitis were significantly higher in confinement-based farms, compared to pasture-based farms.

Chapter 5 through to 7 utilised a targeted lipidomics dataset that contained 185 lipid classes, including glycerophospholipids, sphingomyelins, and triacylglycerols. Chapter 5 investigated the association between the lipidomic data and age or parity. The glycerophospholipids that contained very-long chain, poly-unsaturated fatty acids, including docosahexaenoic acid (C22:6n-3; **DHA**), docosapentaenoic acid (C22:5n-3; **DPA**), and eicosapentaenoic acid (C20:5n-3; **EPA**) were lower in older or high-parity cattle.

Chapter 6 investigated the farm-level associations between lipids and housing system. The dataset used in Chapter 6 also included a standard metabolite blood panel. The results indicated that glycerophospholipids associated with omega-3 fatty acids were decreased in cows from confinement-based systems, compared to cows in pasture-based systems, regardless of stage of production. Glycerophospholipids associated with omega-6 fatty acids were increased in confinement-based cows at peak milk, compared to pasture-based cows at peak milk.

Chapter 7 is the final chapter and utilised the combined lipidomic and standard metabolite panel to investigate metabolic associations with the hazards of culling and mortality. The analysis of this chapter was particularly challenging, owing to a larger than predicted right-censoring of cows (reducing the power of the analysis), the high correlation of the lipid data, and the potential influence of survival bias. The lipid data was generally a very poor predicator of survival when sampled from dry-cows, while results from peak-milk cows produced many

associations between lipids and survival, and these associations differed for different parity groups.

The thesis ends with a general discussion on the key findings, future research direction and concluding remarks.

A brief note on the nomenclature of dairy housing system used throughout this thesis: Chapters 2, 3 and 5 refer to confinement-based housing systems as total-mixed ration (**TMR**) systems. While this definition was strictly true for all enrolled farms, it is not universally true that all confinement-based systems use a TMR. Following suggestions from the peer-review process of Chapter 6, we adopted the acronyms of **CONFINE** for farms using intensive, confinement-based housing systems, and **PAST** for those using extensive, pasture-based cattle, based upon the housing system of the lactating herd. These acronyms are used in Chapters 4, 6 and 7.

NOTES

This thesis refers to supplementary files and data not suitable for print. These files are available as a Figshare repository at https://doi.org/10.6084/m9.figshare.29456534.

NON-STANDARD ABBREVIATIONS

BCS = body condition score; BW = body weight; CONFINE = confinement-based housing systems; DHA = docosahexaenoic acid; DPA = docosapentaenoic acid; EPA = eicosapentaenoic acid; PAST = pasture-based housing system; TMR = total-mixed ration

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CHAPTER 1

Glycerophospholipids in dairy cow health and longevity: A review

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OVERVIEW OF CHAPTER 1

This Chapter reviews the current literature on the glycerophospholipid lipid class, and its associations with outcomes that may influence the longevity of dairy cattle. The glycerophospholipid class and their general structure and properties is first introduced before addressing the specific topics of glycerophospholipids that contain omega fatty acids, and the subclasses of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine. The oxidative pathway theory of aging, 1-carbon metabolism, the endocannabinoid system, and phosphoinositides are discussed. This chapter provided background information and the rationale to perform lipidomic investigations into cow longevity, as presented in Chapters 5 through to 7.

ABSTRACT

This review examines the role glycerophospholipids (**PL**) in dairy cow health, with specific focus on phosphatidylcholine (**PC**), phosphatidylethanolamine (**PE**), phosphatidylinositol (**PI**) and phosphatidylserine (**PS**). Increasing parity of cows is associated with lower concentrations of plasma PL that contain very long-chain omega-3 fatty acids, including docosahexaenoic acid and eicosapentaenoic acid, which are precursors for prostaglandin synthesis, and have anti-inflammatory roles. Low concentrations of these PL could plausibly contribute to the increased risk of disease, reproductive failure and mortality in older cows. The bioavailability and metabolism of fatty acids may differ among supplements that are predominately neutral lipids such as triacylglycerol-rich oils, and those bound to PL including pasture, whole or ground oilseeds, and fish meal. Hepatic lipidosis can occur during the transition period if there is insufficient very-low density lipoproteins (**VLDL**) production in the liver to transport lipids into blood circulation. The PC are the primary PL of VLDL and are produced by two main pathways in the liver, the cytidine diphosphate-choline pathway that uses choline as a substrate, and the phosphatidylethanolamine *N*-methyltransferase pathway that uses PE and methyl-donors as substrates. Co-supplementation strategies that target both pathways may increase PC production over a one-pathway supplementation strategy. The PI are phosphoinositides precursors, which have broad physiological roles including regulating inflammatory processes and may offer targets for novel treatment and management of disease. Both the PI and PE are precursors to endocannabinoids, important regulators of energy metabolism, immune function and reproduction in mammals. Early findings on the endocannabinoid system in transition dairy cows yielded results that diverge from non-ruminant models. The PS expression on cytoplasmic membranes signals apoptosis, coagulation and contributes to sperm-oocyte recognition. As lipidomic diagnostics become increasingly available, understanding the metabolism of PL will continue to develop and promises to offer novel strategies for optimizing cattle health and longevity.

Keywords

Phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, endocannabinoid, omega fatty acids, aging

INTRODUCTION

Lipid metabolism influences all aspects of dairy production and health. Lipids are broadly classified into eight categories; glycerophospholipids (**PL**), fatty acyls or fatty acids (**FA**), glycerolipids, sphingolipids, sterol lipids, phenol lipids, saccharolipids and polyketides (Sud et al., 2007). The PL typically contribute around 41-50% (w/w%) of serum or plasma lipids, with cholesteryl esters (44-50%), unesterified cholesterol (5-9%), free FA (1-2%) and triacylglycerols (trace-3%) also present (Yamdagni and Schultz, 1970; Raphael et al., 1973; Bitman et al., 1984). Though much dairy research has been performed on free or non-esterified fatty acids (**NEFA**), until recently there has been comparatively little emphasis on the PL class. Advances in and availability of bioinformatics and lipidomics are enabling researchers to precisely quantify low-abundance lipids, including the FA composition of specific PL classes (Zehethofer and Pinto, 2008; Li et al., 2014; Han and Gross, 2021; Liu et al., 2025). Subsequently, specific PL and their FA compositions are increasingly recognised as being significantly associated with dairy cows health and longevity (Humer et al., 2016; Rico et al., 2021; Sheedy et al., 2026). These roles include hepatic lipid export during the transition period, aging processes, reproduction and modulation of inflammatory responses. The purpose of this narrative review was to 1) introduce readers to basic glycerophospholipid biochemistry, 2) consolidate current knowledge on the major PL classes in adult dairy cows, 3) to identify emerging evidence on the role of PL in circulation and tissue in relation to cattle longevity through their associations with age, health and reproduction, and 4) highlight areas for further research. Unless otherwise stated, all references are to adult cattle.

This review should be considered in the context of dietary precursors for FA, especially the essential FA that are not only potent anti-inflammatory agents, antioxidants but precursors for critical hormones that have profound effects on metabolism. For details on other lipid categories and classes on dairy production, we direct readers to reviews on sphingolipids (McFadden et al., 2019), essential or omega FA (Palmquist, 2009; Rodney et al., 2015; Moallem, 2018; Veshkini et al., 2023) and endocannabinoids (Myers et al., 2021; Zachut et al., 2025).

OVERVIEW OF GLYCEROPHOSPHOLIPID BIOCHEMISTRY

The basic structure of a PL is the glycerol backbone that has three attachment sites denoted as sn-1, sn-2 and sn-3. Generally, the sn-1 position is esterified to saturated FA, sn-2 to unsaturated FA and sn-3 always attached to a phosphate moiety (Yamashita et al., 2014). Provided that both the sn-1 and sn-2 positions are attached to FA, ester linkages between the sn-3 phosphate and head groups of choline, ethanolamine, inositol or serine create the phosphatidylcholine (**PC**), phosphatidylethanolamine (**PE**), phosphatidylinositol (**PI**) and phosphatidylserine (**PS**) lipid classes, respectively (Figure 1). For ease of discussion, this review will examine the influence of each of these PL on cattle health longevity in turn, while recognising this categorisation is a necessary simplification of the interconnected nature of PL metabolism and that the functional boundaries of PL are not discrete (Figure 1).

The two most abundant PL are PC and PE and have an overall neutral charge that are composed of a positive head group balanced by the negative phosphate at sn-3 (zwitterionic), whereas PI and PS are overall negatively charged with neutral head groups. The consequence of having hydrophilic head groups and long, hydrophobic FA tails allow PL to form the cellular phospholipid bilayers and lipoprotein micelles for which they are most well recognized. There can be profound differences in the relative abundance of PL classes between the two leaflets of a membrane bilayer, as well as among the cytoplasmic and intracellular organelle membranes. These differences contribute to the unique structural and functional properties of cells and their compartments (Holthuis et al., 2003; Harayama and Riezman, 2018). The PS are a notable example, with essentially all cytoplasmic membrane PS on the cytosolic side and none exposed to extracellular space (Leventis and Grinstein, 2010; Fairn et al., 2011).

The enzymatic breakdown of dietary PL and triglycerides in the rumen to free-FA is rapid, and most PL in the body must therefore be synthesized *de novo* from glycerol-3-phosphate (Dawson and Hemington, 1974; Jenkins et al., 2008; Moate et al., 2008). Figure 1 shows the most common pathways for PL production, but notably omits lysophospholipids, the subcellular compartmentalisation of enzymatic reactions and FA composition or remodelling pathways, all of which are important aspects of lipid metabolism.

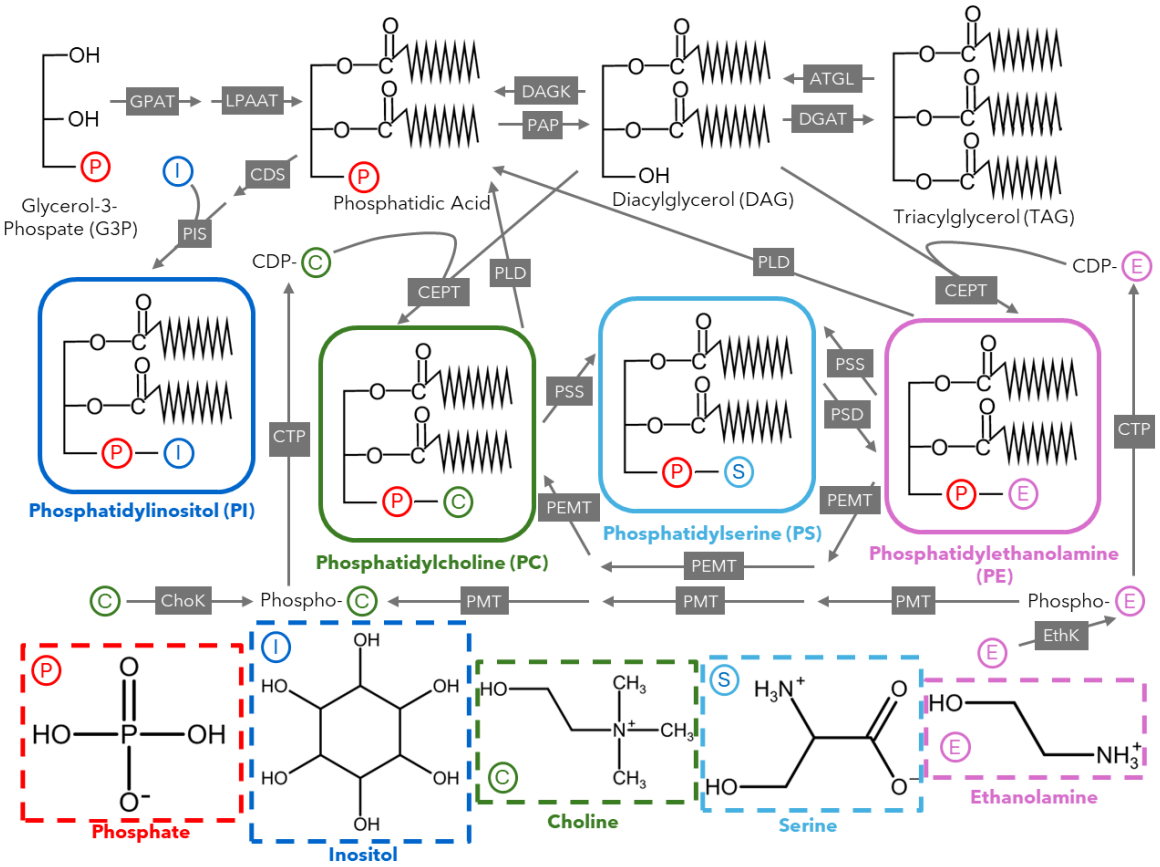

Figure 1: Glycerophospholipid metabolism depicting the most common pathways for production of phosphatidic acid, phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylethanolamine (PE). Enzymes are in grey boxes; fatty acyl chains are simplified as wavy chains esterified to the glycerol backbone. Phosphate and the specific head groups are depicted by the circled coloured letter described in the bottom of the figure. Intracellular locations and lysophospholipids are not shown.
ATGL - adipose triglyceride lipase; CDP - cytidine diphosphate; CDS - phosphatidate cytidylyltransferase; CEPT - choline/ethanolamine phosphotransferase; ChoK - choline kinase; CTP – cytidylyltransferase; DAGK - diacylglycerol kinase; DGAT - Diacylglycerol acyltransferases; EthK - ethanolamine kinase; GPAT - glycerol-3-phosphate acyltransferase; LPAAT - lysophosphatidic acid acyltransferases; PAP - phosphatidate phosphatase; PEMT - phosphatidylethanolamine-N-methyltransferase; PIS - phosphatidylinositol synthase; PLD - phospholipase D; PMT - Phosphoethanolamine methyltransferases; PSD - phosphatidylserine decarboxylase; PSS - phosphatidylserine synthase.
Figure adapted from KEGG glycerophospholipid metabolism and Cayman Chemical lysophospholipid signalling poster (map00564) (Kanehisa et al., 2025; Cayman Chemical)

Fatty acids that esterify to PL can be obtained from the diet, rumen microbe *de novo* synthesis or *de novo* synthesis by the cow. Although the fat content in pasture-fed cows may contain more than 80% as the essential omega-3 FA alpha-linolenic acid (C18:3;n-3 **ALA**), the extensive lipolysis and biohydrogenation of fats by rumen microbial activity result in duodenal flow of mostly (~90%) saturated and mono-saturated FA (Scollan et al., 2001; Jenkins et al., 2008; Glasser et al., 2013). Despite this, the cow requires sufficient ALA and linoleic acid (C18:2; n-6) to reach the duodenum, as these FA can only be obtained from the diet and are important precursors for the bioactive very-long chain omega FA (see section: Glycerophospholipids and long chain polyunsaturated fatty acids).

The PL are transported by the circulatory system in water-soluble lipoproteins that are composed of cholesterols, triacylglycerols, PL and specialised proteins called apolipoproteins. Lipoproteins are categorized in increasing density order as: chylomicrons, very low-density lipoproteins (VLDL), intermediate density lipoproteins, low density lipoproteins and high-density lipoproteins (Bauchart, 1993). Generally, triacylglycerol content decreases and PL increases with increasing lipoprotein-density (Raphael et al., 1973; Grummer and Davis, 1984). The VLDL are typically reported as very low in ruminants, that is <5% of lipoproteins in plasma or serum (Raphael et al., 1973; Grummer and Davis, 1984). Duran et al. (2021) reported approximately 15-20% using a high resolution polyacrylamide gel electrophoretic assay, and suggested an insensitivity of previous assays to the relatively high saturated FA content, due to extensive rumen biohydrogenation, in bovine VLDL compared to monogastric animals. Regardless, the VLDL are the primary method to transport triacylglycerols from the liver into circulation and compromised VLDL export may contribute to lipid accumulation in the liver during the transition period (See section *Phosphatidylcholine: Hepatic lipidosis)*.

GLYCEROPHOSPHOLIPIDS AND LONG CHAIN POLYUNSATURATED FATTY ACIDS

The PL are important donors and transporters of FA and are subsequently involved in FA metabolism (Engelmann and Wiedmann, 2010). Of specific relevance to dairy cow health and production are the very long-chain polyunsaturated FA (**LC-PUFA**), including the omega FA of docosahexaenoic acid (C22:6n-3; **DHA**), eicosapentaenoic acid (C20:5n-3; **EPA**) and arachidonic acid (C20:4n-6; **ARA**) (Palmquist, 2009; Moallem, 2018). The omega FA are active in signalling pathways, including the eicosanoid and inflammatory pathways (Goerig et al., 1985; Funk, 2001; Palmquist, 2009) and are positively associated with cattle health

(Silvestre et al., 2011; Moallem et al., 2020; Veshkini et al., 2023), reproduction (Rodney et al., 2015; Sinedino et al., 2017; Moallem, 2018; Moallem et al., 2020), inter-generational health (Gulliver et al., 2012; Roque-Jiménez et al., 2021) and aging (Sheedy et al., 2025). To avoid replication of work from other authors, this review limits discussion to the specific implications of LC-PUFA when esterified to PL and not their metabolism after disassociation from PL, for which we refer readers to other reviews (Palmquist, 2009; Rodney et al., 2015; Moallem, 2018; Veshkini et al., 2023).

Aging

The risk of mortality and disease increase with increasing parity of dairy cows (Lean et al., 2023a) and could be related to age-associated metabolic changes. There are few cattle studies that have investigated lipid changes specifically associated with increasing age or parity. In non-ruminant studies, the 'oxidative-stress' theory of aging suggests that reactive oxygen species damage biological molecules and promote cellular senescence (Pamplona et al., 2002; Hulbert et al., 2007). Relatedly, the 'cell membrane' or 'membrane pacemaker' theory of aging mechanistically recognizes an association between declining membrane integrity with the progression of age (Hulbert, 2005; Calhoon et al., 2015; Das, 2021). In support of these theories is the observation that long-lived species, such as humans and horses, have relatively low concentrations of membrane PUFA compared to short-lived species such as mice and guinea pigs (Hulbert et al., 2007; Zimniak, 2011; Alba Naudí et al., 2013; Mariona Jové et al., 2013; Zaloga, 2021) and FA double bonds are at greater risk of peroxidation compared to saturated FA (Holman, 1954; Bielski et al., 1983; Niki, 2009; Yin et al., 2011).

Peroxidation of membrane lipids is associated with signalling for oxidative cell death (Dixon and Olzmann, 2024) and decreased membrane fluidity (Choe et al., 1995). Membrane fluidity is an important component of membrane integrity and is increased by increasing the PC:PE of membranes (Fajardo et al., 2011) and, contrary to the oxidative-stress theory of aging, decreasing the FA saturation of PL (Brenner, 1984; Stubbs and Smith, 1984; Rawicz et al., 2000). In non-ruminant studies, increasing age is generally associated with PL with increased saturated FA and hence more rigid cell membranes and increased potential for dysfunction (Hegner, 1980; Prisco et al., 1991; Hashimoto et al., 1999; Das, 2021). Observations that challenge the oxidative-stress theories of aging are inconsistent associations between LC-PUFA levels and longevity within a single species, FA saturation decreasing cell membrane fluidity, and inconsistent responses to antioxidative interventions that theoretically should

reduce membrane peroxidation and dysfunction (Buffenstein et al., 2008; Calhoon et al., 2015; Iakovou and Kourti, 2022).

There are currently very few studies specifically investigating PL composition and aging in cattle. A multi-site, cross-sectional lipidomics study that purposively recruited 1st, 2nd, 3rd and >4th lactation animals reported that EPA and DHA associated PC, PE and PI had the largest relative change in concentration out of the 185 analysed lipids, and decreased with increasing age (n=1492 cows) (Sheedy et al., 2025). Similarly, serum PUFA-PL were significantly lower in early lactation multiparous cows (n=20 cows) compared to heifers (n=10 cows) (Humer et al., 2016). At face-value, these results of lower LC-PUFA in older cattle support the oxidative-stress theories of aging in dairy cattle, however longitudinal studies will be required to address issues of survivorship bias across parity groups. Lower concentrations of PL containing EPA and DHA in older cows may also impact biological fitness by; increasing the endocannabinoid system activity and impairing reproductive efficiency (see Phosphatidylinositol: precursor to endocannabinoids) or decreasing flux through the phosphatidylethanolamine *N*-methyltransferase pathway during the transition period. This decreased flux may increase the risk of hepatic lipidosis (see Phosphatidylethanolamine: Precursor to PC), a condition also associated with increased age.

Glycerophospholipid omega acid supplementation

Supplementation with the essential omega-3 FA ALA (C18:3;n-3) or with marine products high in the omega-3 LC-PUFA of EPA and DHA consistently have positive effects on cattle health and reproduction (Rodney et al., 2015; Moallem, 2018; Veshkini et al., 2023). Supplementation with FA that are esterified to PL have altered bioavailability and metabolic activity when compared to FA esterified to triacylglycerols in non-ruminants (Murru et al., 2013; Schuchardt and Hahn, 2013; Alijani et al., 2025) and dairy cows (Spain et al., 1995), and may be a superior dietary form for supplementation. Oil-based products high in omega-3 content, such as flaxseed (ALA ~50% total FA) or fish oil (combined EPA and DHA ~50% total FA) are the most common omega-3 supplements available for cattle diets (Moallem, 2018), and deliver FA that are predominately esterified in triacyclglycerols (Elgersma, 2015; Makay et al., 2026). There are limited studies in cattle that directly compare whether different FA dietary forms could alter health and longevity.

Temperate pastures are naturally high in ALA esterified to structural PL and ALA is 50-89% of total FA (Glasser et al., 2013; Elgersma, 2015; NASEM, 2021), but there are no studies that

isolate the effect of PL-ALA from pasture with the triacylglycerol-ALA provided in oil seeds. Leduc et al. (2017) performed a meta-analysis exploring lactation performance with different dietary forms of flaxseed, including free oil (n=22 studies), flax hulls (n=8) and whole seed (subdivided into intact: n=32, mechanical processed: n=35 and extruded: n=42). Mechanically processed flax had the greatest energy correct milk and feed efficiency, while protected flax (including seed and oil forms) and flax hulls had the greatest milk ALA concentrations. While the review reports clear differences in production outcomes with dietary forms of flaxseed, the extent that can be attributed to differences in PL and triacylglycerol content remains unknown, and the impact on cattle health was not assessed.

Algae biomass and fish meal, compared to algal or fish oils, provide FA with greater amounts esterified to PL (Mika et al., 2016; Makay et al., 2026). In a series of experiments, Spain et al. (1995) reported different EPA, DHA and ARA plasma profiles between rumen-unprotected fish meal and rumen-unprotected fish oil supplementation, suggesting bioavailability differs with the dietary form of FA. Heravi Moussavi et al. (2007a; b) explored milk production, liver, uterine and ovarian responses to total mixed ration diets supplemented with either fish oil or three levels of fish meal (n=6 cows per treatment) and reported broadly equivalent and improved responses in follicular counts and size, endometrial FA profiles, dry matter intake, milk production, glucose and insulin responses between the fish meal and fish oil treatments compared to the control diet. Stamey et al. (2012) reported that rumen-protected algal biomass supplementation, compared to rumen-protected algal oil (n=4 cows, 4x4 Latin square), had greater DHA content in plasma and milk after 7-days of feeding, and AbuGhazaleh et al. (2009) reported that variation in the FA profile of milk between algae biomass supplementation and fish oil (n=6 cows per treatment). An alternate marine source with a high abundance of LC-PUFA esterified to PL is krill meal or oil (Burri and Johnsen, 2015), which may have superior bioavailability according to studies in non-ruminants (Ulven and Holven, 2015; Lindqvist et al., 2023), but has yet to be explored in ruminants. It is important to note that nations have variably prohibited protein-meal derived from marine sources (European Union, 2001; Animal Health Australia, 2023).

Ultimately, it remains unknown if the dietary form of LC-PUFA supplementation in cows is clinically relevant to health and longevity; costs, local availability, rumen-bypass techniques, ability to incorporate into rations, effect on milk production and quality must be considered in determining optimal interventions.

PHOSPHATIDYLCHOLINE

Choline is the head group in phosphatidylcholine (**PC**) which is the most abundant PL in the body, and in mammals typically comprises 40-50% of cellular phospholipids (Vance, 2015; van der Veen et al., 2017) and 65-70% of plasma phospholipids (Phillips and Dodge, 1967; Liu et al., 2025) (Figure 2). The PC class is the primary PL that establishes the lipid bilayers of cell membranes and lipoproteins due its large, non-polar head group and its generally cylindrical shape (Harayama and Riezman, 2018). Subsequently, most dairy research on PC relates to its structural role.

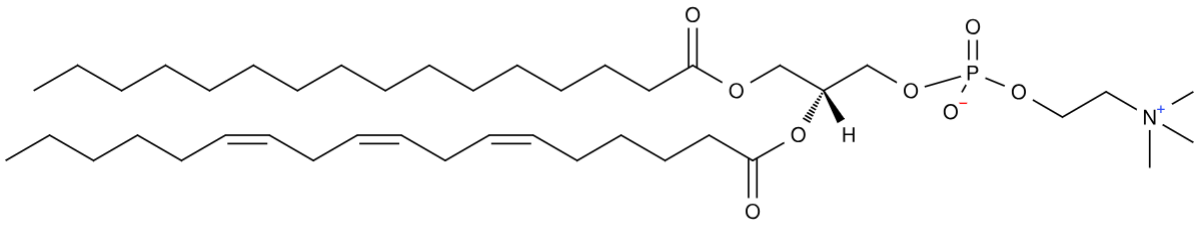

Figure 2: Phosphatidylcholine with palmitic at sn-1, alpha linolenic (n-3) at sn-2 and phosphocholine at sn-3. Common name: PC(16:0/18:3(6Z,9Z,12Z)). Image sourced from lipid maps: LMGP01010601

Hepatic lipidosis

Hepatic lipidosis, also known as fatty liver, is major metabolic disease of cattle during their transition period, and is associated with decreased milk production, cattle health and reproductive performance (Reid, 1983; Gerloff et al., 1986; Bobe et al., 2004). During the transition period from late pregnancy to early lactation, there is a homeorhetic drive to mobilize energy stored in adipose tissue to meet the increased energy demands of lactation (Bauman and Currie, 1980; Bobe et al., 2004). Fatty acids stored in adipose tissue circulate in blood to the liver as NEFA, where they may undergo oxidation, partial oxidation and export as ketones, or lipogenesis to be re-esterified with triacylglycerols and exported from the liver in VLDL. If VLDL production and export from the liver is restricted, NEFA and triacylglycerol levels in hepatocytes increase, and hepatic lipidosis and dysfunction may develop (Reid, 1973; Bobe et al., 2004).

The PL content of plasma VLDL is comprised mostly of PC (70%), with minor contributions from sphingomyelin (11%), PI (10%), PE (4%) and other lipid species in murine studies

(Hamilton and Fielding, 1989; Ågren et al., 2005). Given the high PC content of VLDL, the rate of PC production in the liver is directly related to VLDL production and transport of triacylglycerol from the liver into circulation (Vance, 2008; Cole et al., 2012). However, the rate of VLDL production in ruminant species is less than in other mammals and may predispose ruminants to accumulate lipids in the liver and to develop fatty liver (Kleppe et al., 1988; Pullen et al., 1990).

In the liver, PC is produced via two main pathways, the cytidine diphosphate (**CDP**)-choline pathway (Kennedy pathway) and the phosphatidylethanolamine *N*-methyltransferase (**PEMT**) pathway that converts PE to PC (Figure 1)(Vance, 2008). The PEMT pathway only significantly contributes to PC production in hepatocytes with 20-30% under normal conditions in murine models (Sundler and Akesson, 1975; DeLong et al., 1999) and is discussed in more detail in the 'Phosphatidylethanolamine: Precursor to PC' section.

An adequate supply of choline is required to support PC synthesis in the liver. Dietary supplementation with rumen-protected choline (**RPC**) is theorized to support the production of PC primarily through activating the CDP-choline pathway (Zhou et al., 2018; Myers et al., 2025), in-turn supporting VLDL production and export (Arshad et al., 2020; McFadden et al., 2020). However, the clinical effect of RPC supplementation is complicated by choline's involvement in other important metabolic pathways including acetylcholine synthesis (Wurtman et al., 2009) and transmethylation pathways that use the universal methyl-donor S-adenosylmethionine (Finkelstein, 1990; Mato et al., 1997) (Figure 3). Specifically, choline can be oxidized to betaine by choline dehydrogenase and then donates a methyl group to homocysteine via betaine aldehyde dehydrogenase to produce methionine and subsequently S-adenosylmethionine (Finkelstein, 1990; Mato et al., 1997) (Figure 3).

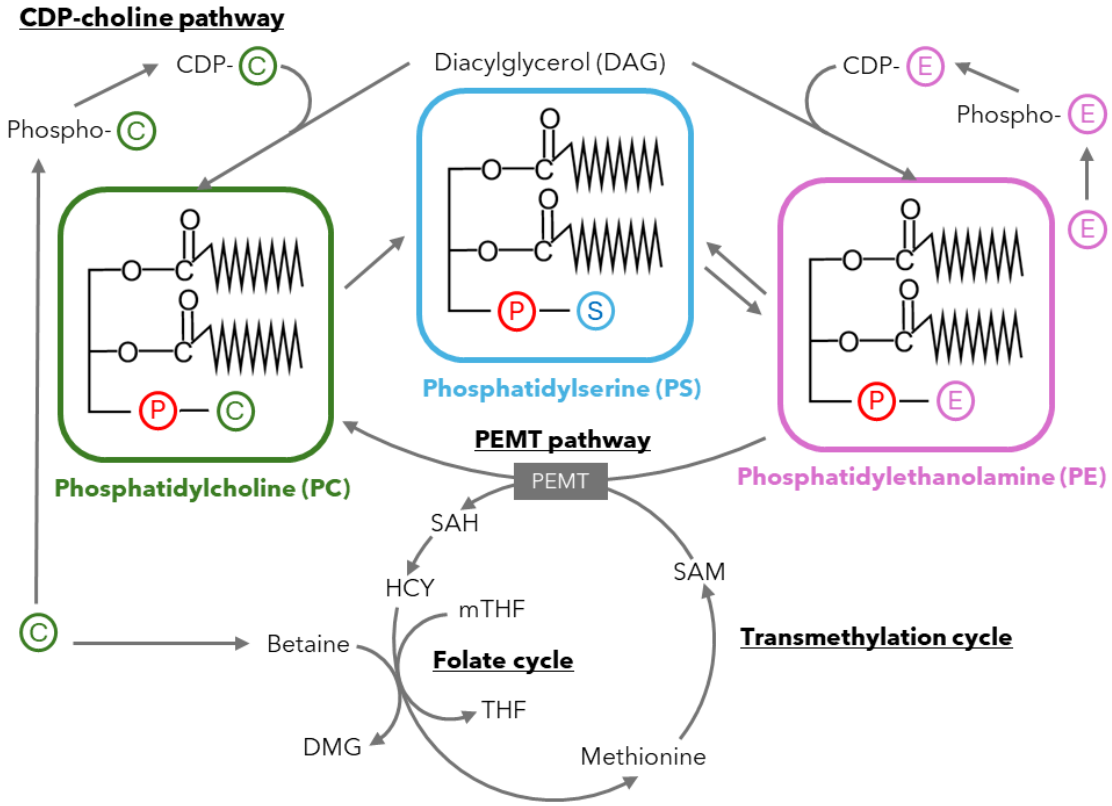

Figure 3: The two main pathways for phosphatidylcholine (PC) production in the liver. The cytidine diphosphate (CDP)-choline pathway predominates, accounting for around 70% of PC production under normal conditions. The CDP-choline pathways progresses from choline (encircled green C), to phosphocholine, CDP-choline and PC. The alternative pathway to produce PC is the phosphatidylethanolamine-N-methyltransferase (PEMT) pathway, which consumes three S-adenosylmethionine (SAM) to convert phosphatidylethanolamine (PE) to PC. Choline may also contribute to the PEMT pathway via the transmethylation cycle.
DMG – dimethylglycine; E (encircled purple) – ethanolamine; HCY – homocysteine; mTHF – methyltetrahydrofolate; P (encircled red) – Phosphate; SAH - S-adenosylhomocysteine; THF – tetrahydrofolate.

A meta-analysis of 21 experiments feeding RPC to transition cows (median 12.9 g choline/d [range 5.6-25.2 g/d], 21 experiments, 66 treatment groups, 1313 cows) was conducted (Arshad et al., 2020). The report found significantly improved feed intake (0.5±0.7[SEM]kg/day at median [12.9g/d] of RPC supplementation), energy corrected milk (1.7±1.6kg/d), milk fat (0.07±0.07kg/d) and milk protein (0.05±0.05kg/d), with numerically reduced risk of retained placenta (odds ratio:0.69, 95%CI: 0.46,1.02) and mastitis (odds ratio: 0.76, 95%CI: 0.55,1.06) (Arshad et al., 2020). However, there was no effect of metritis, milk fever, displaced abomasum and ketosis (Arshad et al., 2020). Perhaps of most relevance to hepatic lipidosis, feeding RPC to transition cows decreased liver triacylglycerol concentrations in some (Cooke et al., 2007; Elek et al., 2008; Zom et al., 2011; Goselink et al., 2013; Lima et al., 2024; Myers et al., 2025), but not all studies (Piepenbrink and Overton, 2003; Zahra et al., 2006; Zhou et al., 2016; Zenobi et al., 2018) and was ultimately not significant in two different meta-analytical reviews (Humer et al., 2019; Arshad et al., 2020). The authors suggested that the natural variation in the concentration of triacylglycerol in the liver during the postpartum period was too great and may have obscured the effects of supplementation in under-powered studies (Humer et al., 2019) and that choline supplementation rates in many studies have often been too low to result in a detectable effect (Arshad et al., 2020). Transcription factors associated with VLDL production and export, including hepatocyte mRNA abundances of critical apolipoprotein and microsomal triglyceride transfer proteins, increase with supplementation of RPC (Goselink et al., 2013; Zhou et al., 2017, 2018; Arshad et al., 2023). The 8th edition of the Nutrient Requirements of Dairy Cattle noted the production and potential health benefits of RPC supplementations, but did not establish a dietary requirement for choline as "it is synthesized by cows and because of potential variability in commercial products" (NASEM, 2021).

PHOSPHATIDYLETHANOLAMINE

Ethanolamine is the small head group of the phosphatidylethanolamine **(PE),** does not spontaneously form bilayers, and comprises 15-25% of cellular phospholipids (Vance, 2015; van der Veen et al., 2017) and around 1.1-3.6% of plasma phospholipids (Phillips and Dodge, 1967; Liu et al., 2025) (Figure 4). The PE are associated with protein biogenesis, oxidative phosphorylation, autophagy, membrane fusion, mitochondrial stability and are an important precursor for other lipids and signalling pathways (Calzada et al., 2016).

The PE are primarily produced by two independent pathways, the CDP-ethanolamine pathway and the mitochondrial phosphatidylserine decarboxylase pathways (Figure 1) (Vance and Tasseva, 2013). The phosphatidylserine decarboxylase pathway preferentially synthesizes PE with PUFA in the sn-2 position, while the CDP-ethanolamine pathway is enriched with mono or di-saturated FA in the sn-2 position (Bleijerveld et al., 2007). The PE also produce the endocannabinoid N-arachidonoylethanolamine (anandamide, **AEA**) and the structurally similar N-acylethanolamines (Liu et al., 2008), which are discussed in 'Phosphatidylinositol – Precursor to Endocannabinoids'.

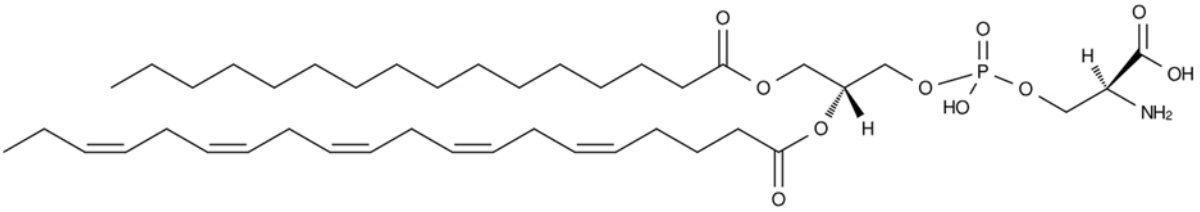

Figure 4: Phosphatidylethanolamine with stearic acid at sn-1, docosahexaenoic acid at sn-2 and phosphoethanolamine at sn-3. Common name: PE[18:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)]. Image sourced from lipid maps: LMGP02010094

Precursors to PC

The PEMT pathway transforms PE to PC via three successive methylation reactions that each consume one universal donor, S-adenosylmethionine (Figure 1). The PEMT pathway preferentially enriches PC with LC-PUFA (Pynn et al., 2011; Myers et al., 2019) and subsequently produces more diverse PC species than the CDP-choline pathway (DeLong et al., 1999). The PEMT pathway is most important for producing PC within hepatocytes, which represent around 20-30% of PC under normal circumstances in murine models (Sundler and Akesson, 1975; DeLong et al., 1999). Selective activation of the PEMT pathway in hepatocytes may increase PC and hence VLDL production, reducing hepatic lipid accumulation in the transition period.

Research on the use of specific methyl-donor supplementation strategies that target PEMT activation and its impact on modulating fatty liver accumulation in dairy cattle is still emerging. There is a growing consensus that specific substrates will preferentially select between PC production pathways. Choline preferentially utilizes the CDP-choline pathway and methionine

the PEMT pathway (Vance and Vance, 1986; Osorio et al., 2014; Chandler and White, 2017; Zhou et al., 2017, 2018). An *in-vitro* study reported increased PEMT activity in Holstein primary liver cells following methionine but not choline supplementation, and increased CDP-choline pathway activity with choline but not methionine supplementation (Zhou et al., 2018). Another *in-vitro* study using neonatal Holstein primary liver cells supplemented with choline reported a simultaneous decrease in PEMT activity and increased VLDL secretion suggesting CDP-choline activation, however, methionine supplementation also decreased PEMT activity but did not affect VLDL secretion (Chandler and White, 2017). The *in-vivo* PEMT gene expression in hepatocytes of transition cows was increased when the cows were given diets supplemented with methionine (Osorio et al., 2014; Zhou et al., 2017), but had no change when diets were supplemented with choline (Zhou et al., 2017). Contrastingly, Potts et al. (2023) did not report any differences in PEMT regulation in hepatocytes between transition cows given a control diet or diets supplemented with either choline or methionine. However, several milk LC-PUFA PC species were increased by more and earlier with methionine supplementation compared to choline supplementation in multiparous cows. More diverse LC-PUFA indicate PE to PC conversion (DeLong et al., 1999), hence the PEMT pathway was facilitated by methionine supplementation. Potts et al. (2023) found no changes in the LC-PUFA PC species in primiparous cows; the authors suggested that primiparous cows had sufficient choline before dietary supplementation to meet PC production without a need to enhance flux through the PEMT pathway.

Overall, the activation of specific PC pathways by different substrates opens the possibility of co-supplementation to target both the CDP-choline and PEMT pathways to optimize PC production and hence VLDL production and reduce lipid accumulation in the liver. The few studies that report co-supplementation offer mixed results. Zhou et al. (2016) saw no additional benefits to milk measures with the addition of choline over methionine supplementation alone (n=20 cows per treatment). Çetin et al. (2022) reported no difference in health, reproduction or a blood metabolite panel including NEFA, BHB, insulin and VLDL, with co-supplementation compared to supplementation with either methionine or choline alone in transition cows (n=8 cow per treatment). Similarly, Potts et al. (2023) reported no consistent difference in blood metabolites including a PL panel and amino acid panel or milk constituents when choline and methionine were provided together in the pre-partum. However, only methionine was supplemented post-partum in this study (n=5 per treatment). Zang et al. (2019) supplemented transition cows with a range of rumen-protected methyl-donors including methionine, choline,

betaine, riboflavin and vitamin B_{12}, in addition to the control diet that already had methionine with 199% and 115% of requirements in the pre- and post-partum diets, respectively. Their study found no difference in DMI and milk yield but decreased accumulation of liver lipids during day 5 to day 14 post-partum. Further studies are necessary to explore co-supplementation strategies that activate both PC production pathways, and possible health or production benefits.

Supplementation with specific FA can also preferentially select between PEMT or CDP-choline pathways for PC production (Pynn et al., 2011). Myers et al. (2019) abomasally infused C22:6 (DHA), C16:0 or C22:0 into late-pregnant, cannulated cows (n=5 per treatment) and observed that the total amount of PC produced was not different between treatment groups, but DHA-infused cows had increased concentration of PC species with diverse LC-PUFA, not only DHA and lower concentration of PC with fewer than 3 double bonds. This result indicated that DHA either did not activate the CDP-choline pathway or that DHA was preferentially incorporated into PE before conversion to PC via the PEMT pathway. Similarly, PEMT activity is associated with circulating PC-DHA in pregnant murine (Chalil et al., 2018) and human models (da Costa et al., 2011). Mechanistically, insufficient LC-PUFA reaching the liver may reduce capacity to activate the PEMT pathway. This is also supported by the observation that PC and PE with LC-PUFA is decreased in periparturient cows with increased liver lipid content (Rico et al., 2021) and DHA supplementation significantly reduces liver fat in human non-alcoholic fatty liver (Lee et al., 2020). Lower plasma concentrations of PL with LC-PUFA are found in multiparous cows compared to nulliparous cows (Sheedy et al., 2025), which may indicate that high parity cows have limited capacity to utilize the PEMT pathway, and could hypothetically increase their risk of fatty liver and associated disease complexes (Lean et al., 2023b). The effect of FA nutrition, choline or methionine supplementation and PC production via the PEMT pathway are worthy of further investigation in periparturient cows (McFadden et al., 2020).

As systemic inflammation has been associated with increased hepatic triacylglycerol accumulation in dairy cattle (Bradford et al., 2009; Eckel and Ametaj, 2016), Javaid et al. (2022) conducted a preliminary investigation to investigate the effect of endotoxin in transition cows. To mimic a transition cows' metabolic status, they used late-lactation cows and induced hyperlipemia using an intravenous triglyceride infusion and feed restriction (n=5 per treatment). The hepatocyte PC:PE ratio was decreased in the endotoxin stimulated cows, suggesting PEMT may have been inhibited. The authors proposed that PE increased as less

was converted to PC via PEMT, and note that the PC:PE ratio was reported as decreased in PEMT knockout mice (van der Veen et al., 2017). A decreased hepatocyte PC:PE ratio increases membrane permeability and rigidity (Fajardo et al., 2011), and hence hepatocyte dysfunction, and is observed in non-alcoholic fatty liver disease of humans (Li et al., 2006; Arendt et al., 2013; van der Veen et al., 2017). The role and clinical significance of endotoxin to inhibit specific PC production pathways remains unclear.

PHOSPHATIDYLINOSITOL

Phosphatidylinositol (**PI**) contains the six-carbon ring inositol head group and constitutes around 10-15% of cellular phospholipids (Figure 5) (Vance, 2015) and around 2% of plasma phospholipids (Liu et al., 2025). The most common of the six naturally occurring stereoisomers of inositol is myo-D-inositol, with one axial hydroxyl group at carbon position two and the remaining hydroxyl groups equatorial, with a turtle-shaped mnemonic often used to aid visualization (Figure 6) (Agranoff, 2009; Irvine, 2016). In contrast with other PL, the FA composition of membrane PI is highly conserved, with upwards of 70% of PI esterified to stearic acid (C18:0) in the sn-1 position and arachidonic acid (C20:4; n-6 ARA) in sn-2 (Barneda et al., 2019). Though PI bovine plasma concentrations appear notably more diverse, with approximately 23% PI(18:0_20:4) and 22% PI(18:0_20:3) (Liu et al., 2025; Sheedy et al., 2026). Owing to the high conservation of ARA, PI are disproportionately significant contributors to eicosanoids and leukotriene pathways compared to their relative abundance (Goerig et al., 1985; Hokin, 1990; Kim et al., 2022).

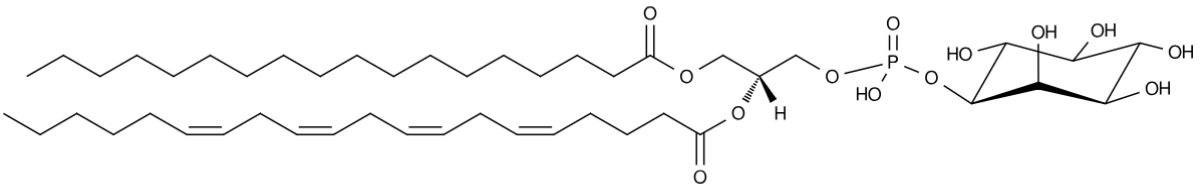

Figure 5: Phosphatidylinositol with stearic acid at sn-1, arachidonic acid (n-6) at sn-2 and phospho-(1'-myo-inositol) at sn-3. Common name: PI(18:0/20:4(5Z,8Z,11Z,14Z)). Image sourced from lipid maps: LMGP06010010

Precursor for Phosphoinositides

The PI are the precursors for all phosphatidylinositol phosphates (phosphoinositides, **PIP**). The PIP have up to three phosphate moieties attached to the inositol ring at carbon 3, 4 or 5, totalling seven unique configurations. Each PIP is tightly regulated by substrate and intracellular location specific kinases and phosphatases (Balla, 2013; Dickson and Hille, 2019). The PIP are extensively bioactive with roles in membrane signalling, regulating vesicular trafficking, ion channels, pumps, and transporters, modulating lipid distribution, and controlling both endocytic and exocytic processes (Di Paolo and De Camilli, 2006; Balla, 2013; Irvine, 2016; Dickson and Hille, 2019). There is growing recognition within medicine of the role PIP have in disease regulation, reflecting their broad activity, however there are still relatively few studies specific to dairy cattle.

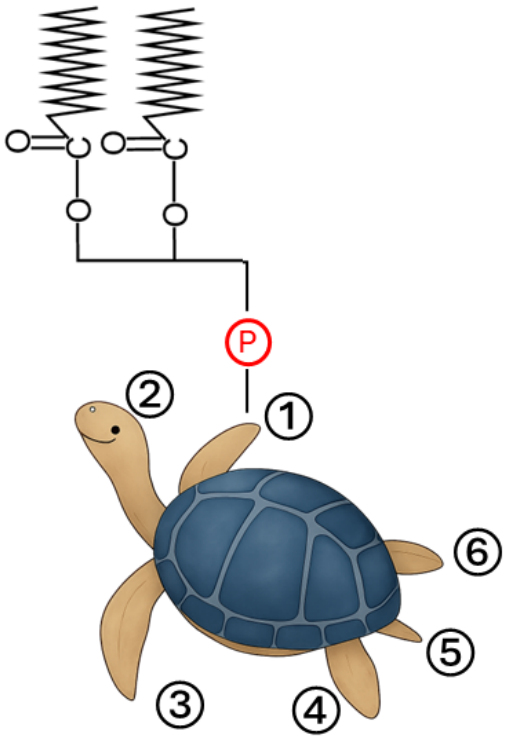

Figure 6: "Argranoff's turtle" is a useful visual mnemonic for inositol. Using the D numbering convention, carbons are numbered anticlockwise beginning with turtle's right-flipper. The turtle is 'right-handed' and so is esterified to the phosphate group of phosphatic acid. The turtle's head is raised (hydroxyl group is axial) and the remaining limbs and tail are flat (hydroxyl groups equatorial). (Turtle image: OpenAI, 2025)

Phosphoinositide-3-kinase (**PI3K**) phosphorylates phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate. The PI3K/protein kinase B (AKT) axis controls cell survival, cycle progression, growth and inflammatory responses, and is perhaps best recognized as frequently altered in human cancers (Vara et al., 2004; Troutman et al., 2012; He et al., 2021). The role of the PI3K/AKT axis in dairy cow inflammatory pathways has recently been investigated in endometritis (Jiang et al., 2024), systemic inflammation and hepatitis (Häussler et al., 2023), and mastitis (Cai et al., 2021).

Bovine endometrial cells exposed to endotoxin *in-vitro* downregulated both the PI3K and AKT pathways, leading to apoptosis (Jiang et al., 2024). The addition of interferon-tau

upregulated PI3K/AKT and alleviated the endotoxin-induced apoptosis in a dose-dependent response, indicating a potential treatment modality for endometritis in cattle (Jiang et al., 2024). Häussler et al. (2023) reported increased hepatic mRNA abundance of AKT genes but not PI3K genes following endotoxin induced systemic inflammation. Cows that were supplemented with a rumen-protected L-carnitine reduced PI3K mRNA following endotoxin exposure, potentially modulating the severity of the pro-inflammatory response. Cai et al. (2021) reported that *in-vitro,* bovine mammary epithelial cells with simulated *Staphylococcus aureus* infection (with lipoteichoic acid treatment) activated the PI3K/AKT signalling pathway and that the pathway could be blocked by inhibiting Toll-Like Receptor 2.

These early studies demonstrated modulation of the inflammatory and apoptotic response to infection and inflammation through the PI3K/AKT axis. This indicates the potential for new treatment paradigms for diseases relevant to dairy cattle, though practical applications may take considerable time to develop.

Precursor to endocannabinoids

The endocannabinoid system (ECS) is an important neuromodulator and regulator of energy metabolism, immune function and reproduction in mammals (Silvestri and Di Marzo, 2013; Lu and Mackie, 2016) and consequently is relevant to dairy cow production. For a more comprehensive exploration of the emerging and complex role of ECS in dairy cattle, we refer readers to two recent review articles (Myers et al., 2021; Zachut et al., 2025).

The two most well-studied endocannabinoids are N-arachidonoylethanolamine (anandamide, AEA; derived from PE associated with ARA) (Liu et al., 2008) and 2-arachidonoylglycerol (**2-AG**, derived from PI associated with ARA) (Murataeva et al., 2014). In addition to being endogenous ligands for cannabinoid receptors, AEA and 2-AG can also contribute to the eicosanoid pathway by transformation to ARA (Ahn et al., 2008). Endocannabinoid synthesis is rapid and on demand, in contrast to classical neurotransmitters that are pre-formed and stored in synaptic vesicles (Silvestri and Di Marzo, 2013; Lu and Mackie, 2016).

Activation of the ECS in murine and human studies is typically associated with increased adipogenesis, lipogenesis, suppression of lipolysis, and increased appetite (Myers et al., 2021) and is accordingly relevant to transition cow research. However, results from early research into the ECS activation of transition cows has generally been contrary to expectations, with a positive association with lipolysis (Zachut et al., 2025). Zachut et al. (2018) observed increased

AEA and 2-AG, and upregulation of pro-inflammatory pathways in the adipose tissue of cows 4 d post-partum that had high body weight loss compared to cows with low body weight loss. Similarly, an *in-vitro* study from Myers et al. (2023) recognized differential regulation of the ECS according to stage of production, with adipose tissue from periparturient cows not responding to ECS stimulus whereas tissue from non-lactating, non-gestating cows had reduced lipolysis and increased adipogenesis with ECS stimulus, as expected from non-ruminant studies. In a review, Zachut et al. (2025) proposed that studies of ECS must consider the gender and reproductive state of their subjects. The paucity of comparative non-ruminant research with female subjects of different reproductive and lactating states may explain why transition-cow studies on the ECS system have results contrary to expectation (Zachut et al., 2025).

The FA composition of diets can modulate ECS activation (Kim et al., 2013; Kra et al., 2022). By decreasing the amount of omega-6 FA in the diet, less ARA is available for esterification to PE and PI and as a result less precursors are available for AEA and 2-AG endocannabinoid production, respectively (Kim et al., 2013). This relation has been supported in a study in dairy cows (Kra et al. (2022), in which the cows were fed a diet supplemented with flaxseed, as a source of omega-3 ALA and reported 49.7% less plasma AEA compared to the control cows. To what degree the production benefits associated with increased dietary omega-3 FA (see section Glycerophospholipid Long Chain Poly Unsaturated Fatty Acids) are directly attributed to the ECS remains to be elucidated (Kra et al., 2022, 2023; Zachut et al., 2025). A plausible link between increased dietary omega-3 FA supplementation, reduced ECS activation and improved reproduction has been made (Zachut, 2020). Increasing omega-3 FA intake is associated with increased reproductive function (Rodney et al., 2015; Sinedino et al., 2017; Moallem, 2018; Moallem et al., 2020), and though levels of endocannabinoids fluctuate throughout a pregnancy, reducing the ECS activity is generally associated with improved reproductive function in ruminants (Weems et al., 2009; Tsutahara et al., 2011; Abolghasemi et al., 2016; Dirandeh et al., 2020).

PHOSPHATIDYLSERINE

Phosphatidylserine has a serine head group and can be produced from either PC or PE by specific phosphatidylserine synthases in the endoplasmic reticulum (Figure 7). The PS can be decarboxylated back to PE via phosphatidylserine decarboxylase (Figure 1) but cannot similarly be directly converted back to PC. The PS are the most abundant anionic PL at around

5-10% of total cell phospholipids (Leventis and Grinstein, 2010; Vance, 2015) and 0.4-1.9% of plasma phospholipids (Phillips and Dodge, 1967; Liu et al., 2025).

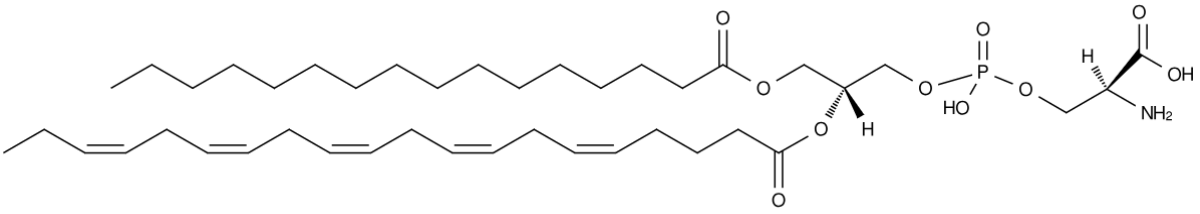

Figure 7: Phosphatidylserine with palmitic at sn-1, eicosapentaenoic acid at sn-2 and phosphoserine at sn-3. Common name: PS[16:0/20:5(5Z,8Z,11Z,14Z,17Z)). Image sourced from lipid maps: LMGP03010902

The PS are unevenly distributed within a cell, with virtually all of the plasma membrane PS on the cytosolic side and none exposed to extracellular space (Leventis and Grinstein, 2010; Fairn et al., 2011). A well-characterized function of PS metabolism is its ability to instigate apoptosis (Fadok et al., 1992; Miyanishi et al., 2007) and, in red blood cell, coagulation (Zwaal et al., 1998; Majumder et al., 2008), which is achieved by flipping the distribution of PS from the inner membrane leaflet to the outer leaflet (Leventis and Grinstein, 2010). The exposed PS becomes a signal for phagocytic cells (Fadok et al., 1992; Miyanishi et al., 2007) and clotting factors (Zwaal et al., 1998; Majumder et al., 2008). Additional functions of PS include contributing to the electrostatic maintenance of membranes (Leventis and Grinstein, 2010) and are associated with many brain functions including neurotransmission and neuroinflammation (Glade and Smith, 2015; Ma et al., 2022). Currently, ruminant PS research exclusively relates PS membrane externalisation.

The mechanistic cause of anaemia associated with cattle infected with *Theileria sargenti* (Japanese bovine theileria) was investigated by measuring the level of PS exposure on red blood cells in infected cattle (Shiono et al., 2003). The relative abundance of exposed PS was associated with the level of parasitic burden and degree of anaemia (Shiono et al., 2003).

A recent murine study showed that sperm are required to externalise PS to be recognised by PS receptors on oocytes for successful fusion and fertilization (Rival et al., 2019). Traditionally, PS externalisation on bovine sperm was a sign of apoptosis, associated with cryopreservation and an indicator of reduced viability (Anzar et al., 2002; Januskauskas et al., 2003; Wu et al.,

2014). However, Haines (2021) reported that capacitated bovine sperm, a required maturation step, exposed more PS compared to uncapacitated sperm (60% vs 18%), and questioned the validity of PS exposure on sperm as a measure of viability.

STUDY LIMITATIONS

Lipidomic studies exploring cattle health and production have increased rapidly with greater access to high-throughput analytical techniques and bioinformatics (Liu and Rochfort, 2023). However, many currently published reports examining PL metabolism and its associations with cattle health or longevity are limited by small study size or observational study designs, restricting inference regarding causality and practical impact. These studies should therefore be considered foundational and hypothesis-generating. Progress toward a more causal understanding of PL metabolism and its potential benefits for cattle health will require adequately powered, controlled intervention studies, ideally conducted under commercial farming conditions and using defined endpoints of relevance to dairy producers and industry.

CONCLUSION

Glycerophospholipids are important regulators of dairy cow health, with influences on hepatic lipid metabolism, inflammatory signalling, aging and fertility. Many of the processes discussed within this review appear to be modified through nutritional management, yet critical questions remain. These include whether specific selection of PC production pathways, that is CDP-choline and PEMT, in the liver can enhance VLDL production and mediate lipid accumulation during the transition period; or the efficacy of different dietary forms of LC-PUFA such as triacylglycerol-rich oils vs. PL-rich formulations to modify the endocannabinoid system, aging and hepatic lipidosis. The exploration of inflammatory pathways associated with PI and PE metabolism, including eicosanoid, phosphoinositides and endocannabinoid systems, is an emerging area of dairy cattle research, but early studies indicate exciting possibilities of new treatment or management modalities for diseases of economic importance, including metritis and mastitis.

Although each PL class was addressed individually in this review, many lipid pathways overlap and an integrated approach to lipid metabolism and research is required to understand which pathways have the most quantitative effects on productive outcomes and which methods of supplementation, or other nutritional and metabolic states might influence the outcomes.

The diversity of enzymatic pathways, FA remodelling, tissue specificity and the production status of cows create an intricate, dynamic and challenging network for investigators. As lipidomic technologies become increasingly available, a deeper understanding of PL biology promises to improve dairy cattle health, productivity and longevity.

NON-STANDARD ABBREVIATIONS

2-AG = 2-arachidonoylglycerol; AEA = N-arachidonoylethanolamine; AKT = protein kinase B; ALA = alpha-linolenic acid; ARA = arachidonic acid; CDP = cytidine diphosphate; DHA = docosahexaenoic; ECS = endocannabinoid system; EPA = eicosapentaenoic; FA = fatty acyls or fatty acids; LC-PUFA = long-chain polyunsaturated fatty acids; NEFA = non-esterified fatty acids; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PEMT = phosphatidylethanolamine *N*-methyltransferase; PI = phosphatidylinositol; PI3K = phosphoinositide-3-kinase; PIP = phosphatidylinositol phosphates; PL = glycerophospholipid; PS = phosphatidylserine; PUFA = polyunsaturated fatty acids; RPC = rumen-protected choline; VLDL = very low-density lipoproteins

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CHAPTER 2

Holstein dairy cows lose body condition score and gain body weight with increasing parity in both pasture-based and total mixed ration herds

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OVERVIEW OF CHAPTER 2

Excessive mobilization of body tissue during the transition period is associated with increased risk of disease, and reproductive failure. The two most common measurements of body tissue reserves are body condition scoring (**BCS**) and body weight (**BW**). However, there is relatively poor correlation between the two measurements. Chapter 2 introduces a novel body-group classification system that combines both BCS and BW measurements; as greater than- or less than the farm of origin median BCS and BW. A large retrospective dataset from 16 prospective trials was utilised to investigate the novel scoring system alongside BCS and BW, and their association with parity and the dairy systems of total mixed ration, confinement-based farms or pasture-based farms. Results showed that parity had stronger effect on BCS and BW than did the system of dairying. The BCS-BW combined metric showed a clear progression from parity 1 cows mostly commonly being in the high BCS/low BW category to parity ≥ 5 cows most commonly being in the low BCS/high BW category.

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Short Communication
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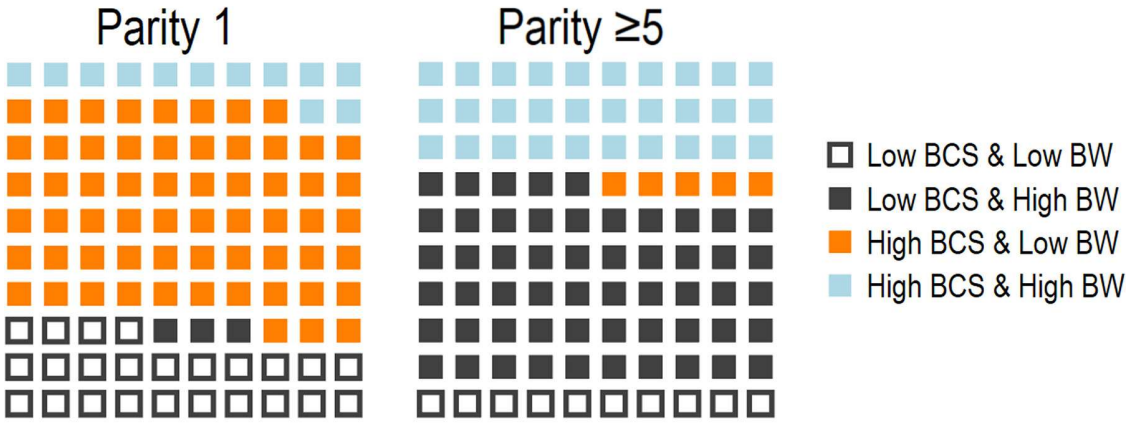
Graphical Abstract



Summary

Body condition scoring (BCS) and body weight (BW) measurements are associated with health and reproductive efficiencies of cows. We used raw data sets from 16 studies to evaluate associations of parity and feeding system (pasture-based or total mixed ration) with BCS at precalving and peak milk, and change in BCS, and BW at peak milk. With increasing parity, there is a general decrease in BCS and increase in BW regardless of feeding system, with most young cows having low BW and high BCS and older cows having high BW but low BCS.

Highlights

- Body condition score decreases and BW increases in older cows.
- Parity had a greater effect on BCS and BW than feeding system.
- Second-parity cows have the lowest pre-calving BCS.

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Abstract: Body condition scoring (BCS) and body weight (BW) are observations associated with labile tissue reserves, health, and reproduction efficiency of dairy cows. The effect of parity (1 through to ≥5) and feeding system (pasture-based and TMR) on BCS and BW were evaluated utilizing raw data sets from 16 retrospective studies that totaled 24,807 Holstein cows across 3 nations (Australia, Canada, and the United States). Linear regression models were used to investigate the 5 outcome variables of precalving BCS, peak milk BCS, change in BCS from precalving to peak milk, and peak milk BW and their respective associations with parity and feeding system. To help control for the influence of calendar time, study treatment protocols when applicable, and genetic change, all outcome variables were center-transformed around each study group mean. Including feeding system as a covariate improved model fit for most outcome variables; however, the relative effect size of parity was generally much greater than feeding system effect size. Parity 2 cows had the lowest precalving BCS of −0.087 [95% confidence interval (CI): −0.107, −0.065] less than the mean, whereas parity 1 cows had the greatest, 0.068 (95% CI: 0.043, 0.092) above mean, regardless of feeding system. Peak milk BCS overall decreased with increasing parity (parity 1 to parity ≥5: −0.13, 95% CI: −0.19, −0.08) and BCS change during the transition period monotonically decreased with increasing parity (parity 1 to parity ≥5: −0.22, 95% CI: −0.26, −0.17). Peak milk BW monotonically increased with increased parity (parity 1 to parity ≥5: 114 kg, 95% CI: 104, 125). A waffle plot was used to present the proportions of cows, by parity, that were partitioned into "low BCS and low BW," "low BCS and high BW," "high BCS and low BW," or "high BCS and high BW" groups. Cows were assigned either a high or low status by being above or below their specific centered study group means, respectively. Considering a null hypothesis of 25% per BCS-BW category, there was a striking change in category from parity 1 cows that were predominantly in the "high BCS and low BW" category (61.2%) to parity ≥5 cows that were predominantly in the "low BCS and high BW" category (55.5%). The study supports studies showing increased weight and change in BCS with increased parity. We highlight the associations among production system, BCS, BW, and parity.

Body condition score in dairy cattle is a rapid visual assessment that is highly associated with available energy reserves, primarily mobile fat reserves (Edmondson et al., 1989). Though different rating systems exist, a low score universally reflects low energy stores or emaciation and a high score with excessive energy stores or obesity (Roche et al., 2004). During early lactation, dairy cows are under a strong homeorhetic drive to mobilize energy reserves to produce large quantities of milk (Bauman and Currie, 1980; Baumgard et al., 2017), resulting in a loss of body condition that typically reaches a nadir after the first 40 to 100 DIM (Gallo et al., 1996). Excessive BCS loss during this period is associated with increased disease incidence, reproductive inefficiencies, and potentially poor cow welfare (Roche et al., 2009). Greater parity dairy cows often experience a greater BCS loss compared with nulliparous cows during the early postparturient period and older cows are also associated with an increased risk of removal from the herd due to reproductive failure, disease, and death (Roche et al., 2009; Pinedo et al., 2010).

Body weight also reflects the protein, fat, and energy reserves of cattle (Botts et al., 1979; Gregory et al., 1998; Thorup et al., 2013). There is an imperfect association between BCS and BW that may be influenced by factors such as rumen fill, stage of pregnancy and lactation, and udder fill as well as composition and location of labile tissue pools. Body weight is also associated with parity, with heifers entering the lactation herd typically at around 80% of their mature BW (Berry et al., 2011; Berry and Evans, 2022). Different feeding systems are also reported to influence BCS and BW, with herds from TMR systems typically having higher total BCS and BW than those from pasture-based systems (Washburn et al., 2002; Roche et al., 2007). However, the influence of feeding system on within-herd BCS and BW during the parturition period is less clearly understood and may be similar between systems (Roche et al., 2007). It is important to understand the association of BCS and BW with parity and management systems as this may provide insight into increased risks for disease, reproductive failure, and survival of older cows and how to manage these risks.

In this study we examine associations of parity with precalving and peak milk BCS, changes of BCS between precalving and peak milk observations, and peak milk BW for Holstein cows in different production systems (pasture-based or TMR) across 3 countries using retrospectively collected data. We hypothesized that BCS and BW of dairy cows are associated with cow parity and that BCS

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and BW are different between the management system types of TMR or pasture-based systems.

The studies used to produce the database for this study were approved by the relevant Animal Care and Use Committees at the time of conduct.

A convenience sample of 16 amalgamated data sets from previous studies were used in this study. In general, commercial dairies were purposively selected for use in the original studies based on good record keeping and a history of performance that suggested capability of maintaining attention to detail congruent with successful trial conduct. The study contributors had conducted large, prospective studies that allowed a rigorous evaluation of the original study hypotheses. Only Holstein or Holstein-Friesian cattle (n = 24,807) were included, as there were few other cow breeds in the database.

Individual study inclusion criteria were being an observational or randomized controlled trial that provided details on parity and either BCS or BW or both. Further details of individual studies that met the criteria are included in Lean et al. (2022a,b). The inclusion criterion for pasture-based study farms was grazing throughout the study period; herds fed partial mixed rations and herds fed concentrates in the parlor were categorized as pasture based. Body condition score data were available from 14 studies, and BW data were available from 8 studies.

The outcome variables investigated were precalving BCS, peak milk BCS and BW, and change in BCS between precalving and peak milk observations. One precalving (−30 to 0 DIM) BCS data point and one peak milk (30 to 110 DIM) BW and BCS data point were entered into the database per cow per study, with only one cow per study. When there were multiple precalving BCS measures per cow, the closest data point to calving was chosen. The peak milk BW and BCS values were determined by the observations obtained at the closest postcalving time point to peak milk, as estimated from herd test or weekly milk yield data. Cows were not required to have a data point for all BW and BCS variables. When an intervention was applied in a herd according to the original study's protocol, treatments within the herd were considered as separate groups for statistical analysis purposes. All outcome variables were center-transformed around the study group mean to control for individual study treatment effects on study outcomes and help mitigate the effects of temporal, genetic, and other clustered, unobserved covariates between each study group.

All statistical analysis was conducted using Stata Version 16 (StataCorp). Initial data evaluation included tabulation of data and visual appraisal of BCS and BW for normality of distribution.

As the database constructed for this study utilized retrospective data, it was possible to compute the statistical power achieved post hoc. Adjustments were made for DIM and feeding system and clustering of study groups, with α set to 0.05. Centered peak milk BCS in heifers was 0.08 (n = 5,165, SD 1.38) and −0.07 for parity ≥5 cows (n = 1,385, SD 0.78). Post-hoc power was calculated as 99.98% (Stata: *power twomeans*). The unit of interest in this study was the cow and statistical significance was set at an α of 0.05.

Ordinary least-squares linear regression and multilevel mixed-effect linear regression (Stata: *regress* and *mixed*, respectively) were used to evaluate the effects of parity and system on BCS, BW, and BCS change between precalving and peak milk. The effects of group were controlled by either assigning group as a random effect in *mixed* or clustered-robust standard errors in *regress*, with the model structure being ultimately determined by minimizing Akaike information criterion (**AIC**). Nesting of group within feeding system was investigated but did not explain group variation beyond a 2-level model. Days prepartum or DIM at time of BCS assignment/BW observation were investigated as covariates, as either continuous, polynomial, categorical, or centered-mean variables. Interactions between system, parity, and time of observation were evaluated. A manual, forward stepwise model was used with model selection determined by minimum AIC. Regression residuals were formally tested for normality with the Shapiro-Francia normality test and heteroskedasticity visually assessed with residual versus fitted value figures.

The Stata code *marginsplot* was utilized to produce graphical summaries of the regression results for cow parity, comparing system where applicable. A waffle plot was used to display the proportions of cows above and below group-centered means for both BCS and BW at peak milk, creating 4 partitions, and each plot was stratified by parity.

The data set mean study size was 1,198 Holstein cows (range: 14–10,958), with a total of 24,807 cows. There were 89.1% (n = 22,112) fed in TMR systems and 10.9% (n = 2,695) pasture based. For the TMR herds, there were 34.7% parity 1 (n = 7,670), 29.9% parity 2 (n = 6,604), 18.8% parity 3 (n = 4,159) 9.4% parity 4 (n = 2,073), and 7.2% parity ≥5 (n = 1,606). In the pasture-based herds, there were 25.6% parity 1 (n = 691), 22.3% parity 2 (n = 600), 21.1% parity 3 (n = 569), 12.7% parity 4 (n = 341), and 18.3% parity ≥5 (n = 494). There were 12.9% (n = 3,198) enrolled cows in AU, 22.5% (n = 5,576) in CAN, and 64.6% (n = 16,033) in the United States. In all cases, the intraclass correlation of study group was best controlled with clustered-robust standard errors in simple linear regression models, according to AIC.

The precalving BCS (−30 to 0 d; mean −7.9, SD 8.1) was evaluated in 12,168 cows across 212 study groups. Parity 1 cows had significantly greater BCS than all other parities, whereas parity 2 cows had significantly less BCS than all other parities, regardless of system of management (Table 1). Parity 3, 4, and ≥5 did not differ significantly from each other (Table 1). The relationship between management precalving BCS and parity for the 2 system types was generally consistent. A notable exception was that pasture-based parity 1 cows had BCS significantly greater than parity >1 pasture-based cows (0.20, 95% CI: 0.11, 0.29) compared with parity 1 cows in TMR systems, which were also significantly greater, but numerically closer to the mean TMR precalving BCS (0.06, 95% CI: 0.04, 0.09; Figure 1). The direct contrast between pasture-based parity 1 and TMR parity 1 was −0.14 (95% CI: −0.24, −0.05). The finding that parity 2 precalving BCS was lower than the centered-mean BCS in both production systems may indicate that neither system is adequately maintaining or recuperating body reserves of parity 1 after postcalving BCS loss nadir. It has been reported that parity 1 cows do not regain lost BCS as quickly as multiparous cows (Berry et al., 2006b; Roche et al., 2007) and that this may be reflected by lower-than-expected BCS of parity 2 cows in our data sets and others (Berry et al., 2011). However, the parity 2 cows in pasture and TMR systems weighed 51.6 kg of BW (95% CI: 41.2, 62.0) greater than parity 1, indicating that BCS and BW are differentially affected by increasing parity.

Body condition score at peak milk was investigated with 15,657 cows in 169 groups. The covariable DIM was dichotomized into 30–70 and 71–110 DIM due to heterogeneity of observation date

Table 1. Effects of parity and management system on measures of BCS at precalving (−30 to 0 DIM) and peak lactation (30 to 110 DIM), the BCS change between precalving and peak milk observations, and BW (kg) at peak lactation[1]

Model parameter	BCS			BW
	Precalving	Peak milk	Change[2]	Peak milk
Parity (1 referent)				
2	−0.31 ± 0.047 (<0.001)	−0.07 ± 0.020 (0.001)[a]	−0.05 ± 0.017 (0.002)	38.26 ± 8.873 (<0.001)
3	−0.22 ± 0.056 (<0.001)[a]	−0.08 ± 0.026 (0.002)[b]	−0.13 ± 0.021 (<0.001)[a]	67.64 ± 10.283 (<0.001)
4	−0.25 ± 0.048 (<0.001)[a]	−0.09 ± 0.039 (0.030)[ab]	−0.14 ± 0.028 (<0.001)[a]	81.08 ± 12.343 (<0.001)
≥5	−0.22 ± 0.065 (0.001)[a]	−0.10 ± 0.041 (0.012)[a]	−0.22 ± 0.025 (<0.001)	96.37 ± 9.804 (<0.001)
System-parity interaction (pasture referent)				
TMR:parity 1	−0.14 ± 0.048 (0.004)	0.04 ± 0.030 (0.192)	—	−6.26 ± 8.526 (0.468)
TMR:parity 2	0.02 ± 0.013 (0.132)	−0.03 ± 0.016 (0.066)	—	20.50 ± 5.369 (0.001)
TMR:parity 3	0.03 ± 0.022 (0.203)	0.02 ± 0.021 (0.428)	—	32.04 ± 5.585 (<0.001)
TMR:parity 4	0.09 ± 0.026 (0.001)	0.00 ± 0.034 (0.921)	—	26.72 ± 10.081 (0.013)
TMR:parity ≥5	0.00 ± 0.031 (0.929)	−0.02 ± 0.036 (0.561)	—	30.30 ± 6.696 (<0.001)
Days (precalving/in milk)	−0.0037 ± 0.001 (0.007)	0.03 ± 0.012 (0.024)[3]	—	0.21 ± 0.039 (<0.001)
Precalving BCS	—	—	−0.64 ± 0.043 (<0.001)	—
Intercept	0.20 ± 0.046 (<0.001)	0.05 ± 0.019 (0.020)	0.09 ± 0.013 (<0.001)	−53.83 ± 8.303 (<0.001)
Cow count	12,168	15,657	2,803	4,694

[a,b]Coefficients sharing a letter in the group label are not significantly different at the 5% level.

[1]All measure outcome variables and day covariates were centered on their respective study group means. Each cell in the table reports regression coefficients ± SE and *P*-values.

[2]Interaction between parity and precalving BCS; parity 2 coefficient = 0.22 ± 0.046 (<0.001); parity 3 coefficient = 0.12 ± 0.057 (0.034); parity 4 coefficient = 0.10 ± 0.073 (0.158); parity ≥5 coefficient = 0.11 ± 0.082 (0.170).

[3]Categorical covariate: 31–70 DIM (referent) and 71–110 DIM.

among studies and to improve model fit according to AIC. There was a consistent decline in BCS with increasing parity, with all parities having significantly lower BCS than the parity 1 heifers and parity ≥5 cows having the lowest body condition of all parities (Table 1). There was a numerically larger decline ($P = 0.06$) in peak milk BCS from parity 1 to parity 2 cows observed in TMR herds (−0.14, 95% CI: −0.20, −0.08) compared with pasture-based herds (−0.09, 95% CI: −0.11, −0.03; Figure 1).

The loss of body condition between precalving and peak milk, with 2,803 cows in 110 study groups, increased monotonically for all parities, with only parity 3 and 4 cows having nonsignificant pairwise comparisons (Table 1). This finding is consistent with previous studies that examined BCS change during the transition period (Roche et al., 2009). Including system or timing of observations did not improve the regression fit. A greater precalving BCS was associated with losing more body condition by peak milk (Table 1). High precalving body condition and excessive loss of BCS during the transition period has been associated with increased incidence of disease, including milk fever and ketosis, and reduced reproductive efficiency (Roche et al., 2009).

The BW at peak lactation (30 and 110 DIM, mean 50.9, SD 16.2) was assessed in 4,694 cows in 32 study groups. Body weight increased monotonically with increasing parity and was significantly different for all pairwise parity comparisons. Despite parity 1 cows not being different with regard to their predicted centered BW across management systems (−6.26 kg, 95% CI: −23.7, 11.12), the interaction of parity and management system shows a greater spread around the centered BW in TMR herds (parity 1 to parity 5: 132 kg, 95% CI: 126, 139) compared with pasture-based herds (parity 1 to parity 5: 96 kg, 95% CI: 76, 116) (Table 1, Figure 1).

The distributions of cows with observations for both BCS and BW at peak lactation were categorized as being above or below the centered group mean for each respective variable and graphically depicted in a waffle plot (Figure 2). Generally, as cows increased in parity they increased in BW but had BCS less than the mean. The null hypothesis is that, by parity, each of the 4 potential BCS-BW categories would contain 25% of cows. There were more than expected parity 1 cows with above mean BCS and below mean BW (61.2%). By contrast, parity ≥5 cows had many more than expected below mean BCS and greater than mean BW (55.5%; Figure 2). Previous studies have focused on individual cows and reported that the correlation between BW and BCS differ with increased parity. As such, the predicted BW change associated with a unit change in BCS differs across parities (parity 1: 44 kg/BCS unit to parity ≥6: 55 kg/BCS unit; Berry et al., 2006a, 2011). However, a consolidated description on a large and diverse population of cows with BCS-BW grouping, stratified by parity, has not previously been presented in a focused manner as reported here. The results were replicated when (1) heifers were removed from the analysis; with concerns they would skew the group mean BW and (2) non-Holstein breeds of cattle were analyzed (data not shown). A study on 50 cows also reported that nulliparous cows had greater BCS and lower BW than multiparous cows throughout the transition period; however, percentage BW loss was independent of parity and was associated with reproduction indices (Sakaguchi, 2009).

The finding of increased proportion of BW and lower BCS, with increased parity may have importance in identifying pathways that influence reproduction and health. The labile protein reserves are considered important determinants of health, reproduction, and production (Bell et al., 2000; Ji and Dann, 2013; Lean and DeGaris, 2021). Characterizing the responses to protein nutrition fed in the dry and transition period has been challenging (Husnain and Santos, 2019); however, nulliparous cows benefited more from increased MP before calving than multiparous cows and cows producing greater than 36 kg/d also increased production with increased MP before calving. Rodney et al. (2016) suggest that

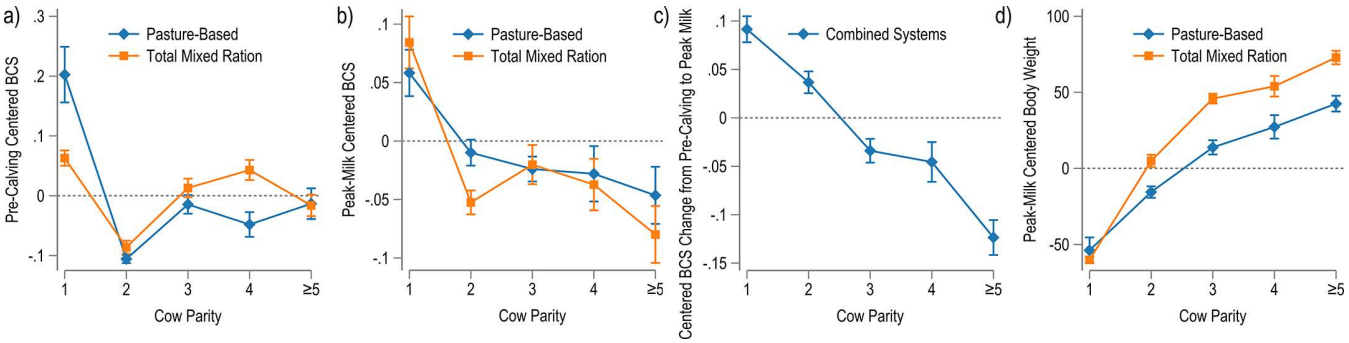

Figure 1. Predictions from linear regression models of (a) precalving centered BCS, (b) peak milk centered BCS, (c) the change in centered BCS from precalving to peak milk, and (d) peak milk centered BW (kg) for different cow parities and management systems are depicted. All outcome variables were center-transformed around study group mean values. Error bars are standard errors.

cows that can adjust to anabolic stimuli, such as additional protein supply, increase yield of milk protein, maintain a higher milk protein percentage, and improve reproductive performance. Yan et al. (2009) analyzed body composition in 146 head of Holstein-Friesian cattle and evaluated some parity effects. Live weight and empty-BW increased with parity; however, there was little difference in ratio of empty-BW to live weight for parity 1 (0.72) vs. parity ≥3 (0.73); the lipid, CP, and ash content (kg) numerically increased with parity. None of those comparisons were statistically significant possibly because the standard deviations were high, especially for lipid (Yan et al., 2009). Given the increased BW with parity, it is unclear what implications the pattern of change in BCS and BW with parity has for labile tissue pools that could influence immune and inflammatory response and provide support for lactation and health.

Limitations to this study include using single time point observations for BCS and BW. It is known that the nadir of body condition loss during early lactation is different across parities (Roche et al., 2009) and is not a simple linear relationship; however, by including DIM as a covariate, we help control for observation time. It was not possible to compare inter- and intraobserver variability for BCS assignment, though all assignments were performed by skilled practitioners with years of cattle experience. We attempted to control for genetic influence on BCS/BW outcome variables by using group-centered means, which requires an assumption that intragroup variation associated with genetics was similar across study groups.

Body condition loss during the transition period is strongly associated with cow parity, with older cows losing a much greater amount of labile energy reserves than younger cattle. Parity 2 cows had lower BCS results than expected at both precalving and peak milk observations. The use of waffle plots to visualize BCS-BW is unique and easily highlights the dramatic change in the body as cows age.

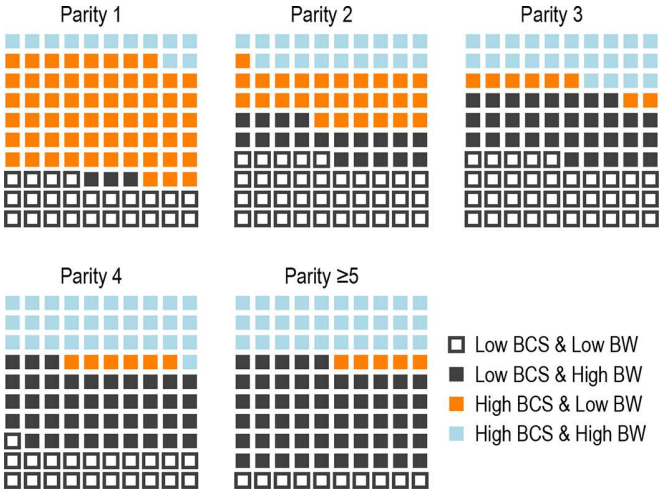

Figure 2. Proportion of cows in each category of BCS (low BCS ≤ group mean, high > group mean) and BW (low BW ≤ group mean, high > group mean) by parity at peak milk.

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CHAPTER 3

Associations among body condition score, body weight, and serum biochemistry in dairy cows

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OVERVIEW OF CHAPTER 3

This chapter expands on the novel body condition score (**BCS**) – body weight (**BW**) scoring system introduced in Chapter 2 by refining the BCS category to be greater than-, equal to-, or less than the median farm of origin BCS. The BW category remained as greater than- or less than median BW. The chapter investigates the body tissue metrics and their association with a standard panel of 15 blood metabolites. There were 739 dry-cows and 690 peak-milk cows with body measurements and serum metabolite data. The analyses performed were linear regressions on BCS and BW, and a polytomous logistic regression for the novel scoring system. Second-parity cows were most often low BCS and low BW, while high BCS and high BW was most common in > 3 parity cows. Albumin was the metabolite most consistently and positively associated with body measurements and milk production. Other important metabolites included, in the dry-period: glucose, which was negatively associated with the probability of being a low BCS/high BW cow, and positively with high BCS/high BW, and; urea, which was negatively associated with the probability of being low BW/low BCS and positively with median BCS/low BW; in the peak-milk period: β-hydroxybutyric acid, which was negatively associated with the probability of being median BCS/low BW and positively with high BCS/high BW, and high BCS/low BW, and; milk production (L/d), which was negatively associated with the probability of being high BCS/low BW and positively with low BCS/high BW. The consistent association between albumin and body tissue metrics suggests that protein metabolism, alongside lipid metabolism, should be considered in tissue mobilisation.

Note on supplementary material

The published article refers to a supplementary document available at https://doi.org/10.6084/m9.figshare.27426771. The tables from the supplementary document are also available in the *Appendix*. Supplementary Table S1 is reported across both Tables A1 and A2, Table S2 is Table A3, Table S3 is Table A4, Table S4 is Table A5, Table S5 is Table A5, and Table S6 is Table A7.

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Associations among body condition score, body weight, and serum biochemistry in dairy cows

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ABSTRACT

Body condition score and BW yield insights into body tissue reserves and diet, and serum biochemical measures reflect the metabolic status of cows. Associations between body composition measures and biochemistry are unclear and investigation may reveal important information on the metabolic and physiological status of cattle with varying levels of labile tissue reserves. Cohorts of 739 nonlactating, late-pregnancy, dry cows (26.9 d prepartum, SD = 12.4) and 690 peak-milk cows (58.0 DIM, SD = 14.5) were selected by stratified (parity: 1, 2, 3, >3) random sampling from 30 farms (15 pasture, 15 TMR) in this cross-sectional study. A single serum, BCS (1–5 scale), BW, and milk-production datum was collected per cow, per cohort between November 2022 and July 2023. Eleven analytes were collected, analyzed, and standardized within group (cohort/breed per farm). Mixed linear models for BCS and BW were specified, with the random effect of group. A 6-point, unordered, categorical body-group classification that combined BCS (greater, equal to, or less than group median; as high, median, or low BCS) and BW (greater or less than group median; as high or low BW) was analyzed by polytomous logistic regression. Effect sizes are listed for a 1 SD increase in the specified analyte, keeping other covariables at their mean value. Dry BCS was positively associated with albumin (0.075 BCS ± 0.014 SE), urea (0.038 BCS ± 0.014 SE), and glucose (0.052 BCS ± 0.014 SE), and negatively with the interaction between cholesterol and days precalving. Dry BW positively associated with albumin (11.03 kg ± 2.48 SE) and negatively with cholesterol (−8.47 kg ± 2.57 SE). Peak-milk BCS was positively associated with albumin (0.47 BCS ± 0.015 SE), BHB (0.048 BCS ± 0.015 SE), and glucose (0.051 BCS ± 0.015 SE). Peak-milk BW was positively associated with albumin (6.94 kg ± 2.35 SE) and negatively with Ca (−7.02 kg ± 2.33 SE). Increasing BW and decreasing BCS was associated with increasing parity, except in dry second-parity cows that had low BCS. The dry polytomous model associated a 1 SD increase in albumin with a 4.89% ± 1.56 SE decreased risk of being low BCS/low BW and 5.87% ± 1.46 SE increased risk of high BCS/high BW. Risk change associated with 1 SD of glucose was −5.61% ± 1.58 SE for low BCS/high BW and 3.17% ± 1.58 SE for high BCS/high BW. For the peak-milk cohort, change in risk was associated with albumin for low BCS/low BW −3.67% ± 1.56 SE, low BCS/high BW −3.22% ± 1.53 SE. Risk change with 1 SD of BHB was −3.36% ± 1.47 SE for median BCS/low BW, 2.86% ± 1.44 SE for high BCS/low BW, and 2.69% ± 1.37 SE for high BCS/high BW. Risk of low BCS/low BW was greatest in second-parity cows, and high BCS/high BW was greatest in dry cows with greater than third parity and third-parity cows in peak milk. There were no interactions between parity and analytes. Albumin was consistently associated with BCS and BW, potentially reflecting innate differences in protein metabolism of cows.

Key words: body condition, parity, albumin, protein, body weight

INTRODUCTION

Cows mobilize body reserves to facilitate the strong homeorhetic drive to produce large quantities of milk in early lactation (Bauman and Currie, 1980). This period of negative nutrient balance typically results in body condition and weight loss that reaches a nadir around 40 to 100 DIM (Westwood et al., 2002; Roche et al., 2009; Hernandez-Gotelli et al., 2023). Cows differ in their ability to partition and mobilize body reserves, with excessive mobilization associated with increased disease incidence, reproductive inefficiencies, and potentially poor cow welfare (Roche et al., 2009). Monitoring BCS

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-25. Nonstandard abbreviations are available in the Notes.

or BW, both measures broadly associated with available nutrient reserves, can allow producers to identify deficiencies in diet management and at-risk groups of cattle.

Though BCS and BW are the most commonly used indirect measurements for body tissue reserves, the correlation between the 2 metrics ranges from low to moderate (Berry et al., 2006a; Roche et al., 2007c) and can be influenced by a multitude of factors including visceral adipose tissue (**AT**; Drackley et al., 2014), stage of lactation or pregnancy, water intake (Moe et al., 1971), gut fill, and parity (Berry et al., 2006b; Lean et al., 2022). Combining BCS and BW into a single classification could be useful if it would increase our understanding of the biological underpinning of labile tissue pools of proteins, fats, minerals, and vitamins. Lean et al. (2022) introduced a novel classification scheme for BCS and BW that categorized cows into 4 groups according to being above or below the within-farm median BCS or BW. Heifers were most prevalent in the high BCS/low BW group with cows increasingly moving toward low BCS/high BW as they aged. Similarly, the risk of disease, mortality, and culling increases with advancing parity (Lean et al., 2023a) and parity is associated with differences in blood metabolites (Lean et al., 2023b). The interrelationship among BCS and BW, cow aging, and blood metabolites is currently unclear.

Biochemistry panels, such as those used in "metabolic profile tests" are a tool to investigate herd health and could provide broad insights into biological pathways associated with BCS and BW measurements (Payne et al., 1970; Van Saun, 2023). Panel analytes that are associated with protein (e.g., albumin, BUN) and energy (BHB, nonesterified fatty acids [**NEFA**], glucose) metabolism are suitable candidates for investigation (Kitchenham and Rowlands, 1976; Busato et al., 2002; Caldeira et al., 2007; Van Saun, 2023). Body condition score has been associated with insulin sensitivity or resistance (De Koster et al., 2015; Samii et al., 2019), lipid metabolism (Ghaffari et al., 2019; Schuh et al., 2019; Wang et al., 2020) and inflammatory markers (Akbar et al., 2015; Ghaffari et al., 2019; Wang et al., 2020) using a combination of transcriptomics, metabolomics, and biochemistry panels. However, serum analytes have often shown weak associations with BCS and BW. It is well established that analytes are strongly associated with the structural effects of a specific farm or feeding system (Krogh et al., 2020; Luke et al., 2020) and indeed this provides the metabolic profile test its utility. However, the strong association of an analyte with farm will obscure subtle associations with BCS or BW and prevent meaningful associations (VanderWeel et al., 2021). Study design and statistical methods capable of removing this effect improve both external validity and assist in understanding BCS and BW biology.

An analysis of cows at 2 extremes in body composition, those heaviest in late pregnancy and lightest at 40 to 100 DIM (Westwood et al., 2002; Roche et al., 2009; Hernandez-Gotelli et al., 2023), provides contrasting insights into the biological processes associated with these states. This approach also avoids the dynamic metabolic changes that occur during the immediate postpartum period (Walter et al., 2022). Hence, the objective of this prospective, multisite cross-sectional study was to investigate associations among BCS, BW, and a combined BCS/BW classification with serum biochemistry and parity, using cohorts of late-pregnancy and peak-lactation cows.

We consequently hypothesized that serum analytes, especially those associated with protein, energy, and calcium metabolism would be associated with BCS, BW, or a combined classification of BCS and BW in late-pregnancy and peak-lactation cows.

We anticipate that the results may highlight productive areas for future investigation into causes for variation in BCS and BW at a biological level and ultimately allow for optimized management of labile tissue pools to maximize milk solids production while reducing risks of adverse health and reproductive events.

MATERIALS AND METHODS

This study was carried out in accordance with the recommendations of the Australian Code for Care and Use of Animals for Scientific Purposes, Scibus Animal Ethics Committee (Scibus # 1022–1024).

Farm Enrollment

Farms were purposively selected to obtain an even distribution of 15 pasture-based (**PB**) farms and 15 housed, TMR farms, based on the lactating herd management system. Farms selection criteria were as follows: a mean lactating herd size of >200 animals, conventional (non-organic), maintained accessible, and electronic records (including a minimum of cow reproduction, birth date, and health treatment details) that were reviewed for suitability before inclusion. Most PB farms (n = 13/15) had kikuyu (*Pennesetum clandestinum*) as a substantial pasture grass in their swards, and all offered grain and supplemental concentrates in the dairy parlor. The TMR farms included freestall barns (n = 4/15, 26%), compost-bedded pack barns (n = 11/15, 73%), and dry-lots (n = 2/15, 13%). Farms, by regional development areas, were in Subtropical Dairy (PB: n = 3, region: n = 371), Dairy New South Wales (PB: n = 11, TMR: n = 4, region: n = 317), Murray Dairy (PB: n = 1, TMR: n = 8, region: n = 861), WestVic Dairy (TMR: n = 1, region: n = 941) and DairySA regions (TMR: n = 2, region: n = 182; Dairy

Australia, 2023). Cattle were not housed at any time of the year in PB farms. Individual farm details are in Supplemental Table S1 (see Notes) and lactating diets are available upon request.

Cow Enrollment

Two cohorts of cows per farm were enrolled, a precalving dry cohort (selected between 50 and 20 d prepartum, based on expected calving dates) and a peak-milk cohort (selected between 40 and 90 DIM). Exclusion criteria included animals on "to be culled," "do not breed," or "to be sold" lists; cows with fewer than 4 functional teats; or cows with or >2 lameness scores at the time of visit (Sprecher et al., 1997). For each score cohort, a disproportionate, stratified, random sampling procedure (Stata Statistical Software, release 16, StataCorp, College Station, TX) was used on lists of eligible cows that were provided by the farm managers. Stratification was on parity (dry cohort: nonlactating heifers, parity 1, parity 2, and parity >2; peak-milk cohort: parity 1, parity 2, parity 3, and parity >3). Each farm was visited once or twice, depending on-farm breeding strategy, with each cohort being completely sampled on a single day. Sampling began November 17, 2022, and concluded June 15, 2023. The parity of dry cows will be reported as their postcalving parity (e.g., a cow that has completed one lactation and will begin her second lactation after calving will be reported as second parity) to provide consistent interpretation of parity between cohorts.

Sample Size Estimate

A sample size estimate was performed for a 2-sample mean test in a cluster randomized design, with the cluster being farm type and an estimated interclass correlation of 0.2, equal group sizes of 15 farms, a normalized effect size for a single metabolite of 1 SD, a Bonferroni adjusted α of 3.8×10^{-3} (α = 0.05 and 13 independently assessed metabolites), 2-sided test, and study power of 80%. The result of this estimation required 3 cows per parity per farm to be sampled: Stata code, power twomeans 0, diff(1) k1(15) k2(15) rho(0.2) a(0.0038). A concurrent study power calculation required 9, 6, 6, and 8 animals per respective parity strata and consequently these counts were used in this study. If insufficient animals were available per parity strata, the eligibility window for days was extended as required.

Sample Collection

Body condition score was recorded on a 5-point scale using 0.25 increments, with 1 indicating emaciation and 5 obesity (Edmondson et al., 1989) and performed by the sampling veterinarians. Training for BCS was provided by an author of Edmondson el. al. (1989), no formal reliability testing was performed. Scorers had scored together on multiple occasions. Body weight was obtained on a split-platform stationary scale (Tru-Test Datamars, Queensland, Australia), with 3 farms preferring to use their own equipment. Breed was collected from on-farm assessment and cow-card information from the respective herd management software.

Blood samples were collected from the coccygeal vein into a 10-mL serum clot activator Vacutainer tube (Becton Dickinson, Franklin Lakes, NJ). Samples were allowed to clot at room temperature in darkness for 45 to 60 min before centrifugation (DM0412; DLAB Scientific Co. Ltd., Beijing, China) at 1500 × *g* for 15 min at room temperature. Aliquots of 1 mL were collected, stored temporarily at −18°C (Engel, Carole Park, QLD) during field travel (<4 d), before long-term storage at −80°C (Isotemp, Thermo-Fischer Scientific, Waltham, MA).

Individual cow milk data were collected from either a third-party herd testing center or milk volume was provided by in-line milk readers. Milk fat and protein were not available for all farms (n = 3). Scheduling of herd-test visits and sample collection was planned to be within a maximum of 2 wk and never on the same day. Milk volume from in-line milk readers was recorded from the day before sampling.

Laboratory Analysis

Serum samples were transported from long-term storage to Agriculture Victoria, AgriBio (Bundoora, VIC, Australia) on dry ice, where they were stored at −80°C. Samples were analyzed for bilirubin, glucose, NEFA, total protein, urea, cholesterol, triglycerides, BHB, albumin, calcium, and magnesium concentrations on a ChemWell 2910 Automated EIA and Chemistry Analyzer (Awareness Technology, Inc., Palm City, FL) using Catachem Inc. (Oxford, CT) reagents, controls, and calibrators per manufacturer's instructions. Globulin concentration was calculated by subtracting albumin concentration from total protein concentration, and the albumin to globulin ratio (**A:G**) was determined.

Statistical Analysis

Initial data evaluation included tabulation and visual appraisal of BCS and BW and biomarker histograms for normality of distribution and to identify potential outliers. The 2 cohorts, dry and peak milk, were analyzed as separate datasets throughout, with cow as the unit of interest. All statistical analyses were performed in Stata 16. Basic descriptive statistics were performed on analytes, including comparing the distribution within

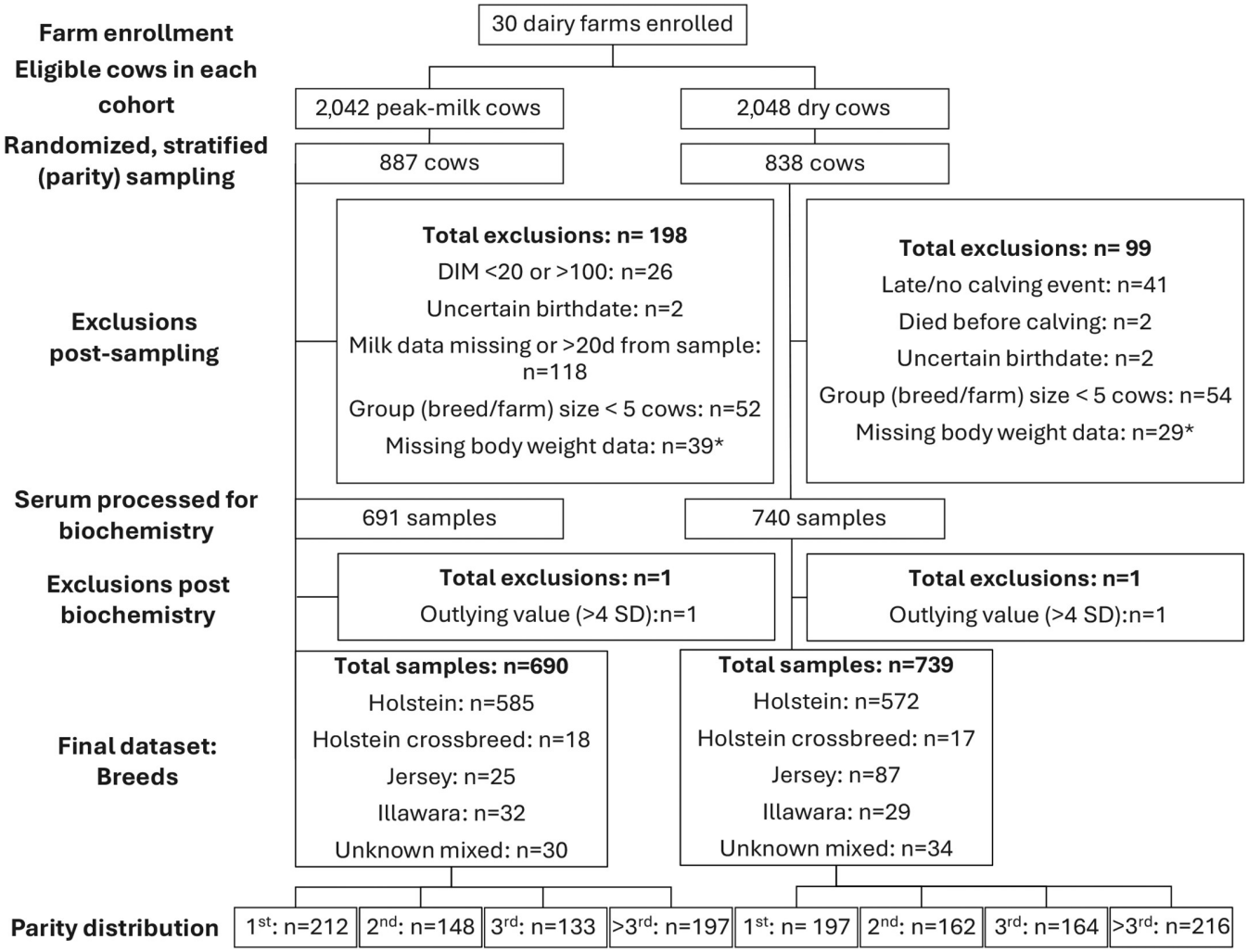

Figure 1. Flow diagram of cow enrollment and subsequent exclusions for the peak-milk and dry-cow cohorts. *Cows with missing BW measurements were not excluded in this figure, although were excluded where appropriate in the analysis.

farming system and individual farms and formally tested with unpaired, Welch's *t*-tests—Stata: ttest *analyte*, by(*farm_system*) welch.

To control the effect of breed and farm-level information (including system and season), analytes and milk data were all z-score normalized (standardized) within breed and farm, hereafter referred to as "group." Any group that contained 5 or fewer animals was excluded. Cows with any analyte greater than 4.0 SD from the mean were removed as extreme outliers or possible laboratory errors (n = 2 cows).

Further data exploration included cluster analysis of the blood metabolites using Stata's cluster *kmeans* (uninformed) and discriminate *lda* (informed) functions. Although the *kmeans* analysis separated distinct biological groups, these were not associated with our outcomes of interest (BCS, BW). Likewise, the discriminant analysis was poorly predictive of these outcomes of interest and thus cluster analysis will not be discussed further.

Mixed-effect linear regression models were specified for BCS and BW for the 2 cohorts. The effect of group was controlled by standardization and as a random effect. A manual, backward stepping approach was used to build the model, with the full model including (as appropriate) the following: all standardized analytes and milk production, parity, and days (precalving or DIM). Variables were removed if P-value > 0.05; confounding was considered if a coefficient value changed by 20% between steps, and interactions and quadratic transformations were explored. Milk protein and fat variables were also analyzed in a reduced dataset (53 cows removed) and discussed where appropriate. The intraclass correlation (by group)

Table 1. Descriptive statistics for serum analytes by housing system for late-pregnancy, nonlactating cows[1]

	PB (n = 335)		TMR (n = 404)		
Biomarker	Mean	SD	Mean	SD	*P*-value
Albumin (g/L)	34.61	3.20	36.59	3.15	<0.001
Globulin (g/L)	42.79	8.66	39.32	8.22	<0.001
Albumin:globulin	0.86	0.25	0.98	0.27	<0.001
Total protein (g/L)	77.40	7.37	75.93	7.55	0.008
BHB (mmol/L)	0.47	0.21	0.44	0.27	0.074
NEFA (mmol/L)	0.70	0.34	0.70	0.38	0.804
Glucose (mmol/L)	3.43	0.46	3.75	0.59	<0.001
Calcium (mmol/L)	2.55	0.17	2.56	0.18	0.537
Magnesium (mmol/L)	0.97	0.11	0.97	0.12	0.567
Cholesterol (mmol/L)	2.68	0.79	2.92	1.03	<0.001
Triglycerides (mmol/L)	0.50	0.18	0.36	0.15	<0.001
Bilirubin (μmol/L)	2.32	1.17	1.63	0.96	<0.001
Urea (mmol/L)	5.32	1.63	4.19	1.37	<0.001
Days precalving	26.63	13.58	27.18	11.52	0.555
Parity	2.79	1.58	2.81	1.64	0.886
BCS (1–5)	3.31	0.44	3.44	0.44	<0.001
BW[2] (kg)	622.88	121.02	697.97	118.22	<0.001

[1]PB = pasture based, NEFA = nonesterified fatty acids.
[2]Reduced sample size: PB, n = 335, TMR, n = 375.

was calculated and can be interpreted as the proportion of variance explained by the random effect structure.

The 4-category cow classification system introduced by Lean et al. (2022) was expanded, with cows assigned to a 6-category BCS/BW classification we refer to as a body classification group (**BCG**). Each BCG contained a BCS class (below, at, or above group median, referred to as low, median, and high BCS, respectively) and a BW class (below or above group median, referred to as low or high BW; median BW cows were randomly assigned to a BW category). The justification for increasing to 6 categories by introducing the "median BCS" category was because the biological interpretation of a median BCS cow could provide additional insight, and we found ~30% of cattle had median BCS. Group mean values for BCS and BW are reported in Supplemental Table S1.

Associations between BCG and analytes were analyzed in the following multinomial (polytomous) logistic regression (Stata: *melogit*):

$$\Pr(Y_i = j \mid \boldsymbol{w}_i) = \frac{\exp\left(\boldsymbol{w}_i'\boldsymbol{\beta}_j\right)}{1+\sum_{k=1}^{J}\exp\left(\boldsymbol{w}_i'\boldsymbol{\beta}_j\right)}, j = 0,1\ldots,J.$$

This model provides a set of probabilities that the *i*th cow will equal the *j*th BCG category given the *i*th cow's set of characteristics $\mathbf{w}_i$, with $\boldsymbol{\beta}_j$ the coefficient vector associated with the *j*th category. The group effect was controlled with the robust variance estimator '*cluster*' option. This model assumes no natural ordering between the BCG categories. A manual, backward stepwise selection was performed with a Wald test that all coefficients are zero for each BCG group (*mlogtest*, *wald*; Scott Long and Freese, 2014), with *P*-values < 0.05 retained in the final model. Quadratic transformations and interactions were evaluated.

We caution that the direct interpretation of multinomial logistic regression coefficients is difficult, because coefficients are specific to the reference group (low BCS/low BW) and change depending on the chosen reference group (including reported *P*-values and 95% CI). Therefore, results are presented as marginal values; the change in risk of belonging to a specific BCG following a SD change in an analyte, holding all other variables constant at their mean value.

RESULTS

A total of 739 dry cows and 690 lactating cows were included in the final analysis. Cows that were initially enrolled and subsequently excluded were due to the following: an uncertain birth date or parity of enrolled cow (n = 4), DIM <20 or >120 (n = 26), calving dates >60 d or did not calve (n = 41), died before calving (n = 2), lactation data missing or >20 d from sampling date (n = 118, 2 TMR and 2 PB farms) and if breed within herd count was less than or equal to 5 (dry; n = 54, lactating; n = 52; Figure 1). The eligible cow and sampled cow counts, by parity and group, are listed in Supplemental Table S2 (see Notes).

The untransformed distribution of analytes by farming system are in Table 1 and Table 2. Regardless of dry or peak-milk cohort, albumin, A:G, and cholesterol

Table 2. Descriptive statistics for serum analytes by housing system for early lactation cows

	PB (n = 306)		TMR (n = 384)		
Biomarker	Mean	SD	Mean	SD	*P*-value
Albumin (g/L)	35.42	4.84	37.14	3.91	<0.001
Globulin (g/L)	42.43	7.02	40.14	7.34	<0.001
Albumin:globulin	0.86	0.22	0.96	0.22	<0.001
Total protein (g/L)	77.89	7.21	77.10	7.23	0.048
BHB (mmol/L)	0.58	0.31	0.55	0.29	0.77
NEFA (mmol/L)	0.71	0.42	0.69	0.38	0.148
Glucose (mmol/L)	3.67	0.57	3.62	0.64	0.047
Calcium (mmol/L)	2.46	0.26	2.46	0.18	0.154
Magnesium (mmol/L)	0.99	0.14	1.07	0.12	<0.001
Cholesterol (mmol/L)	4.13	1.13	5.31	1.52	<0.001
Triglycerides (mmol/L)	0.31	0.11	0.21	0.09	<0.001
Bilirubin (μmol/L)	2.00	0.97	1.56	1.02	<0.001
Urea (mmol/L)	5.49	2.05	5.72	1.67	0.075
Milk volume (L)	26.96	7.82	41.01	12.90	<0.001
Protein[1] (%)	3.15	0.41	3.00	0.30	<0.001
Protein yield (kg)	0.84	0.24	1.20	0.37	<0.001
Fat[1] (%)	3.62	1.08	3.63	1.21	0.895
Fat yield (kg)	0.99	0.43	1.45	0.64	<0.001
DIM	60.61	16.03	55.94	12.74	0.002
Parity	2.72	1.66	2.65	1.53	0.534
BCS (1–5)	2.76	0.40	2.92	0.52	<0.001
BW[2] (kg)	552.92	106.28	648.72	89.98	<0.001

[1]Reduced sample size: PB, n = 300; TMR, n = 337.
[2]Reduced sample size: PB, n = 303, TMR, n = 348.

were higher and globulin, total protein, triglycerides, and bilirubin were lower in the TMR systems compared with the PB systems. Within the dry cohort, glucose (3.75 vs. 3.43 mmol/L, $P < 0.001$) was greater and urea (4.19 vs. 5.32 mmol/L, $P < 0.001$) were lower in the TMR systems compared with PB systems. In the peak-milk cohort, magnesium (1.07 vs. 0.99 mmol/L, $P < 0.001$) was greater in the TMR systems. Cows were heavier and in greater body condition in the TMR herds and produced more milk volume, fat yield, and protein yield (Tables 1 and 2).

Mixed Linear Regression Models

Dry Cohort. There were positive associations between BCS and an SD change in the serum analytes of albumin (0.07 BCS, 95% CI: 0.043–0.098), urea (0.037 BCS, 95% CI: 0.009–0.065), and glucose (0.050 BCS, 95% CI: 0.022–0.079; Table 3). The BCS was lower the further away from respective calving date (0.006 BCS, 95% CI: 0.003–0.009). There was an interaction between days from calving and serum cholesterol such that cholesterol was negatively associated with BCS in cows that were recently dried-off and became decreasingly associated as cows approached calving (with ultimately no fixed effect of cholesterol at calving date). Heifers had the highest BCS in the dry period, with second-parity cows being in the lowest condition (Table 3). The intraclass correlation for group was 0.261 (95% CI: 0.167–0.383).

The final mixed linear regression for BW indicates that an SD increase in albumin was associated with an 11.0 kg (95% CI: 6.13–15.9) increase in BW, while a SD increase in cholesterol was associated with an 8.7 kg (95% CI: 3.5–14.0) decrease in BW (Table 4). Cows continue to gain BW with increasing age, with each parity different from another ($P < 0.01$; Table 4). There were no significant interactions or nonlinear associations. The intraclass correlation for group was 0.618 (95% CI: 0.491–0.731).

Peak-Milk Cohort. Within the peak-milk cohort, there were positive associations between BCS and an SD change in albumin (0.047 BCS, 95% CI : 0.017–0.077), BHB (0.048 BCS, 95% CI: 0.019–0.077), and glucose (0.051 BCS, 95% CI: 0.021–0.081), and negative associations with milk volume (0.083 BCS, 95% CI: 0.046–0.119; Table 5). The >3 parity cows had the lowest BCS scores, though only statistically lower than the heifers ($P < 0.05$). The intraclass correlation for group was 0.331 (95% CI: 0.215–0.473). In the reduced dataset and allowing only one milk parameter in the full model, protein yield (0.04 BCS 9, 95% CI: 0.12–0.086) and fat yield (0.041 BCS, 95% CI: 0.006–0.075) were negatively associated, protein percent (0.053 BCS, 95% CI: 0.023–0.083) was positively associated with BCS. Fat percent was unassociated with BCS.

Body weight was positively associated with an SD change in albumin (6.94 kg, 95% CI: 2.33–11.56) and milk production (6.19 kg, 95% CI: 0.56–11.83), and negatively with calcium (7.02 kg, 95% CI: 2.46–11.58;

Table 3. Mixed linear model of BCS with serum analytes and parity for nonlactating, late-pregnancy dry cows and random effects of breed and farm[1]

Parameter	Coefficient	SE	*P*-value	95% CI	
Fixed					
Albumin	0.075	0.014	<0.001	0.047	0.102
Urea	0.038	0.014	0.007	0.010	0.065
Glucose	0.052	0.014	<0.001	0.024	0.080
Cholesterol × DBC	−0.0018	0.0005	<0.001	−0.003	−0.001
DBC	0.006	0.001	<0.001	0.004	0.009
Parity: 1	Referent[A]	—	—	—	—
2	−0.138[B]	0.042	0.001	−0.217	−0.052
3	−0.087[AB]	0.042	0.035	−0.167	−0.002
>3	−0.031[A]	0.038	0.421	−0.107	0.046
Constant	3.279	0.056	<0.001	3.170	3.389
Random					
Var (breed/farm)	0.047	0.013	—	0.027	0.081
Var (residual)	0.132	0.007	—	0.119	0.147

[A,B]Parity coefficients sharing a letter were not different at the 5% level, holding all other variables at their mean.
[1]All serum analytes are z-standardized within breed/farm such that a 1-unit increase is a 1-SD increase. DBC = days before calving; Var = variance.

Table 6). Cows of greater parity had greater BW than those of lesser parity, although parities 3 and >3 were not statistically different at the 5% level. The intraclass correlation for group was 0.625 (95% CI: 0.486–0.747). In the reduced dataset and allowing only one milk parameter in the full model, only protein yield was weakly associated with BW (5.21 kg, 95% CI: −0.48 to 10.80). Fat yield, fat percent, and protein percent were not associated with BW.

The intraclass correlations across all linear mixed models indicated that roughly 25% to 30% of the variance in BCS and 60% of the variance in BW was explained by the random effect structure of farm and breed.

Multinomial (Polytomous) Logistic Regression Models

The cow count for each BCG group, by parity, is reported in Supplemental Tables S3 and S4 (see Notes). The multinomial logistic regression results for both cohorts are in Supplemental Tables S5 and S6 (see Notes). For the dry cohort, by parity group, the highest probability BCG were as follows: low BW/high BCS in heifers (36.21%, 95% CI 27.67–44.76); low BW/low BCS in second lactation (36.16%, 95% CI: 26.32–46.00); high BW/high BCS in third lactation (27.33%, 95% CI: 18.77–35.90); and high BW/high BCS in greater than third lactations (39.00%, 95% CI: 31.00–46.99; Figure 2). There were no significant interactions or nonlinear transformations for the logistic regression model.

For the lactating cohort, within each parity group, the highest probability BCG were as follows: median BCS/low BW in heifers (37.94%, 95% CI: 27.42–48.46); low BCS/low BW in second-parity cows (26.83%, 95% CI: 18.08–33.13); high BCS/high BW in third-parity cows (33.53%, 95% CI: 25.09–41.97); and low BCS/high BW in greater than third parity cows (34.23%, 95% CI: 26.12–42.33; Figure 3). There were no significant interactions or nonlinear transformations for the logistic regression model.

There was a monotonic increase in the risk of being high BW, regardless of BCS category or cohort as cows increased in parity.

Dry Cohort. The final model for the dry cohort included albumin, glucose, and urea, with parity and days precalving. An SD increase in serum albumin was associated with a 4.89% (95% CI: 1.82–7.96) decreased probability of being in the low BCS/low BW category and a 5.88% (95% CI: 3.00–8.75) increased probability of being in the high BCS/high BW category (Figure 4). Increasing serum glucose by 1 SD was associated with a 5.61% (95% CI: 2.51–8.72) decreased probability of being low BCS/high BW and a 3.17% (95% CI: 0.07–6.27) increased probability of being high BCS/high BW. An SD increase in urea was associated with a 4.15% (95% CI: 1.57–6.74) decreased probability of being low BCS/low BW and a 2.25% (95% CI: 0.09–4.41) increase of being median BCS/low BW.

Peak-Milk Cohort. The final model for the lactating cohort included albumin and BHB, parity, and milk volume. An SD increase in albumin was associated with a decreased risk of low BCS/low BW (−3.67%, 95% CI: −6.73 to −0.60) and low BCS/high BW categorization (−3.22%, 95% CI: −6.22 to −0.22). An SD increase in BHB was associated with a 3.35% (95% CI: 0.47–6.25) decrease in median BCS/low BW and an increased risk of high BCS/high BW (2.69%, 95% CI: 0.01–5.37) and high BCS/low BW (2.86%, 95% CI: 0.04–5.67) categorization. An increase of 1 SD in milk volume was associated with a 5.39% (95% CI: 1.14–9.64) increased risk of being

Table 4. Mixed linear model of BW with serum analytes and parity for nonlactating, late-pregnancy dry cows and random effects of breed and farm[1]

Parameter	Coefficient	SE	*P*-value	95% CI	
Fixed					
Albumin	11.03	2.48	<0.001	6.17	15.90
Cholesterol	−8.74	2.67	<0.001	−13.96	−3.51
Parity: 1	Referent	—	—	—	—
2	81.87	7.55	<0.001	67.08	96.67
3	143.23	7.46	<0.001	128.62	157.85
>3	172.55	6.84	<0.001	159.14	185.96
Constant	557.42	15.39	<0.001	527.26	587.59
Random					
Var (breed/farm)	6,728	1,736	—	4,057	11,157
Var (residual)	4,153	225	—	3,734	4,619

[1]All serum analytes are z-standardized with breed/farm such that a 1-unit increase is a 1-SD increase. All parity coefficients were significantly different at the 1% level, with other variables held at mean values. Var = variance.

low BCS/high BW and a 7.11% (95% CI: 4.10–10.13) decreased risk of being high BCS/low BW (Figure 5).

DISCUSSION

This cross-sectional study was designed to investigate associations among BW, BCS, and a combination of the 2 poorly correlated metrics, with serum analytes and parity of cows at precalving and peak lactation. The novel BCS/BW classification provides additional insights to the body tissue reserves of cows. Parity and serum albumin were consistently associated with all body measurements, regardless of cohort or model.

Variation associated with farm-level factors (including season, housing system, region) were eliminated through random effects models, centering, and z-score standardization. All remaining variation in analytes, BCS/BW measurements can then be attributed at the cow level (or random error), enabling comparison across farms. Although this method prevents investigating specific farm-level fixed effects, it controls for unknown and unmeasured farm-level factors while increasing the degrees of freedom (compared with a fixed-effect model) and hence study power. We note that a causal relationship cannot be determined in a cross-sectional study; consequently, these observations are reported as associations. We identified analytes that were associated with body measurements and often with relatively small effect sizes. These associations, although independently valid, may identify more substantial associations if maintained over repeated observations and time. Furthermore, by enrolling multiple farms (n = 30) from 2 different production systems and using a large sample size (n = 1,429), the external validity is enhanced. New and existing studies will determine whether such associations are substantive and important determinants of BCS and BW.

Parity

Body weight increased monotonically with parity in the dry cohort, whereas the peak-milk cohort increased BW until the third lactation (which was not different to the >3 parity group). This plateau of BW in older cows at peak lactation, despite a difference during the dry period, is likely a result of increased mobilization of body tissue reserves in older cattle. Indeed, the reduction in predicted BCS (between the dry and peak-milk linear regression models) for the 3 and >3 parity cows were 0.51 and 0.62, respectively. This phenomenon of older cows being heavier and of lower body condition at peak milk has previously been described (Roche et al., 2009; Lean et al., 2022). Higher parity cows are generally higher producing, but at the cost of greater BCS loss in the early postpartum period (Buckley et al., 2000; Lee and Kim, 2006; Khan et al., 2011; Gärtner et al., 2019). After controlling for the effect of milk production, our models indicate that older cows will still have lower BCS than younger stock. Although the effects of milk production are characterized, other biological pathways that cause older cows to lose more BCS than younger cows remain unclear. There were no significant interactions between tested analytes and parity that increased the model fit, indicating that although analyte levels frequently differ across parities (data not shown), the strength of association between an analyte and a body measurement is unchanged across parities. For example, although the risk of having high BHB is greater in a >3 parity cow than a heifer, the association between high BHB and BCS and BW is the same for all parities.

The second-parity dry cows were most likely to be low BCS/low BW, as has been reported elsewhere (Berry et al., 2006b; Roche et al., 2007a; Lean et al., 2022). This phenomenon follows from first-parity cows failing to gain BCS post BCS-loss nadir as efficiently as older cows

Table 5. Mixed linear model of BCS with serum analytes and parity for peak-milk cows and random effects of breed and farm[1]

Parameter	Coefficient	SE	*P*-value	95% CI	
Fixed					
Albumin	0.047	0.015	0.002	0.017	0.077
BHB	0.048	0.015	0.001	0.019	0.077
Glucose	0.051	0.015	0.001	0.021	0.081
Milk volume	−0.083	0.019	<0.001	−0.119	−0.046
Parity: 1	Referent[B]	—	—	—	—
2	−0.072[AB]	0.045	0.112	−0.160	0.017
3	−0.073[AB]	0.049	0.135	−0.168	0.023
>3	−0.123[A]	0.046	0.007	−0.213	−0.033
Constant	2.920	0.059	<0.001	2.805	3.036
Random					
Var (breed/farm)	0.069	0.021	—	0.039	0.124
Var (residual)	0.139	0.008	—	0.125	0.155

[A,B]Parity coefficients sharing a letter were not different at the 5% level, holding all other variables at their mean.

[1]All serum analytes and milk production are z-standardized within breed/farm such that a 1-unit increase is a 1-SD increase. Var = variance.

(Berry et al., 2006b; Roche et al., 2007a). First-parity cows enter lactation at around 80% of their mature BW (Berry et al., 2005) and must continue to accrete resources to skeletal growth throughout that lactation. Possible reasons for limited tissue accretion in first-lactation heifers include the competing resource demands for skeletal growth while producing enough milk to avoid culling, as well as restricted feed access due to low social hierarchy associated with their lower BW and young age (Phillips and Rind, 2002; Deniz et al., 2021).

Albumin

Albumin was consistently and significantly positively associated with higher BW and higher BCS in the mixed linear and logistic regression models across both cohorts. Additionally, in the peak-milk logistic model, albumin was negatively associated with low BCS regardless of BW. These observed associations between BCS and BW metrics and albumin may reflect differences in the hepatic inflow of protein and AA for albumin synthesis, the innate ability to synthesize albumin, or a reduction in irreversible loss of albumin after its production. We briefly explore these possibilities.

Serum albumin concentration is increased with crude protein intake and with increased DMI (Payne et al., 1970; Lee et al., 1978; Law et al., 2009; Bobbo et al., 2017). Because the relationship between feed intake and BCS and BW is already well established, we attempted to minimize the effect of different diets offered across the different farms and farming systems through study design. Standardization and centering of all analytes on group and controlling for other factors that may vary DMI, including parity, days pregnant or DIM, and production level were included in the final models. However, individual DMI was not monitored and could vary within a group through competition with feed scarcity, limited bunk space, ill health, or other adverse events. Hence body measurements could still reflect the relative difference in DMI despite our efforts to limit this effect. The low BCS/low BW and high BCS/high BW associations with albumin may follow a sustained decrease or increase in dietary protein intake, respectively. Agenäs et al. (2006) investigated 3 severely undernourished herds (BCS 1.0) using serum analyte reference values and reported that albumin, urea, creatinine, and fructosamine were significantly associated with malnourishment. Although this observation supports our results, our cattle were not malnourished (Tables 1 and 2). Given the control measures in our study design, the association between body measurements and albumin is unlikely to be fully explained by differences in diet, and serum albumin has previously been reported to differ within a farm, despite similar protein intake (Rowlands, 1978).

Variation in cows' microbial protein production, which contributes 40 to 90% of absorbed protein, could underlie the observed associations between albumin and BCS and BW (Castillo-Lopez and Domínguez-Ordóñez, 2019; Li et al., 2019; NASEM, 2021; Lima et al., 2023). Recently 18% to 30% of variation in milk protein production was explained by measures of rumen-microbiome diversity (Xue et al., 2020; Lima et al., 2023). The differences in ruminal microbiomes and efficiency of protein production (Golder et al., 2018) may be partly reflected in the moderate heritability of serum albumin (h^2 = 0.19, Peterson et al., 1982; h^2 = 0.13, Cecchinato et al., 2018; h^2 = 0.27, Luke et al., 2019).

Albumin is a negative acute phase protein that decreases in concentration during periods of inflammation by downregulation of hepatic synthesis to putatively increase the availability of glucogenic AA in support of gluconeogenesis (Bertoni et al., 2008; Ceciliani et al.,

Table 6. Mixed linear model of BW with serum analytes and parity for peak-milk cows and random effects of breed and farm[1]

Parameter	Coefficient	SE	*P*-value	95% CI	
Fixed					
Albumin	6.94	2.35	<0.001	2.33	11.56
Calcium	−7.02	2.33	<0.001	−11.58	−2.46
Milk volume	6.19	2.87	0.031	0.56	11.83
Parity: 1	Referent	—	—	—	—
2	59.78	7.07	<0.001	45.93	73.62
3	104.85[A]	7.70	<0.001	89.76	119.93
>3	114.09[A]	7.25	<0.001	99.88	128.31
Constant	529.27	15.21	<0.001	499.46	559.08
Random					
Var (breed/farm)	5,363	1,528	—	3,068	9,376
Var (residual)	3,206	181	—	2,869	3,582

[A]Parity coefficients sharing a letter were not different at the 5% level, holding all other variables at their mean.
[1]All serum analytes and milk production are z-standardized within breed/farm such that a 1-unit increase is a 1-SD increase. Var = variance.

2012). Akbar et al. (2015) investigated serum albumin concentration in association with BCS and indicators of inflammation, including via liver biopsies. Cows with low BCS at calving (3.5 on a 10-point scale) had significantly lower albumin concentration throughout the study period (−4 wk to 6 wk around calving) compared with the mid (4.5/10 BCS) and high BCS (5.5/10 BCS) groups. There were no interactions between albumin and measures of inflammation that included haptoglobin, serum amyloid A, and globulin. The authors noted a higher A:G in the medium BCS and hypothesized a less pronounced inflammatory status and better liver function for this group of animals. Our results found no significant effect associated with A:G and any BCG. Similarly, Roche et al. (2013) reported increased proinflammatory liver enzymes in over-conditioned animals; however, albumin was again lowest in the low BCS group. Although it is plausible to use albumin or indexes that include albumin to identify individual cattle with, or at risk of, clinical disease (Bertoni and Trevisi, 2013; Cattaneo et al., 2021), we hypothesize that the negative-phase protein aspect of albumin is not strongly associated with BCS and BW. Rather, the consistent associations of albumin with increased BCS observed within studies (Akbar et al., 2015; Roche et al., 2013) indicate that protein accretion into tissue pools is reflected in higher serum albumin.

Serum albumin concentrations are reduced with parasite infections (e.g., fascioliasis or ostertagiosis) through reduced hepatic albumin production, reduced appetite, and increased irreversible loss in the intestine (Fox, 1997; Forbes et al., 2004; Taylor et al., 2007). Parasitism is also associated with lower BW and BCS (Forbes et al., 2004; Taylor et al., 2007). Although we expect the effect of parasitism to be minimal for our enrolled herds based on the age of cattle and possibility of anthelmintic treatments, this remains a plausible source of irreversible loss of albumin in infected cattle.

Glucose

In the linear models, glucose was positively associated with BCS for both dry and lactating cows but was not associated with BW. In the dry cohort logistic model, there was a monotonically positive association between glucose and BCS categories within high BW cows, but there were no associations between BCS categories in low BW cows. The differing associations between BCS and BW highlight that these are very different biological measures.

Prepartum cows in high BCS have reduced insulin responsiveness and sensitivity, and hence higher blood glucose (De Koster et al., 2015; Karis et al., 2020; Ghaffari et al., 2023). A possible reason for glucose only being associated with BCS in the high BW category of cows, and not in low BW cows, may be attributed to visceral AT. Visceral AT is more readily metabolizable than subcutaneous AT, is not recognized in BCS measurements, and represents the largest proportion of fat in the body (~66% in dry cows; De Koster et al., 2015). As such, visceral AT can contribute to BW differences without differences in BCS (Drackley et al., 2014). Visceral AT is also associated with increased insulin resistance (Drackley et al., 2014) and could provide different energy metabolism regulatory environments between the 2 BW categories in dry cows.

Glucose metabolism is considerably different in early lactation compared with the dry period due to the marked irreversible loss of glucose in the noninsulin dependent drive by the mammary gland (Bell, 1995; De Koster and Opsomer, 2013) and is influenced substantially by the

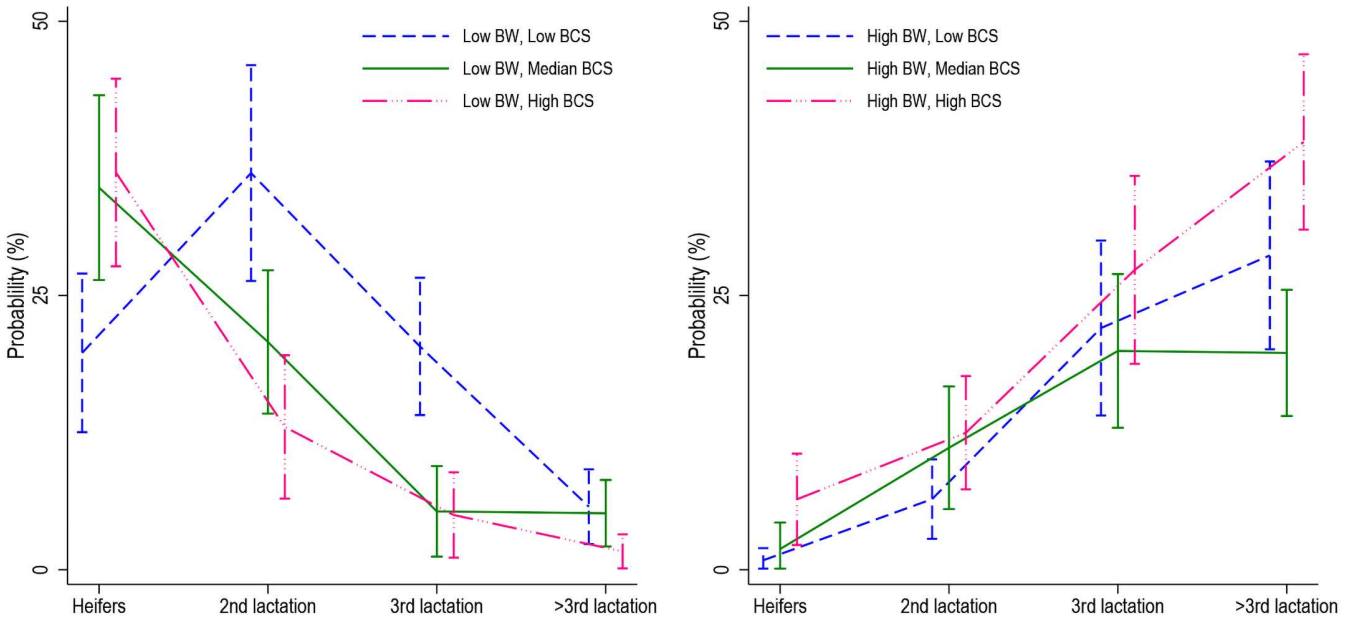

Figure 2. The change in probability of having a specific body condition group associated with parity for cows in the dry cohort with all other variables in the multivariable polytomous model held constant at their mean values. Left: Low BW cows at 3 BCS levels; right: high BW cows at 3 BCS levels. The error bars are 95% CI.

somatotropic axis (Bauman, 1999). The reported linear models controlled for the effect of milk production, yet a positive association between BCS and glucose remained. This indicates that the association of reduced insulin sensitivity and responsiveness with BCS is maintained in lactating cows. Busato et al. (2002) similarly found high-BCS cows and cows that do not lose BCS during the early postpartum period have higher serum glucose.

The results from the peak-milk logistic model indicate that, unlike the dry cohort, glucose is not associated with BCG. We hypothesize that following a period of negative nutrient balance, the differences in the mass of the rapidly metabolizable visceral and other AT between high and low BW cows is expected to be relatively small when compared with dry cows. As such, the regulatory environment for energy metabolism associated with these lipid reserves would be less significant in peak-milk cows.

BHB

The BCS of cows at peak milk production was positively associated with BHB in the linear model. The peak-milk logistic regression model reported a positive association with high BCS, regardless of BW. During the early postpartum period, BHB is produced in increased quantities by the liver as labile tissue pools are mobilized to provide peripheral tissue with energy (Krebs, 1966; Lean et al., 1992; Bobe et al., 2004). Peak concentrations of BHB are expected at around 21 DIM (Dohoo and Martin, 1984; Westwood et al., 2000; Akbar et al., 2015), with the mean of our cohort at 58 DIM. Consequently, the observed positive associations between BCS and BHB at peak milk are likely to reflect the continued availability of labile fatty acids in high-BCS cows at peak milk, high milk production (Westwood et al., 2000), possible hypophagia related to high BCS, or immune activation (Horst et al., 2021). Because nutrient balance or immunosuppression improves with DIM, the association between BHB and body tissue measures may be reduced or different compared with early postpartum. Associations between high BCS and high BHB in early lactation have been reported elsewhere (Busato et al., 2002; Akbar et al., 2015; Schuh et al., 2019).

Urea

Serum urea was positively associated with precalving BCS, while the logistic model indicated low BCS/low BW cows were more likely to have low urea. All peak-milk models showed no association with urea. Ureagenesis occurs mostly in the liver to primarily oxidize AA surplus to protein-synthesis requirements and to detoxify ammonia of predominately rumen origin (Staples et al., 1992). Serum urea therefore is positively related to protein intake (Staples et al., 1992; Law et al., 2009). As such, cows in low BCS may be catabolizing comparatively less protein, processing less rumen ammonia, or had reduced intake. These reasons have similarities to the

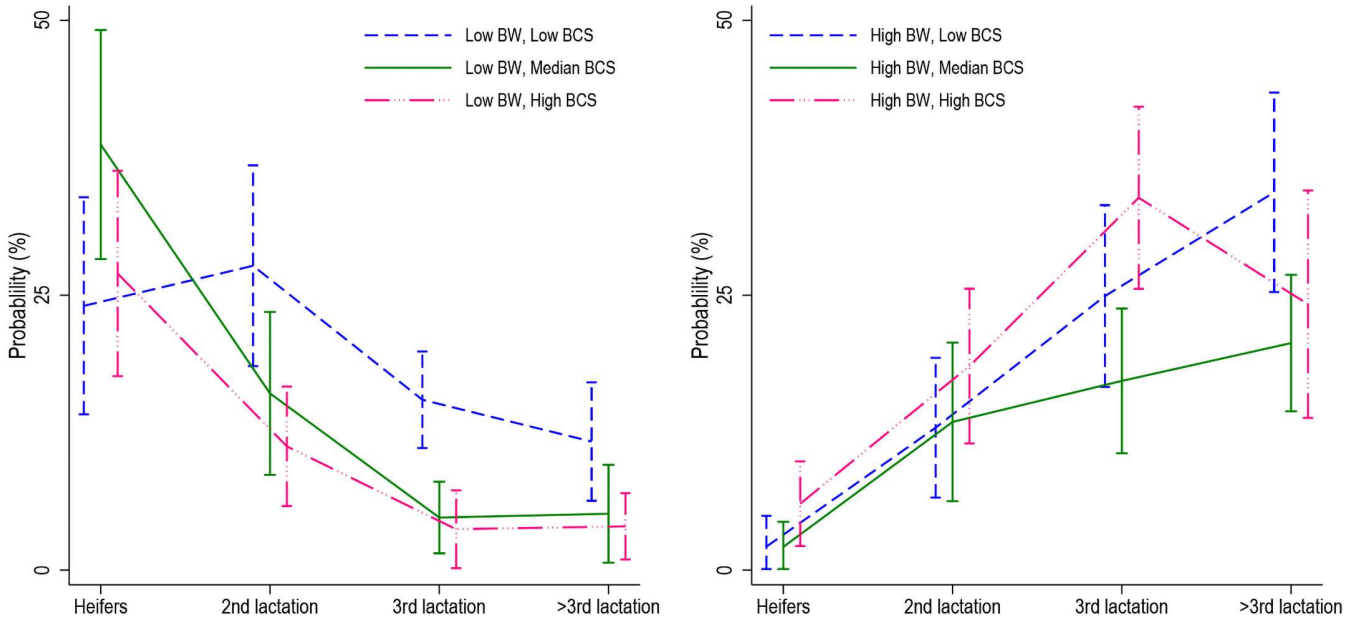

Figure 3. The change in probability of having a specific body condition group associated with parity for cows in the peak-milk cohort with all other variables in the multivariable polytomous model held constant at their mean values. Left: Low BW cows at 3 BCS levels; right: high BW cows at 3 BCS levels. The error bars are 95% CI.

earlier albumin discussion and will not be repeated here. The microbiome may be an important source of differentiation in the urea cycle in cows as suggested by Prahl et al. (2022), who reported that cows with high MUN had high plasma urea and rumen ammonia, but did not differ in their liver hepatic urea synthesis rate.

Serum urea concentrations increase from a nadir around 3 wk precalving to a plateau around 2 to 5 wk postcalving (Stephenson et al., 1997; Stockdale, 2006), because both dietary intake and protein catabolism increase with lactational demands. Our results support this, with TMR systems reporting a larger difference in urea between the dry and peak-milk cohorts compared with the PB farms. Stockdale (2006) reported cows in greater BCS (6 vs. 4 on an 8-point scale) had greater serum urea at around 15 d before calving, but there was no difference between BCS groups from calving to the study end at 10 wk of lactation. Similarly, Gobikrushanth et al. (2019) reported no difference in serum urea and changes in BCS between precalving (9 d) and postcalving (35 d). It is unknown why the association between BCS and urea is not present in early lactation, but it may reflect increased water intake and clearance rates.

Cholesterol

Cholesterol was negatively associated with BCS and BW early in the dry period, but this relationship declined to nonsignificance as calving approached in the BCS linear model. Serum cholesterol decreases as cows approach calving and subsequently rebounds to a steady state at around 6 to 12 wk postpartum (Ruegg et al., 1992; Kessler et al., 2014; Schuermann et al., 2019), which is supported by our results (Tables 1 and 2). Although chronically overfed cows develop high cholesterol levels (Dänicke et al., 2014; Arroyo et al., 2017), studies with sudden feed restriction consistently show a homeostatic response to the nutrient deficiency by increasing serum cholesterol and hepatic gene expressions involved in lipid metabolism (Laeger et al., 2012; Gross et al., 2015; Leduc et al., 2021). Dry-cow diets are less energy dense than lactating diets, such that introducing a dry-cow diet mimics a brief feed restriction trial. We hypothesize that high-BCS cows at dry-off date were low-milk-producing cows (hence accumulating body reserves), and consumed less feed compared with high-production cows. Therefore, the transition to a dry-cow diet had less relative change in total energy intake for the high-BCS cows. It then follows that high-BCS cows would have a relatively reduced homeostatic increase in serum cholesterol. As cows approached calving date, the adaptation to the dry-cow diet was complete and cholesterol levels normalized across the body type groups.

Both low serum cholesterol and over-conditioned cows have been associated with increased risk of periparturient disease (Kaneene et al., 1997; Sepúlveda-Varas et al., 2015) and a decreased risk of pregnancy, expression of estrus at first ovulation, and interval from calving to

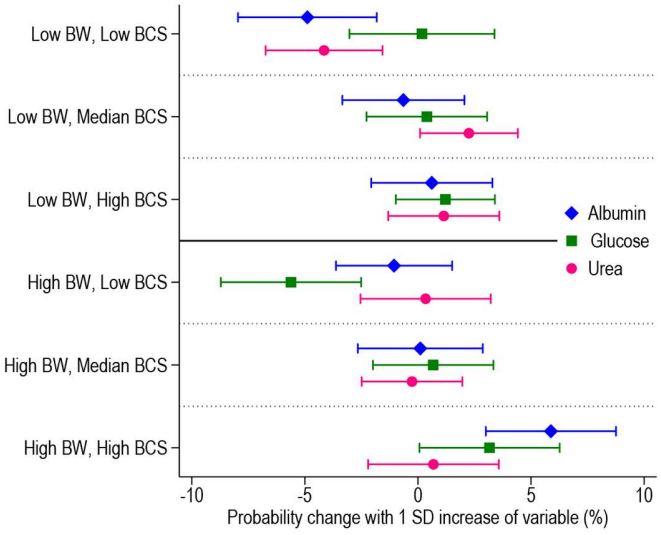

Figure 4. The change in probability of being in a specific body condition group associated with a 1-SD change in the shown variable for cows in the dry-cow cohort, with all other variables in the multivariable polytomous model held constant at their mean values. The error bars are 95% CI.

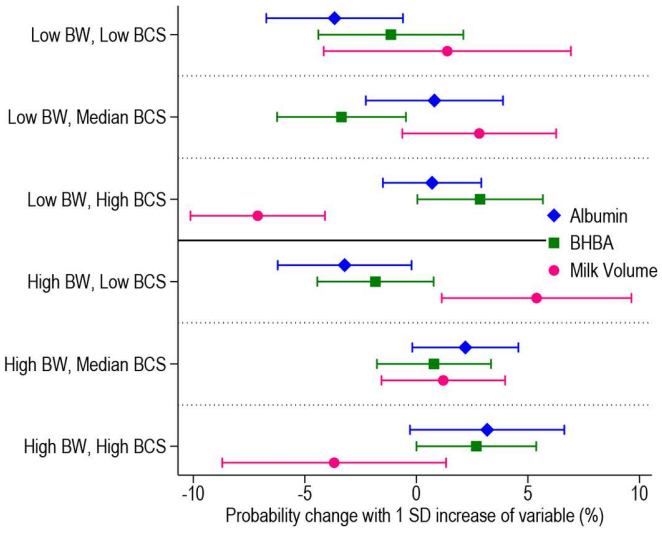

Figure 5. The change in probability of being in a specific body condition group associated with a 1-SD change in the shown variable for cows in the peak-milk cohort, with all other variables in the multivariable polytomous model held constant at their mean values. The error bars are 95% CI.

pregnancy or conception (Kappel et al., 1984; Ruegg et al., 1992; Moss, 2001; Westwood et al., 2002). Our study indicates cholesterol and BCS are correlated, but any causal relationship between these measures and the negative health or production outcomes remains to be fully elucidated. Attempts to alter precalving serum cholesterol through dietary manipulation in the dry period had moderate effects; however, the response in cattle health and reproduction has not been reported (Andersen et al., 2008; Newman et al., 2016).

Calcium

Calcium was negatively associated with BW in the peak-milk linear model. Bone tissue is a noted endocrine organ that regulates energy metabolism in murine, human, and more recently in dairy cattle models (Lee et al., 2007; Ferron and Lacombe, 2014; Lean et al., 2014; Martinez et al., 2018), predominately through action of the osteoblast-produced hormone osteocalcin. In general, higher BW cows will have higher skeletal mass than lower BW cows and this difference may produce a different regulatory environment for energy and calcium metabolism. The result of heavier cows having lower calcium at peak milk suggests these animals have relatively less reaccretion of bone tissue following the demineralization associated with the immediate postpartum period and therefore also potentially aberrant energy metabolism. Although BW is associated with differences in osteocalcin concentrations in human studies, this association has not yet been reported in dairy cows (Kord-Varkaneh et al., 2017). The effect of variable feed intake due to BW differences, after accounting for milk production, could also influence serum calcium levels.

Milk Measures

In the logistic model, milk volume was positively associated with low BCS/high BW and negatively with high BCS, regardless of BW. In the linear models, milk production was negatively associated with BCS and positively with BW. The opposite association of BW and BCS with milk production provides further compelling evidence that these 2 body measures are poorly correlated. Labile body tissue reserves after calving are catabolized to enable the strong homeorhetic drive for milk production under conditions of negative energy and nutrient balance (Bauman and Currie, 1980). Subsequently cows at peak milk with high BCS are presumed to have mobilized less tissue and possess a relatively muted homeorhetic drive for milk production. The association of high BW with increased production is well characterized (ARC, 1984; NASEM, 2021). Reduced protein and fat yield and increased protein percentage were similarly associated with BCS, which further supports the hypothesis that high-BCS cows were not mobilizing tissue toward milk production. Other studies support our finding of high milk volume and solids production associated with increased loss of body condition (Buckley et al., 2000; Roche et al., 2007b; Gobikrushanth et al., 2019).

The results support other work on BCS loss during the postpartum period; although excessive BCS loss is associated with disease risk and poor reproduction, maintaining or even increasing BCS during this period is not necessarily a purposeful goal given the association with poor milk production (Lean et al., 1994; Buckley et al., 2000; Herdt, 2000; Gobikrushanth et al., 2019). Rather, efforts should be on achieving optimal BCS after the nadir and before dry-off (Roche et al., 2009; Mineur et al., 2020) and providing appropriate transition diets that support milk production and prevent diseases associated with excessive rapid tissue mobilization (Lean and DeGaris, 2010).

Albumin was positively associated with higher milk volume and milk protein yield in our study and others (Rowlands et al., 1977; Bobbo et al., 2017) and was also positively associated with body measurements. However, these milk-production parameters were negatively associated with BCS. This relationship may indicate potential confounding, which is not possible to establish in a cross-sectional study design and therefore careful consideration is required in any future studies that wish to measure milk production, blood metabolites, and BCS/BW.

Bias and Further Research

Selection bias could be a factor in this study and cannot be quantified. Selective culling and mortality events could introduce an increasingly substantial selection bias as cows age. By way of example, high milk production and early postpartum BCS loss are related (Buckley et al., 2000; Lee and Kim, 2006) and older cows must have had sufficiently high production to have avoided being culled. Hence, the observed association between high parity and low BCS at peak milk could be, partly or wholly, an artifact of older cows having been selectively protected from culling through high production, and not related to parity or age per se.

The novel combination of BCS and BW into a single classification provides different insights into the underlying biology of either BCS or BW in isolation. The utility of this classification in explaining production and health outcomes of interest to the dairy industry should be further investigated.

CONCLUSIONS

We explored associations among serum analytes, parity, and milk production with BCS, BW, and a novel combined classification of BCS and BW. It is important to consider that the associations between measures of body tissue and serum analytes identified were cross-sectional, but if repeated over time could reflect very substantial differences in metabolism among cattle. Older cows were more often heavier and in lower BCS than their younger peers, particularly at peak milk. Albumin was consistently and positively associated with body measurements and milk production parameters, and milk production was negatively associated with BCS. We speculate that the observed associations between metabolites that reflect protein and energy metabolism, including albumin, glucose, urea, cholesterol, BHB, and calcium, within a cohort and body tissue reserve measures (BCS and BW) may indicate that protein metabolism importantly differs among cattle. Further investigation into albumin and protein metabolism may identify biological pathways that support high milk production while reducing BCS-related diseases and loss.

NOTES

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Nonstandard abbreviations used: A:G = albumin to globulin ratio; AT = adipose tissue; BCG = body classification group; DBC = days before calving; NEFA = nonesterified fatty acids; PB = pasture based; Var = variance.

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CHAPTER 4

Reproduction, mastitis, and lameness in confinement and pasture-based systems: Associations with parity

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OVERVIEW OF CHAPTER 4

This chapter utilises a large database composed of data collected from herd management software from the enrolled farms to explore associations between parity, housing systems, and reproductive, mastitis, and lameness risks. The database was purpose-built for the Dairy UP program and is constructed in structured query language (**SQL**), with robust and documented data-auditing rules. The utility of the database is exemplified in this chapter and is also used for the survival analysis in Chapter 7. The database will be utilised beyond this thesis to provide excellent quality data for further research, and to provide flexible benchmarked reports for farm producers.

Reproductive risk was assessed through 100 d in-calf rates, Weibull parametric survival models and competitive risk models; mastitis and lameness through days in milk of first event, crude incidence rates, Weibull parametric survival models and competitive risk models. Collectively, the results showed that increased parity was strongly associated with worse reproductive performance, and increased hazards of mastitis and lameness. Comparatively, the effect of the housing system was minor or non-existent for most metrics, except for mastitis risk, which was higher in confinement-based cows compared to pasture-based farms across all parity levels.

This chapter suggests that to increase the welfare, health and sustainability of the dairy industry, it is important that we increase our understanding of the effects of aging in dairy cows.

ABSTRACT

Dairy housing systems can be broadly classified as either confinement (CONFINE) or pasture-based (PAST). System-level differences in diet, housing, management, and milk production may influence health and reproduction of cows and the interaction of parity with these outcomes. This multi-site prospective, observational study investigated risks of pregnancy, mastitis, and lameness for different parities and housing systems (CONFINE and PAST). There were 15 CONFINE and 14 PAST farms enrolled, with health, reproduction, and production data collected from each farm between Feb 2022 to Jan 2026. Linear regression, logistic regression, and Weibull parametric survival models were used to assess the effects of parity, production system, and season on measures of pregnancy, mastitis and lameness. Robust sandwich estimators controlled within-farm correlation. The 100 day in-calf rate (100DICR, logistic regression) was not significantly different between PAST (34.0%; 95%CI: 28.0, 40.0) and CONFINE (32.9%; 95%CI: 28.9, 36.9) farms. The hazards of pregnancy (HPREG) assessed with a Weibull model were not different between PAST (1.04 HR, 95%CI: 0.82, 1.25) and CONFINE [1.08 hazards ratio (HR); 95%CI: 0.70, 1.45] farms. There was a monotonic decrease in the 100DICR and HPREG with increasing parity regardless of system. Cows breeding during Summer or Spring in PAST farms and Spring in CONFINE farms had reduced reproductive efficiency compared to other seasons. The effect of season was greater in PAST compared to CONFINE. There were 34,890 mastitis events across 129,039 lactation records. The crude incident rate of mastitis in CONFINE was 42.6 per 100 cows per 365 cow-days (95%CI: 41.9, 43.3) and 34.9 (95%CI: 33.7, 36.3) in PAST. The hazard of mastitis was numerically greater in CONFINE (2.85 HR, 95%CI: 1.10, 4.61) compared to PAST (2.13 HR, 95%CI: 1.62, 2.64) farms. The hazard of mastitis increased monotonically with parity. There were 9,495 cases of lameness across 125,362 lactation records. The crude incident rate was 8.75 per 100 cows per 365 cow-days (95%CI: 8.43, 9.07) in CONFINE and 10.7 (95%CI: 9.97, 11.4) in PAST. The hazard of lameness was similar in CONFINE (1.04 HR, 95%CI: 0.24, 1.84) and PAST (1.19 HR, 95%CI: 0.92, 1.44) farms. The hazards of lameness were unchanged in the first three (PAST) and two (CONFINE) parities but increased with parity thereafter. The association of increasing parity was far greater on health and reproductive outcomes than that of housing system. These results suggest that to support cow health and longevity we must address the impact of increased parity on health and reproduction of cows.

Keywords

Housing, lameness, mastitis, reproduction, survival analysis

INTRODUCTION

Dairy housing systems can be broadly classified as either confinement (**CONFINE**) or pasture-based (**PAST**) systems. The CONFINE systems may include free-stall barns, compost-bedded pack barns, and dry-lots, and provide shelter with or without restricted outdoor access. Feed is typically provided as a total mixed ration and delivered directly to the cows. In contrast, PAST systems rely primarily on grazed pastures for their main source of forage, with grain concentrates or partial mixed rations often provided for greater dry matter intake and dietary balance. These housing systems differ in many aspects, including feeding strategies, herd management, climate control, and pathogen exposure. These differences may influence cattle health, reproductive performance, and ultimately longevity and welfare (Washburn et al., 2002; Burow et al., 2011; Arnott et al., 2017; Lean et al., 2023a; b).

The risk of clinical mastitis was higher in CONFINE systems compared to PAST (Barkema et al., 1999; Washburn et al., 2002), though more contemporary comparative studies are lacking. There was no significant effect of housing system on the odds of developing clinical mastitis (n = 27,857 lactational records) in a meta-analytical review, though not all included studies recorded full lactation lengths (Lean et al., 2023b). Mastitis pathogens isolated from clinical cases differ between housing system, suggesting divergent risk profiles; coliform bacteria are most often isolated in CONFINE systems, and environmental streptococcus infections are most common in PAST (Barkema et al., 1999; Rowe et al., 2024).

Lameness is often reported as greater in CONFINE systems compared to PAST (Haskell et al., 2006; Olmos et al., 2009; Arnott et al., 2017; Lean et al., 2023b). Lameness is not a distinct disease entity, but rather the clinical expression from various underlying pathological conditions, and is therefore influenced by many factors, including parity (Lean et al., 2023b), cattle handling (Chesterton et al., 1989; Ranjbar et al., 2016), herd size (Barker et al., 2009), walking surfaces (Barker et al., 2009; Ranjbar et al., 2016), and diet (Bergsten, 1994; Bramley et al., 2013). As many of these risk factors are expected to differ between dairy housing systems, it is reasonable to expect the incidence of lameness could also differ.

Reproductive failure is a leading reason for culling in dairy cows (Hadley et al., 2006; Pinedo et al., 2010). A large meta-analysis (n = 28,077 lactational records) reported lower odds of becoming pregnant in a lactation (OR = 0.56 ± 0.12 SE), lower hazards of pregnancy (HR = 0.67 ± 0.05), and increased hazard of not being bred (HR = 3.09 ± 1.05) in CONFINE systems

compared to PAST systems, after accounting for milk yield (Lean et al., 2023a). However, the pregnancy to first insemination was not significantly different between housing systems (Washburn et al., 2002; Lean et al., 2023a), indicating that both management decisions and bovine physiology are important.

Understanding housing system-specific risks for health is vital for both welfare of cattle and public support of the dairy industry (Arnott et al., 2017; Mee and Boyle, 2020). In Australia, the dairy industry is undergoing a rapid shift from predominately PAST systems towards more CONFINE systems, driven by motivations of business expansion, ability to protect from climate extremes, and decreasing availability of irrigation water (Rogers et al., 2022). It is important to proactively identify any housing system-specific health and reproductive risks that may exist. As the transition to CONFINE is relatively recent in Australia, and with considerable interest globally in different housing systems, studying Australian farms offers a unique opportunity to evaluate outcomes in contemporary facilities and management practices.

The objective of this prospective, observational, multi-site study was to explore relationships between parity, dairy housing system, and outcomes related to reproduction, mastitis, and lameness.

MATERIALS AND METHODS

Farm enrolment

Farms were purposively selected to obtain a balanced study design of 15 PAST and 15 CONFINE farms, based upon the housing system of the lactating herd for a separate series of observational studies (Sheedy et al., 2026). These same farms have been used opportunistically in this analysis. Selection criteria were a mean lactating herd size of > 200 animals, conventional (non-organic), maintained accessible electronic records (including a minimum of individual cow pregnancy diagnosis, inseminations, calving, abortion, parity, birthdate, and health treatment details) that were reviewed for suitability before inclusion in the study. After enrolment, one PAST farm relocated and did not contribute to the study. The PAST farms had ryegrasses (*Lolium* spp.) and most had kikuyu (*Cenchrus clandestinus*, previously *Pennisetum clandestinum;* n = 13/14, 93%) as a substantial pasture for part of the year. All PAST cows offered grain-based supplemental concentrates in the dairy parlour (mean 7.1 kg ± 2.1 SD). The CONFINE farms included free-stall barns (n = 4/15, 26%), compost-bedded pack barns (n = 11/15, 73%), and dry-lots (n = 2/15, 13%), with some farms using more than one CONFINE

system. All farms bred cattle year-round. Cattle were not housed at any time of the year in PAST farms, and none had undercover loafing areas for the lactating herd. The mean lactating herd size for PAST farms was 330 (range:140-760) and 1,720 (range:360-9,100) in CONFINE farms. Robotic milking was used on 1 PAST and 1 CONFINE farm. Mean daily milk production was 22.24 L (± 4.22 SD) in PAST farms and 34.83 L (± 3.8 SD) in CONFINE farms. Individual farm details are in the Appendix Table A1 and wet chemistry of lactational diets in the Appendix Table A8.

Data collection

A centralized relational database was constructed in structured query language (**SQL**) specifically for the Dairy UP project (https://dairyup.com.au) and was utilized in this report. The enrolled farms operated six different herd management software: EasyDairy (n = 15, Easy Dairy Automation Systems, Shepparton, VIC), DelPro (n = 6, DeLaval, Port Melbourne, VIC), DairyComp305 (n = 3, Valley Ag Software, Visalia, CA), DairyPlan (n = 3, Gesellschaft für Entstaubungsanlagen; GEA, Melbourne, VIC), DairyWIN (n = 1, Massey University, Palmerston North, NZ), and LelyT4C (n = 1, Lely, Truganina, VIC). Backups of herd management software or customized reports were collected every 2-3 mo. Each herd management software required specific "extract, transform, and load" documentation to preprocess data to be parsed to the central database. Data quality rules flagged potential errors prior to loading them to the database for individual review; examples being suspicious calving, culling, or dry-off timings. Farm managers were contacted regularly to provide additional detail on potential errors. All reported data were collected prospectively from February 2022 to January 2026.

Statistical analysis

Statistical analysis was performed in STATA 16.1 (StataCorp. 2020. Stata Statistical Software, College Station, TX). In all analyses, each farm contributed equally regardless of farm size by performing inverse proportional weighting (STATA code: *pweight*). Parity was grouped as 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, and > 4^{th} lactation. The housing system covariate in our analysis represents not only differences in feeding, housing, and management practice, but also the greater mean-milk production of the CONFINE farms. Multi-level modelling was not performed as farm was the unit of analysis and housing system does not vary at the farm level. Using farm as a random intercept would have removed the variation associated with housing system. The effect of within farm correlation was instead controlled by using and reporting

cluster-robust sandwich estimators of variance which relax the assumption of independence between observations [STATA code: vce(cluster *farmid*)].

A sample size analysis was performed for a clustered hazards analysis with a HR of 1.5 between feeding systems, a survival probability of between 30% (estimate for reproductive failure at 305 d) and 65% (estimate for no mastitis at 305 d), alpha 0.05, power of 80%, an intraclass correlation of 0.05 [STATA code: power logrank 0.3, cluster hratio(1.5) alpha(0.05) m1(1000) rho(0.05)]. Results indicated between 14 and 26 total farms would be sufficient.

Reproduction

The effect of housing system, parity, and season on the reproductive outcomes of the 100 day in-calf rate (**100DICR**) and hazards of pregnancy (**HPREG**) were analysed.

Rules and assumptions

As the completeness of reproductive data varied across farms and the specific herd management systems, the following rules and assumptions were required:

(1) If a successful pregnancy examination was recorded, but not linked to a specific insemination event, the most recent insemination before the examination (between 25 d and 250 d) was assumed to be successful (n = 1683/100340, 1.68%).

(2) If an inter-calving interval existed without a recorded breeding, the date of conception was assumed to be 279 d prior to calving (n = 935/100340, 0.93%). If this assumption caused the DIM of conception to be < 15 d, this record was excluded (n = 13/100340, 0.01%), with the assumption that conception at ≥ 16 d was the limit of biological plausibility (Zain et al., 1995).

(3) If there was no insemination event to estimate the DIM of a successful pregnancy test and no inter-calving interval available, the record was excluded (n = 34/100340, 0.03%)

(4) If a cow aborted and was recorded to begin a new lactation, but was without a recorded DIM to pregnancy, this record was excluded (n = 564/100340, 0.56%).

Season was included as a categorical variable in the reproductive models. Season was defined as the time of year when each cow first became eligible for breeding, defined as 60 d post-calving. Seasons were Summer (December – February), Autumn (March – May), Winter (June – August), and Spring (September – November).

The 100DICR was analysed in a logistic regression model with independent variables of housing system, parity, season, and interaction terms.

For the hazards analysis, the event of interest was the first successful pregnancy of a lactation. Cows were right censored at "do not breed" or "to be culled" decision dates, sold or died event dates, or if never pregnant, the most recent date of either an insemination or a negative pregnancy examination, that is, their last known pregnancy status. Cows that did not meet any of the above criteria were right-censored at the lesser of the cow's DIM of the farm's most recent data collection date or 500 DIM.

A Weibull parametric model was used to analyse the HPREG by housing system, parity, season, and interactions (STATA code: *streg, distribution[Weibull]*). The general Weibull hazard function used in this report was specified as:

$$h\left(t|x\right) = p\, e^{(x\beta)}\, t^{p-1}$$

Where $h(t|x)$ is the hazard of an event at DIM t, conditional on covariates x including housing system, parity and, where applicable, season, and their interactions; β is the vector of regression coefficients; p is the Weibull shape parameter that determines the baseline hazards (StataCorp, 2019). Hazard ratios (**HR**) are reported and are to be interpreted multiplicatively. A HR > 1 indicates increased hazards and < 1 indicates decreased hazards, compared to a referent group.

A competitive risk model was used to graphically present the time to pregnancy within housing system and parity. The competing risks to pregnancy were being sold, dying, or becoming a "do not breed", or "to be culled" cow (STATA code: *stcompet*). If competing risks are not accounted for, the cumulative incidence can be substantially overestimated (Fine and Gray, 1999).

Health events - mastitis and lameness

The hazards of mastitis (**HMAST**) and the hazards of lameness (**HLAME**), their respective DIM at first health event, and crude incidence rates were calculated.

Rules and assumptions

The following rules and assumptions were used regarding health event analyses.

(1) A repeat health event within 14 d was considered the same event. Cows could contribute more than one health event per lactation.

(2) Two farms did not record mastitis events on their herd management system and were excluded from this analysis (PAST n = 2). Five farms recorded less than 0.5% cases per lactation and were excluded for presumed under-reporting (PAST n = 4, CONFINE n = 1).

(3) Routine hoof treatments were not considered lameness events. Hoof specific lameness conditions, bone fractures, swollen joints, and hock injuries causing lameness were included in the condition.

(4) Cows were right censored at the earliest date of dry-off (or re-calving if no dry-off date was recorded), sale, death, at 500 DIM, or at the DIM of a farm's last data collection date.

To compare the DIM at first health event between housing system and parity, multivariable linear regression was performed with housing system, parity, and their interactions forced into the model (STATA code: *regress*). Crude incidence rates, which do not account for farm parity structure, were calculated as cases per 100 cows per 365 cow-days at risk (STATA code: *stptime*). The incidence rates of mastitis in the first 30 d of lactation were reported as cases per 100 cows per 30 cow-days at risk. The incidence rate ratio between CONFINE and PAST for the above incidence rates were also reported (STATA code: *iri*).

A Weibull parametric model was used to analyse the hazards of the respective health events by housing system and parity, with interactions, as described in the reproduction analysis methods. A competitive risk model was used to graphically present the time to these health events within housing system and parity. The competing risks to a health event within a lactation were being sold, dying, and drying-off.

Regression coefficients for all reproductive and health analyses are presented in the Tables 1 – 3. The marginal change between the categorical variables of parity and season (where applicable), by housing system are presented graphically, with the values and confidence intervals reports in the Appendix Tables A10, A11 and A12.

Table 1: Coefficients for the 100 d in-calf rate (100DICR) logistic regression model and hazards of pregnancy (HPREG) Weibull parametric survival analysis, with independent variables of housing system, parity, and the season a cow became eligible for breeding, and their interactions.

	100DICR				HPREG			
	Coef.	SE	P-value	95%CI	Coef.	SE	P-value	95%CI
System (ref: PAST)								
CONFINE	0.234	0.234	0.305	(-0.218, 0.697)	0.283	0.183	0.123	(-0.077, 0.643)
Parity (ref: 1st)								
2nd	0.062	0.074	0.408	(-0.084, 0.207)	0.042	0.081	0.607	(-0.117, 0.200)
3rd	-0.165	0.110	0.133	(-0.381, 0.050)	-0.106	0.098	0.283	(-0.298, 0.087)
4th	-0.274	0.111	0.013	(-0.490, -0.057)	-0.215	0.091	0.018	(-0.393, -0.037)
> 4th	-0.585	0.100	<0.001	(-0.781, -0.389)	-0.417	0.120	<0.001	(-0.651, -0.182)
Season (ref: Summer)								
Autumn	0.185	0.195	0.343	(-0.197, 0.566)	0.231	0.118	0.051	(-0.001, 0.463)
Winter	0.573	0.203	0.005	(0.175, 0.970)	0.262	0.112	0.020	(0.042, 0.483)
Spring	0.228	0.191	0.233	(-0.147, 0.603)	-0.018	0.105	0.866	(-0.223, 0.188)
System#Parity								
CONFINE#2nd Parity	-0.156	0.113	0.169	(-0.378, 0.066)	-0.152	0.095	0.111	(-0.338, 0.035)
CONFINE#3rd Parity	-0.091	0.139	0.513	(-0.364, 0.182)	-0.132	0.110	0.232	(-0.348, 0.085)
CONFINE#4th Parity	-0.263	0.144	0.068	(-0.544, 0.019)	-0.237	0.113	0.036	(-0.458, -0.016)
CONFINE#> 4th Parity	-0.216	0.129	0.095	(-0.469, 0.038)	-0.305	0.145	0.035	(-0.589, -0.021)
System#Season								
CONFINE#Autumn	-0.008	0.208	0.969	(-0.416, 0.400)	-0.136	0.127	0.285	(-0.386, 0.114)
CONFINE#Winter	-0.419	0.217	0.053	(-0.844, 0.005)	-0.284	0.140	0.043	(-0.559, -0.009)
CONFINE#Spring	-0.138	0.224	0.537	(-0.577, 0.301)	-0.060	0.125	0.633	(-0.304, 0.185)
Constant	-0.740	0.222	0.001	(-1.175, -0.306)	-8.430	0.274	<0.001	(-8.968, -7.893)

Note that both the logistic regression and Weibull model coefficients (Coef.) are on the log-scale and are non-linearly related to the odds ratio and the hazards ratio, respectively. To obtain estimates for the odds ratio or the hazards ratio, the sum of the relevant β coefficients must be exponentiated: exp(Σβ). In both models, the referent (ref) is PAST, 1st parity, Summer-bred cows.
The baseline hazards for HPREG was: ln(p) = 0.468 (95%CI: 0.417, 0.520)
CI = confidence interval; CONFINE = confinement-based dairy system; PAST = pasture-based dairy system; SE = standard error

Post-hoc study power

The intraclass correlation at the farm level was consistently approximately 0.20 (data not shown), indicating substantial farm heterogeneity. A post-hoc sensitivity power analysis was performed for a clustered survival analysis assuming a HR of 1.5 between feeding systems, a 70% probability pregnancy by 305 DIM, alpha 0.05, 14 PAST and 15 CONFINE farms, and varying levels of intraclass correlations [STATA code: power logrank 0.3, cluster hratio(1.5) alpha(0.05) k1(14) k2(15) rho(0.05 0.1 0.2) m1(1000)]. The estimated study power was 87%, 59% and 34% for intraclass correlation of 0.5, 0.10 and 0.20, respectively. Given the empirically observed intraclass correlation was approximately 0.20, the effective power to detect a true HR of 1.5 with the current study design was approximately 34%. Consequently, some non-significant findings between feeding systems should be interpreted with caution.

RESULTS

Reproduction

There were 100,340 lactational records (PAST n = 11,728; CONFINE n = 88,612) available for analysis of reproductive outcomes.

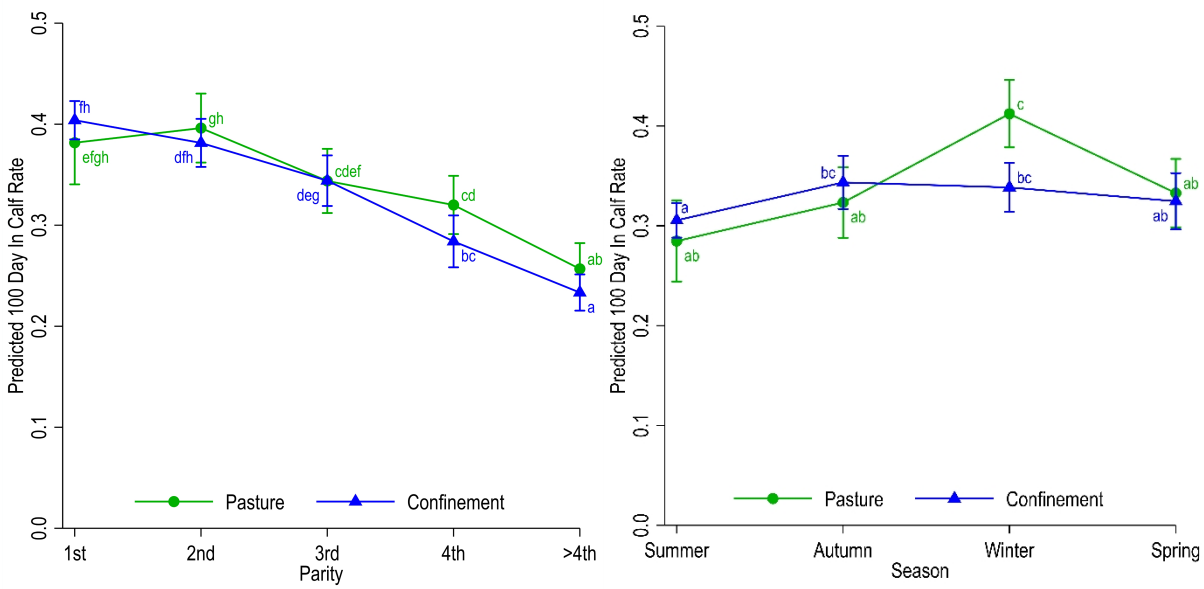

Figure 1: Predicted 100 d in-calf rate (100DICR) by production system and parity (left) and season (right), following multivariable logistic regression. Season is the time of year a cow first became eligible for breeding. Markers sharing a letter are not significantly different at the 5% level, within a subplot. Error bars are standard errors.

The 100DICR logistic regression coefficients are reported in Table 1. After adjustment for parity and season, the predicted 100DICR was 33.95% (95%CI: 27.97, 39.94) in PAST compared to 32.93% (95%CI: 28.94, 36.92) in CONFINE farms (P = 0.781; Figure 1). There was a monotonic decline in the predicted 100DICR with increasing parity regardless of housing system (Figure 1), except for 2nd parity PAST cows that had a numerically greater 100DICR than other PAST parity groups.

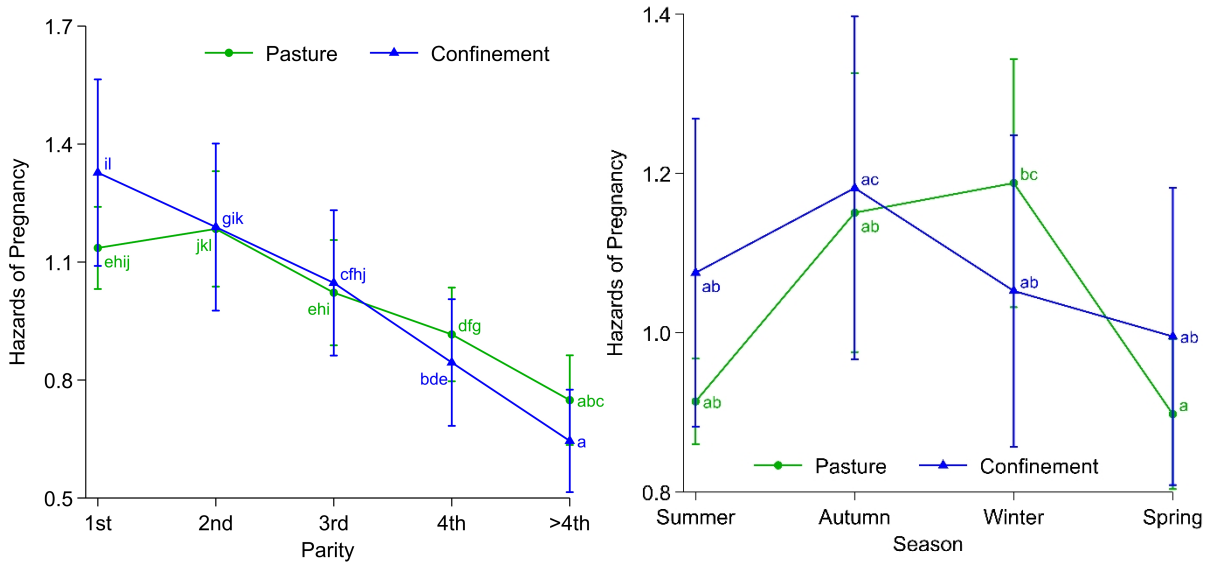

Figure 2: Predicted relative hazards of pregnancy (HPREG) by dairy system and parity (left) and season (right), following a Weibull parametric survival analysis. Season is the time of year a cow first became eligible for breeding. Markers sharing a letter are not significantly different at the 5% level, within a subplot. Error bars are standard errors. Referent group is pasture, 1st parity, Summer.

The season of first breeding had a greater effect on the 100DICR in PAST farms than CONFINE farms (Table 1, Figure 1). The PAST cows had both the highest and lowest 100DICR across the seasons, 41.22% (95%CI: 34.61, 47.83) in Winter and 28.47% (95%CI: 20.5, 36.39) in Summer.

The HPREG survival analysis coefficients are reported in Table 1. Survival analyses showed that CONFINE had a not significantly (P = 0.800) greater HPREG (1.08 HR, 95%CI 0.70, 1.45) than PAST (1.04 HR, 95%CI: 0.82, 1.25; Table 1). There was a monotonic decrease in the hazard ratio with increasing parity, with an exception in 2nd parity PAST cows, which had a numerically greater HPREG compared to other PAST parities (Figure 2). The greatest HPREG were for CONFINE heifers (1.33 HR, 95%CI: 0.86, 1.79) and the least were in CONFINE >

4th parity cows (0.65HR, 95%CI: 0.39, 0.90). There were no significant differences ($p<0.05$) between the HPREG at each parity level across housing systems (Figure 2).

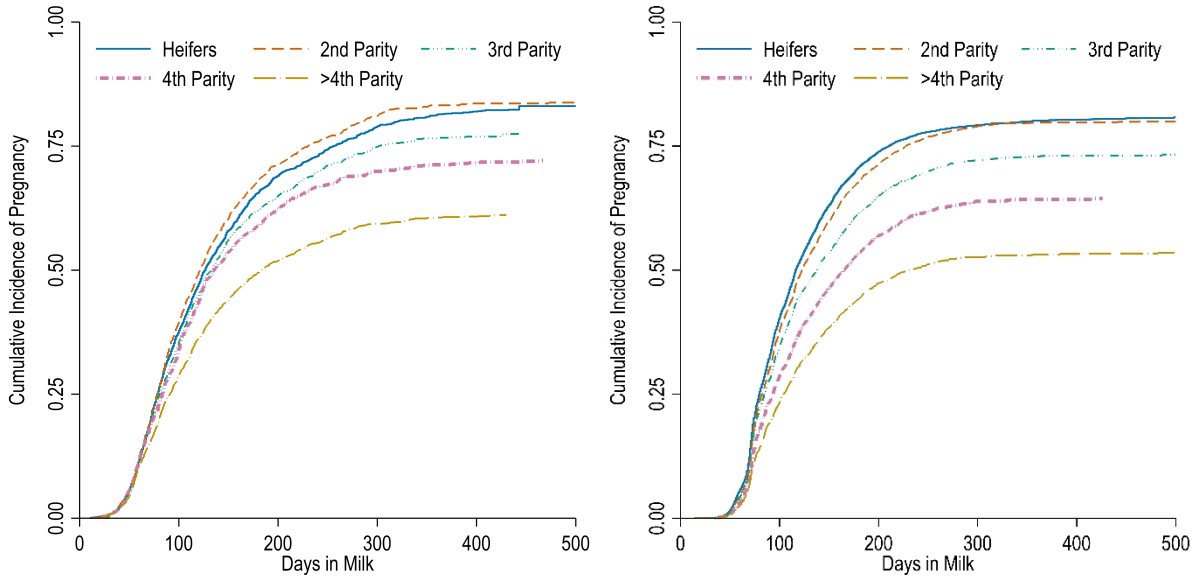

Figure 3: Competitive risk models showing the cumulative incidence of pregnancy by parity in pasture-based systems (left) and confinement-based systems (right). The competing risks were being sold, dying or declared as "do not breed" or "to be culled". Cows that were never pregnant were right censored at their most recent date of known pregnancy status (either an insemination or a negative pregnancy examination). Cows not meeting any of the above criteria were right-censored at the lesser of the cow's days in milk (DIM) of the farm's most recent data collection date or 500 DIM.

The season that a cow became eligible for breeding influenced the HPREG in PAST farms by a greater magnitude than CONFINE farms (Figure 2, Table 1). Spring and Summer had a lower HPREG (0.90 and 0.91 HR, respectively) compared Autum and Winter (1.15 and 1.19, respectively) in PAST farms. In CONFINE farms, Spring had the lowest HPREG (1.00 HR, 95%CI: 0.63, 1.36), Autumn the highest (1.18 HR, 95%CI: 0.76, 1.60), with the Winter and Summer HPREG between these two measures.

The cumulative incidence of pregnancy, with competing risks of being sold, dying or declared as "do not breed" or "to be culled" cows is shown in Figure 3. Of cows that begin a lactation, the CONFINE > 4th parity cows had the lowest cumulative incidence of becoming pregnant (52.6% at 305 DIM) and PAST > 4th parity cows the second lowest (59.3%). The

greatest cumulative incidence of pregnant cows at 305 DIM in PAST farms were 2nd parity cows (81.6%) and in CONFINE farms were heifers (79.3%) and 2nd parity cows (79.1%).

Mastitis

There were 34,890 mastitis events recorded across 129,039 lactations. The crude incidence rate of mastitis in CONFINE farms was 42.6 per 100 cows per 365 cow-days (95%CI:41.9, 43.3) and in PAST farms was 34.9 per 100 cows per 365 cow-days (95%CI: 33.7, 36.3). The incidence rate ratio of mastitis cases in CONFINE:PAST was 1.22 (95%CI: 1.22, 1.22, $P < 0.001$). When focusing on cases within the first 30 DIM, the crude incidence rate was 4.80 per 100 cows per 30 cow-days (95%CI: 4.58, 5.02) in CONFINE farms and 6.76 per 100 cows per 30 cow-days (95%CI: 6.28,7.29) in PAST farms, with a resulting CONFINE:PAST incidence rate ratio of 0.71 (95%CI: 0.71, 0.71, $P < 0.001$).

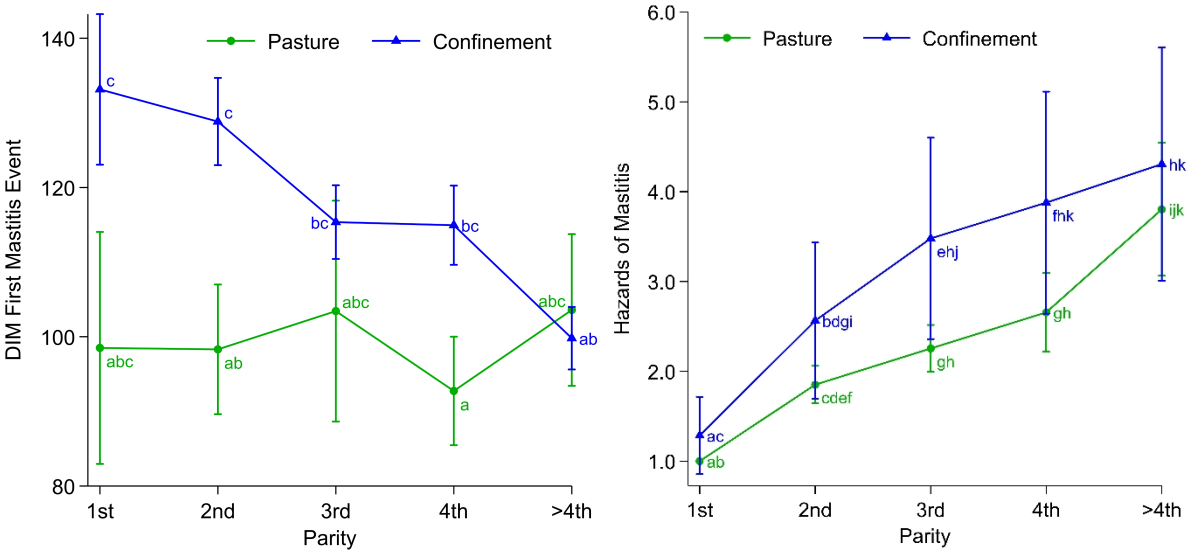

Figure 4: The predicted days in milk (DIM) of the first mastitis event for different parities and production system, following linear regression (left). The relative hazards of mastitis (HMAST) for different parities and production system, following Weibull parametric survival analysis (right). Markers sharing a letter are not significantly different at the 5% level, within a subplot. Error bars are standard errors. HMAST referent group is pasture, 1st parity.

Of cows recorded with a mastitis event, there was no difference in the DIM of the first event across parity groups in PAST systems (Table 2, Figure 4), averaging 100.0 DIM (95%CI: 91.1, 108.8). On CONFINE farms, there was a monotonic decrease in the DIM at first mastitis event

from 133.1 DIM (95%CI: 112.8, 153.5) in heifers to 99.8 DIM (95%CI: 91.4, 108.2) in > 4th parity cows.

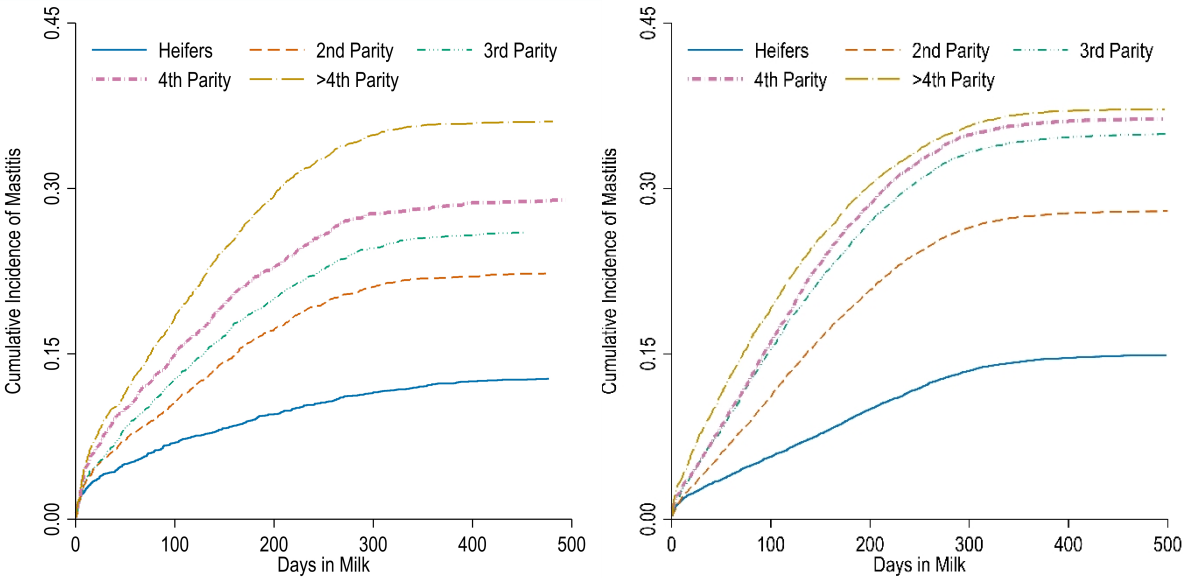

Figure 5: Competitive risk models showing the cumulative incidence of mastitis by parity in pasture-based systems (left) and confinement-based systems (right). The competing risks were being sold, dying, or dry-off (or re-calving if dry-off data not present). Cows were right-censored at the earlier of 500 days in milk (DIM) or the DIM at a farm's last data collection date.

The HMAST survival analysis coefficients are reported in Table 2. The HMAST was not significantly different (P = 0.341) in CONFINE farms (2.85 HR, 95%CI: 1.10, 4.61) compared to PAST farms (2.13 HR, 95%CI: 1.62, 2.64) after controlling for parity, though the CONFINE cows had numerically greater HMAST at every parity level (Figure 4). There was a monotonic increase in HMAST with increasing parity, regardless of housing system (Figure 4). The cumulative incidence of mastitis events is shown Figure 5, with competing risks of dry-off, sold, or dying. In the PAST farms, cows in the first 30 DIM have a greater incidence of mastitis than CONFINE farms. After 30 DIM, the incidence rate appears linear over a lactation until around 300 DIM as cows begin to be dried-off. The cumulative incidence of mastitis increases with increasing parity in PAST farms. In contrast, the CONFINE farms cumulative incidence increased from heifers to 3rd lactation, but is broadly similar between 3rd, 4th, and > 4th parities.

Table 2: Coefficients for the days in milk (DIM) of first mastitis event (linear regression) and the hazards of mastitis (HMAST) Weibull parametric survival analysis, with independent variables of housing system, parity, and their interactions.

	DIM first mastitis event				HMAST			
	Coef.	SE	P-value	95%CI	Coef.	SE	P-value	95%CI
System (ref: PAST)								
CONFINE	34.65	18.18	0.068	(-2.71, 72.00)	0.251	0.333	0.451	(-0.402, 0.904)
Parity (ref: 1st)								
2nd	-0.19	20.64	0.993	(-42.63, 42.24)	0.616	0.118	<0.001	(0.384, 0.848)
3rd	4.94	24.80	0.844	(-46.04, 55.91)	0.813	0.117	<0.001	(0.583, 1.043)
4th	-5.77	14.09	0.686	(-34.73, 23.20)	0.977	0.166	<0.001	(0.652, 1.302)
> 4th	5.09	19.98	0.801	(-35.99, 46.16)	1.336	0.200	<0.001	(0.943, 1.729)
System#Parity								
CONFINE#2nd Parity	-4.12	22.92	0.859	(-51.24, 43.00)	0.075	0.135	0.580	(-0.189, 0.339)
CONFINE#3rd Parity	-22.72	26.29	0.395	(-76.76, 31.31)	0.183	0.156	0.241	(-0.123, 0.488)
CONFINE#4th Parity	-12.44	16.91	0.469	(-47.20, 22.33)	0.127	0.203	0.530	(-0.270, 0.524)
CONFINE#> 4th Parity	-38.43	22.05	0.093	(-83.76, 6.90)	-0.127	0.247	0.608	(-0.611, 0.357)
Constant	98.50	15.25	<0.001	(67.17, 129.84)	-6.403	0.329	<0.001	(-7.047, -5.758)

Note that Weibull model coefficients (Coef.) are on the log-scale and are non-linearly related to the hazards ratio. To obtain estimates for the hazards ratio, the sum of the relevant β coefficients must be exponentiated: exp(Σβ).
In both models, the referent (ref) is PAST, 1st parity cows.
The baseline hazards for HMAST was: ln(p) = -0.258 (95%CI: -0.328, -0.188)
CI = confidence interval; CONFINE = confinement-based dairy system; PAST = pasture-based dairy system; SE = standard error

Lameness

There were 9,495 cases of lameness across 125,362 lactational records. The crude incidence rate of recorded lameness in CONFINE farms was 8.75 per 100 cows per 365 cow-days (95%CI: 8.43, 9.07) and in PAST farms it was 10.66 per 100 cows per 365 d (95%CI: 9.97, 11.42). The CONFINE:PAST incidence rate ratio for lameness was 0.82 (95%CI: 0.82, 0.82; $P < 0.001$), showing less incidence of lameness in CONFINE farms.

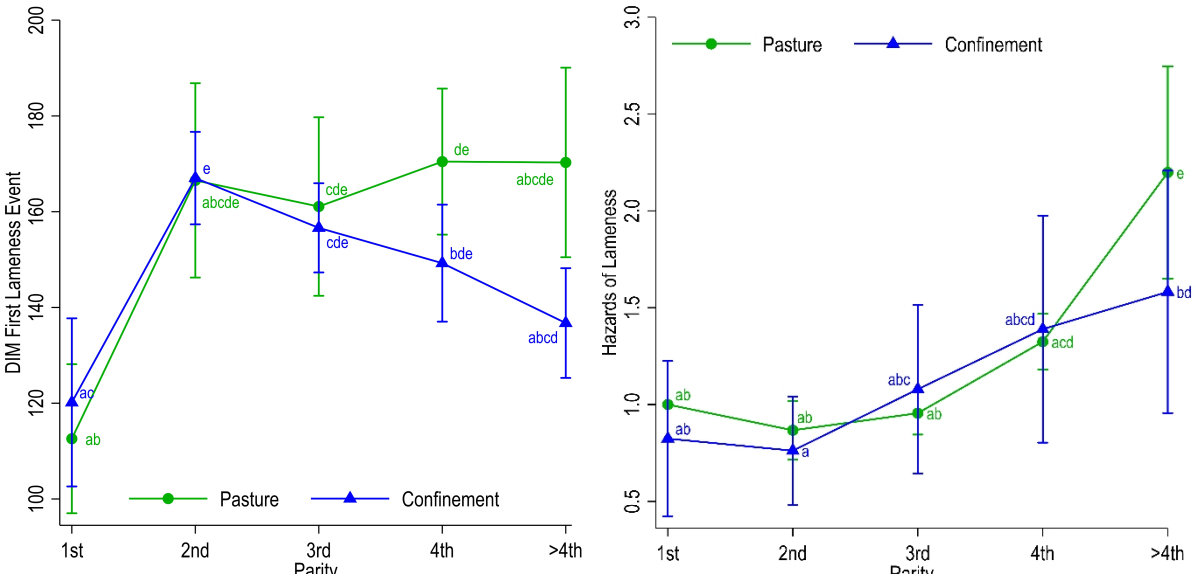

Figure 6: The predicted days in milk (DIM) of the first lameness event for different parities and production system, following linear regression (left). The relative hazards of lameness (HLAME) for different parities and production system, following Weibull parametric survival analysis (right). Markers sharing a letter are not significantly different at the 5% level, within a subplot. Error bars are standard errors. HLAME referent group is pasture, 1st parity.

The coefficients for the linear regression of DIM to first lameness event are reported in Table 3. Of cows that were recorded as lame, the parity-controlled DIM of first lameness was not significantly ($P = 0.448$) different between CONFINE and PAST farms (-9.31 d, 95%CI: -34.28, 15.66). The first case of lameness occurred earliest in heifers regardless of housing system (PAST: 112.6 DIM, 95%CI: 81.1, 144.1; and CONFINE: 120.2 DIM, 95%CI: 84.6, 155.7; Figure 3).

Table 3: Coefficients for the days in milk (DIM) of first lameness event (linear regression) and the hazards of lameness (HLAME) Weibull parametric survival analysis, with independent variables of housing system, parity, and their interactions.

	DIM first lameness event				HLAME			
	Coef.	SE	P-value	95%CI	Coef.	SE	P-value	95%CI
System (ref: PAST)								
CONFINE	7.59	22.95	0.744	(-39.89, 55.07)	-0.194	0.482	0.688	(-1.138, 0.751)
Parity (ref: 1st)								
2nd	53.95	28.09	0.067	(-4.17, 112.07)	-0.143	0.163	0.380	(-0.461, 0.176)
3rd	48.50	22.58	0.042	(1.80, 95.20)	-0.046	0.112	0.682	(-0.264, 0.173)
4th	57.88	22.50	0.017	(11.34, 104.41)	0.281	0.093	0.003	(0.099, 0.463)
> 4th	57.70	31.99	0.084	(-8.48, 123.88)	0.788	0.218	<0.001	(0.361, 1.214)
System#Parity								
CONFINE#2nd Parity	-7.11	34.64	0.839	(-78.76, 64.56)	0.064	0.308	0.835	(-0.540, 0.668)
CONFINE#3rd Parity	-12.04	29.42	0.686	(-72.90, 48.81)	0.315	0.359	0.379	(-0.388, 1.019)
CONFINE#4th Parity	-28.82	24.83	0.258	(-80.18, 22.54)	0.241	0.335	0.471	(-0.415, 0.898)
CONFINE#> 4th Parity	-41.12	33.83	0.237	(-111.11, 28.87)	-0.135	0.393	0.730	(-0.905, 0.634)
Constant	112.59	15.23	<0.001	(81.09, 144.08)	-8.024	0.377	<0.001	(-8.763, -7.284)

Note that Weibull model coefficients (Coef.) are on the log-scale and are non-linearly related to the hazards ratio. To obtain estimates for the hazards ratio, the sum of the relevant β coefficients must be exponentiated: exp(Σβ).
In both models, the referent (ref) is PAST, 1st parity cows.
The baseline hazards for HLAME was: ln(p) = -0.055 (95%CI: -0.141, 0.030)
CI = confidence interval; CONFINE = confinement-based dairy system; PAST = pasture-based dairy system; SE = standard error

After controlling for parity, the HLAME were not statistically different (P = 0.731) in CONFINE farms compared to PASTURE farms (PAST ref. -0.145 HR, 95%CI: -0.340, 0.731). The HLAME were similar in the first 3 lactations on PAST farms, and the first 2 lactations on CONFINE farms, with each subsequent lactation increasing the HLAME within the respective systems (Figure 6, Table 3).

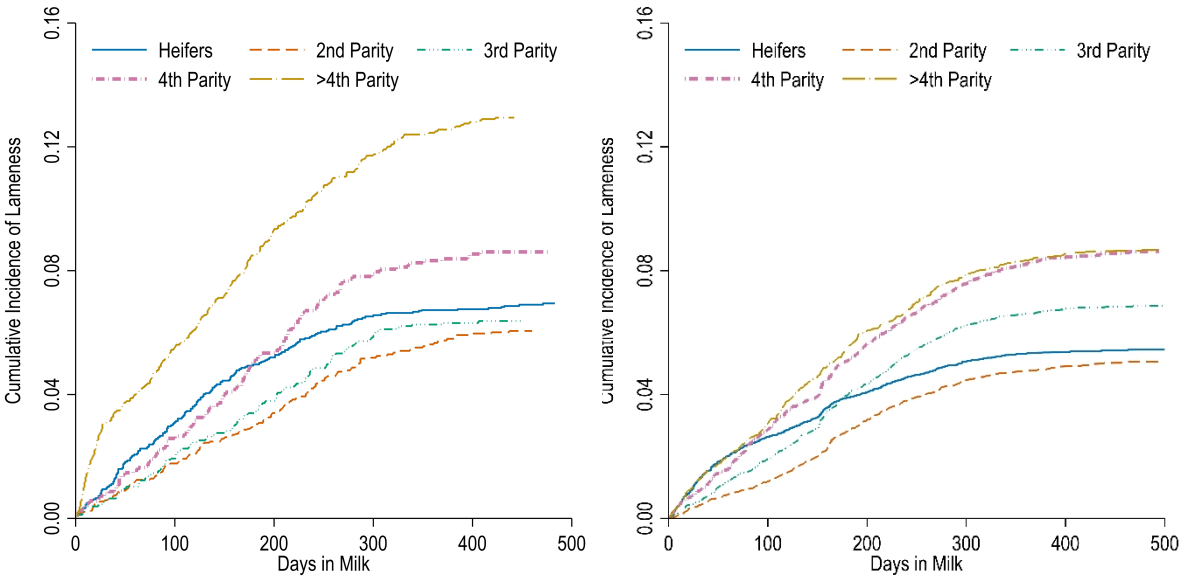

Figure 7: Competitive risk models showing the cumulative incidence of lameness by parity in pasture-based systems (left) and confinement-based systems (right). The competing risks were being sold, dying, dry-off or re-calving. The competing risks were being sold, dying, or dry-off (or re-calving if dry-off data not present). Cows were right-censored at the earlier of 500 days in milk (DIM) or the DIM at a farm's last data collection date.

The cumulative incidence of lameness events under competing risks is shown in Figure 7, with competing risk of dry-off, being sold or dying. The cumulative incidence was greatest in > 4th parity PAST farms (11.8% at 305 DIM). Compared to other parity groups, heifers in both housing systems had relatively high risk of lameness early in their lactation (steep curve) that became relatively low risk as lactation progressed, observed as a flattening of the survival curve and crossing the curves of other parities. In both housing systems, the cumulative incidence was lowest in 2nd parity cows by 305 DIM (PAST: 5.2%, CONFINE: 4.5%).

DISCUSSION

We compared the effect of parity and housing systems on reproduction, mastitis, and lameness from contemporary dairy farms. In all cases, increasing parity had a more profoundly negative association with the reported outcomes than the housing system. Comparing hazards across each parity level, the HPREG and HLAME was remarkably similar between housing systems, the HMAST was consistently greater in CONFINE systems. The high farm-level intraclass correlation limited study power to detect differences between PAST and CONFINE farms, and results should be interpreted accordingly.

Reproduction

The HPREG between housing systems were remarkably similar after controlling for parity and season of breeding. A meta-analytical study that investigated housing systems and pregnancy outcomes reported contrary results, with a lower hazards and odds of pregnancy in CONFINE systems (Lean et al., 2023a). However, the temporal dissociation between PAST (mean study year: 2013) and CONFINE (mean study year: 2008) farms that were included in the meta-analysis may partly explain the lower reproductive outcomes in their analysis, assuming there has been an improvement over time in reproductive management, genetic merit and, potentially, the decreasing influence of Arlinda Chief and his offspring in herds (Adams et al., 2016).

The mean 100DICR of 34.0% in PAST and 32.9% in CONFINE systems was consistent with Australian standards with a reported median 100DICR of 34 - 36% (Morton, 2021). This 100DICR is relatively high compared to reports from overlapping dairy regions, ranging from 28.6% - 34.7% between seasons in ten farms (Rynia et al., 2023), and 28.5% from eleven farms (Brooks et al., 2021). The season in which a cow became eligible for breeding influenced reproductive efficiency and was moderated by housing system. Reproductive efficiency was unsurprisingly lowest in Spring and Summer, given the well-established negative effects of heat stress on reproductive outcomes in dairy cattle (Rensis and Scaramuzzi, 2003). Notably though, PAST farms had a greater magnitude of change in the 100DICR and HPREG across seasons. We speculate this is due to the limited ability on PAST farms to mitigate climatic extremes, specifically heat stress. None of our enrolled PAST farms had undercover loafing areas for the lactating herd, though paddocks with tree-shade were available. In contrast, all CONFINE farms provided shade, and some the compost-bedded pack barns and free-stall barns had fans and wetters installed to aid evaporative cooling.

The effect of housing system had a less profound effect on reproductive success than the effect of parity. Reproductive efficacy decreased with increasing parity in both the 100DICR and HPREG, with the only exception of 2nd parity PAST with increased reproductive success. Studies have indicated that the odds of pregnancy to first insemination is only moderately influenced by parity (Harman et al., 1996; Grimard et al., 2006; Kim and Jeong, 2019; Lean et al., 2023a), a position supported by our results. The 100DICR was less influenced by parity than the HR (which measures time to event across an entire lactation), and the cumulative incidence of pregnancy was indistinguishable across parities in early lactation (Figure 3). These results suggest that low hazards of pregnancy in high parity cows are likely due to a combination of physiology and competing risks, including increased culling, mortality, and decisions to stop breeding older cattle occurring earlier than in younger cattle.

Health events

Before discussing mastitis and lameness, it is important to address a study limitation. Recorded health events were all producer reported, not externally validated, and based on individual farm treatment protocols. As such, a higher treatment or incidence rate may reflect increased monitoring for disease, lower threshold for treatment, or higher disease prevalence. For the results to be valid, we must assume that monitoring and thresholds for treatment are not systemically different between housing systems.

Mastitis

The HMAST were higher in CONFINE farms but occurred later in a lactation than PAST farms. In terms of magnitude, the greatest difference between the housing systems and the HMAST was between parities 2, 3, and 4, with CONFINE farms at almost double the HR among these parities. Coliform bacteria are consistently reported as the most common clinical mastitis pathogen in compost bedded pack and free-stall barns (Oliveira et al., 2013; Eckelkamp et al., 2016; Freu et al., 2023; Rowe et al., 2024). This was anecdotally supported by our CONFINE producers who self-reported a high incidence and severity of coliform mastitis, though confirmatory laboratory testing was either not performed or unavailable for analysis. A report on mastitis in Australian intensive farms supports the high incidence of coliform bacteria, with *Klebsiella* spp. (28.1%) and *E.coli* (15.0%) the two most commonly isolated bacteria from clinical mastitis samples (Rowe et al., 2024). In contrast, the most common clinical mastitis pathogens isolated from PAST Australian farms were *Streptococcus uberis* (35.0%) (Rowe et al., 2024). At the time of reporting, Australia does not have a

commercial J5 vaccine available. The J5 vaccine targets coliform mastitis, though the exact mechanism of action remains elusive (Rainard et al., 2021), and reduces the incidence of severe mastitis (Rainard et al., 2021; Mata et al., 2023). Reducing severe mastitis could reduce treatment frequency, with current recommendations being not to treat coliform mastitis in cows that are otherwise free of systemic effects, from a cost and antimicrobial stewardship perspective (Lago et al., 2011; Jong et al., 2023). If the J5 vaccine were made available to Australian producers, it is possible that mastitis requiring treatment on CONFINE farms could be substantially reduced.

Although the overall rate of mastitis was greater in the CONFINE systems, the risk and rate of mastitis in the first 30 d of lactation was higher in PAST cows. This difference suggests that the management of late-lactation or non-lactating cows in PAST farms increased the risk of early lactation mastitis compared to CONFINE farms. We do not have sufficient information at the farm-level to investigate specific factors influencing mastitis; hence the following discussion is speculative. Dry cow housing facilities and their management are associated with the odds of early lactation mastitis (Green et al., 2007). The management of CONFINE dry cows varied between farms, with some dry-cows held on pasture out-paddocks and others in confinement housing, whereas the PAST cows were universally held on pasture and most (13/14) used out-door calving pads. Soil-bedded calving pads can be particularly challenging to manage during periods of high rainfall, which could increase the risk of mastitis in early lactation of PAST farms. Biting fly management is associated with mastitis in the first 30 d of lactation (Green et al., 2007) and may differ across feeding systems. Producers routinely used blanket dry-cow therapy and internal teat sealants at dry off, consequently, dry-off mastitis prevention protocols were unlikely to substantially contribute to differential incidence of mastitis between housing systems in this report.

The HMAST increased monotonically with parity, as has been reported elsewhere (Barkema et al., 1998; Zadoks et al., 2001; Green et al., 2007; Lean et al., 2023b). There are multiple potential causes for the increased risk of mastitis with parity such as chronic infection refractory to treatment (Barkema et al., 2006), or physical changes to the udder including increasing udder size (Klaas et al., 2004), changes to teat shape (Guarín and Ruegg, 2016), teat-end damage (Pantoja et al., 2020), and the distance of the teat end to the floor (Shanks and Spahr, 1982; Slettbakk et al., 1995). Heifers had increased risk of mastitis in early lactation compared to multiparous cows (Barkema et al., 1998; Berry and Meaney, 2005), findings that were not supported by our results. There was no difference across

parities in the DIM of first mastitis event in PAST cows (Figure 4), though the cumulative incidence functions show > 50% of heifer mastitis events occurred in the first 100 DIM (Figure 5). The CONFINE heifers first experienced mastitis at later DIM compared to all other CONFINE parities (Figure 4) and the incidence of mastitis in CONFINE heifers appeared to be constant throughout a lactation (Figure 5). The difference in heifer mastitis between housing systems may again suggest differences in non-lactating cow management.

Lameness

The crude and cumulative incidence rate of lameness requiring treatment were greater in PAST compared to CONFINE systems. This result is contrary to reports of reduced lameness with pasture access (Haskell et al., 2006; Olmos et al., 2009; Chapinal et al., 2013; Arnott et al., 2017), though others have reported no difference or less lameness with CONFINE systems (Baird et al., 2009; Barker et al., 2009). The mean herd size of our PAST farms was greater than many previous comparative studies (Barker et al., 2009; Olmos et al., 2009; Chapinal et al., 2013) and herd size is associated with increased risk of sole ulcers, white line disease, and digital dermatitis (Barker et al., 2009). The association between herd size and lameness could be due to increased walking distance, maintenance of tracks or handling of cattle (Chesterton et al., 1989; Laven and Lawrence, 2006; Ranjbar et al., 2016). The PAST farms all practiced feeding grain-based concentrates in the dairy parlour, which can increase the risk of ruminal acidosis and subsequently laminitis and white line disease compared to TMR diets (Bergsten, 1994; Bramley et al., 2013).

Though the HLAME were generally low for heifers, those that become lame did so early in a lactation, as previously reported (Dewes, 1978; Singh et al., 1993; Webster, 2001). We speculate that contributing factors to early heifer lameness include the need to adapt to walking distances, time spent standing on concrete flooring (Chesterton et al., 1989; Laven and Lawrence, 2006; Barker et al., 2009; Ranjbar et al., 2016), social hierarchy and dominance interactions (Dewes, 1978; Singh et al., 1993), and adjusting to an energy dense diet (Bergsten, 1994; Livesey et al., 1998).

Biases and study limitations

Selection bias, specifically survivorship bias, is an implicit complication when analysing parity yet is rarely acknowledged. We only observe cows that have avoided removal, including reasons that are directly associated with our outcomes of interest: reproduction, mastitis, and lameness. Cows that survive to later lactations must have been sufficiently protected from

removal due to these outcomes, and subsequently high-parity cows are a survival-biased subset of the population that have protective features. The observation that increasing parity is very strongly associated with worsening reproductive and health, despite the survivorship-bias, may indicate that the negative effects of parity in dairy cattle are even greater than observed. A reasonable counterargument can be posed: surviving cows must have adequate milk production to have avoided culling for low production, and if we make a strong assumption that high milk production reduces reproduction (Leblanc, 2010) and increases health risk (Fleischer et al., 2001), then cows surviving to later lactations will be high producing cows and hence be high-risk. To further complicate the issue of survivorship-bias, both scenarios can simultaneously be valid.

In further evaluating the effect of survivorship-bias on housing system, the enrolled CONFINE farms had increased culling pressure (data not shown; largely driven by the age of first calving being ~6 months earlier than PAST). The increased culling pressure could allow CONFINE farms to more selectively remove poor performing, high health-risk, and high reproductive-failure risk cows than PAST farms could. With greater selection pressure in CONFINE systems, the remaining older CONFINE cows may be of comparatively lower-risk than the equivalent parity in PAST farms, further complicating the interpretation between housing systems and parity. While we cannot fully resolve survivorship-bias, acknowledging its presence is important when interpreting parity associated outcomes.

This analysis used data collected opportunistically from a separate observational study (Sheedy et al., 2026). The ability to detect significant differences between feeding systems was limited by the higher than anticipated intraclass correlation at the farm level and the number of farms included in the study. A post-hoc clustered survival power analysis indicated that approximately 50 farms per feeding system would be required to detect a hazard ratio of 1.5 for reproductive outcomes, assuming such a difference truly existed.

CONCLUSION

Pregnancy and lameness risk was not substantially different between PAST and CONFINE systems, while the mastitis was consistently greater in CONFINE cows. Increasing parity was associated with worse reproductive performance, increased mastitis, and increased lameness. Notably, the impact of parity on these measures far exceeded the effect of the dairy housing system that the cows belonged to. These findings suggest that supporting

cow health and longevity may be best achieved by managing and researching the effects of aging in dairy cows.

NON-STANDARD ABBREVIATIONS

100DICR = 100 day in-calf rate; CONFINE = confinement based dairy system; DIM = days in milk; HLAME = hazards of lameness; HMAST = hazards of mastitis; HPREG = hazards of pregnancy; HR = hazard ratio; PAST = pasture-based dairy system; SQL = structured query language; TMR = total mixed ration

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CHAPTER 5

A large, multi-site lipidomic investigation of parity and aging in dairy cows

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OVERVIEW OF CHAPTER 5

The remaining three Chapters of this thesis utilise a targeted lipidomics dataset to investigate factors relevant to longevity of cows. Plasma samples were collected from 30 commercial dairy farms (15 pasture-based, 15 confinement-based) across two production cohorts (dry cow, peak-milk). A total of 185 lipids species were quantified (including glycerophospholipids, sphingomyelins, and triglycerides). In Chapter 5, the cross-sectional association between lipids species and age (d) or parity were investigated. The variation in lipid profile due to the farm of origin was carefully controlled for. The most consistent and significant lipids findings were glycerophospholipids that contained very long-chain omega-3 fatty acids, including eicosapentaenoic acid (**EPA**; C20:5n-3) and docosahexaenoic acid (**DHA**; C22:6n-3), had lower blood concentrations with increased age or parity. Given the known positive associations between omega-3 fatty acids and reproduction and health in dairy cattle, we postulated that the reduced concentration of these lipids in older cattle could be partly causal to reduced reproductive performance and increased health risks.

Note on supplementary material

The published article refers to a supplementary document available at https://doi.org/10.6084/m9.figshare.26352787. The tables and figures from the supplementary document are also available in the *Appendix.* Supplementary Table S1 is reported as Table A13, Table S2 as Table A14, Figure S1 as Figure A1, Figure S2 as Figure A2, and A2, Figure S3 as Figure A3, Figure S4 as Figure A4, Figure S5 as Figure A5, Figure S6 as Figure A6. Supplemental material "Final model Specifications" is in the *Appendix* page 276.

Supplemental Tables S3 and S4, and the datasets are available from the above Figshare link and the thesis supplementary repository at https://doi.org/10.6084/m9.figshare.29456534.

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A large multisite lipidomic investigation of parity and aging in dairy cows

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ABSTRACT

Efforts to optimize the longevity of dairy cows are hindered by the increased risk of adverse health events, culling, or dying on farm with increased parity. Lipidomics provides a platform to help identify important biomarkers and biological pathways associated with increased parity and associated aging. A large multisite (15 pasture-based, 15 TMR farms) cross-sectional study collected plasma samples from nonlactating, late pregnant, dry cows (n = 696, ~27 d prepartum) and peak milk cows (n = 796, ~58 DIM) in a disproportionate stratified random sampling frame (parity: 0, 1, 2, >2 for dry cows; 1, 2, 3, >3 for peak milk cows). A total of 185 lipid species, comprising the lipids classes of phospholipids, sphingomyelins (SM) and triacylglycerols, were quantified in a targeted, liquid chromatography-MS approach. Dry and peak milk cohorts were analyzed separately throughout. Variation in lipid profiles were mostly attributed to farm of origin (36%–41% of variation), with feeding system explaining 13% to 21% and parity explaining 6% to 9%, according to ANOVA simultaneous component analysis modeling. Multiple linear regression and orthogonal partial least squares (O-PLS) investigated the association of the lipid profile with age (d), whereas discriminant analysis compared first parity with >3 parity cows in O-PLS discriminant analysis, random forest, and support vector machine models. Rankings of the most important lipid species for each model type were compared. Phospholipids with 40 carbon atoms and 6 double bond equivalents (40:6) were consistently decreased with increasing parity and age across both dry and peak milk cohorts. These lipids most likely contained stearate (18:0) and docosahexaenoic acid (DHA, C22:6n-3), an n-3 fatty acid. Additionally, phospholipids with 40:5 and 38:6, lysophosphatidylcholine (17:0), SM(35:1), and SM(35:2) were commonly identified lipids that decreased in concentration with parity and age. Docosahexaenoic acid has been associated with improved cattle health, reproduction, and milk production and quality. This study raises the hypothesis that reduced DHA levels in older cows may be an important factor increasing susceptibility to adverse health events, reduced reproductive performance, and herd removal. Studies that supplement DHA or its precursors can test this hypothesis and may be important in optimizing longevity of cows.
Key words: lipids, parity, n-3 fatty acids, docosahexaenoic acid

INTRODUCTION

Optimal longevity of dairy cows is critical for farm profitability, cattle welfare, environmental sustainability, and the social license of the industry (Dallago et al., 2021). However, increased parity is associated with increased risk of adverse health events, being culled, or dying on farm (Lean et al., 2023a,b). To optimize longevity, it is critical to understand the underlying biology of increased parity. Lipidomics, the large-scale study of lipids, is a burgeoning and appropriate platform to help identify biomarkers and biological pathways associated with increased parity and associated aging (Wenk, 2005; Almeida et al., 2021).

For example, the phospholipid (**PL**) lipid class, which comprises of a phosphate containing hydrophilic core and 1 or 2 attached fatty acids (**FA**), are well recognized for their structural importance in the bilayers of cellular compartments, but are themselves highly diverse with wide-ranging functions that include associations with many diseases and aging (Harayama and Riezman, 2018; Dai et al., 2021). High levels of PL with PUFA are negatively associated with maximal age in many species, potentially due to high rates of peroxidation of PUFA and subsequent risk of oxidative damage (Hulbert

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et al., 2007; Zimniak, 2011; Naudí et al., 2013; Jové et al., 2013; Zaloga, 2021). Conversely, n-3 FA also protect against age-related disease, including cardiovascular, inflammatory, and neurodegenerative disease in humans and model organisms (Ruxton et al., 2004; Shahidi and Ambigaipalan, 2018). In dairy cows, serum long-chain PL were significantly lower in early lactation multiparous cows compared with heifers (Humer et al., 2016). Another important lipid class, sphingolipids, have been associated with regulating insulin resistance, hepatic lipidosis, and inflammation in dairy cows, conditions that are associated with increased parity (Rico et al., 2015, 2018; McFadden and Rico, 2019).

Though using a lipidomic platform for the investigation of metabolic disease in dairy cows is increasingly common (Hailemariam et al., 2014; Imhasly et al., 2015; Humer et al., 2016; Rico et al., 2021), there are no lipidomic investigations specific to parity and age in dairy cows. Considering that dairy cow longevity is often determined by a removal decision made by farm management and potentially unrelated to age, the association between age and the lipid profile of dairy cows could be profoundly different to those being uncovered in nonproductive animal models. It is vital that we develop our understanding of lipid metabolism specific to the unique challenges of high-producing dairy cows and its relationship with longevity.

The objective of this multisite cross-sectional study was to investigate the associations among parity and age with plasma lipid classes of phospholipids, sphingomyelins (**SM**), and triacylglycerols using a targeted liquid chromatography-MS (**LC-MS**) platform.

We hypothesize that concentrations of plasma lipids will differ with parity and age of cows.

MATERIALS AND METHODS

The study was carried out in accordance with the recommendations of The Australian Code for Care and Use of Animals for Scientific Purposes. Protocols were approved by the Scibus Animal Ethics Committee (Scibus #1022-1024) and The University of Sydney Animal Ethics Committee (Project # 2022/2247).

Farm Enrollment

Farms were purposively selected to obtain an even distribution of 15 pasture-based farms and 15 intensively housed TMR farms, based upon the management system of the lactating herd. Selection criteria for the farms were as follows: having a mean lactating herd size of >200 cows, a nonorganic production system, and maintaining good, accessible electronic records. Most pasture-based farms (n = 13/15) had kikuyu (*Pennisetum clandestinum*) as a substantial pasture grass in their swards, and on all these farms, cows were offered grain-based supplemental concentrates in the dairy parlor. The TMR farms included freestall barns (20%), compost-bedded pack barns (53%), and dry lots (13%). Pasture farms did not house their cattle at any time during the year.

Cow Enrollment

Two cohorts of cattle per farm were selected; a precalving dry cohort (50–20 d prepartum, based on expected calving dates), and a peak milk cohort (40–90 DIM). Exclusion criteria for both cohorts were cattle on "to be culled," "do not breed," or "to be sold" lists, cows with <4 functional teats, or lameness score >2 at the time of visit (Sprecher et al., 1997). For each cohort, a disproportionate stratified random sampling procedure (Stata Statistical Software, Release 16, StataCorp, College Station, TX) was used on lists of eligible cows that were provided by the farm managers. Stratification was on parity (dry cohort: nonlactating heifers, parity 1, parity 2, and parity >2; peak milk cohort: parity 1, parity 2, parity 3, and parity >3). For clarity of reporting between the 2 cohorts, we report the dry cohort parity strata by their immediate postparturition parity.

Sample Size Estimate. A sample size estimate was performed for a 2-sample mean test in a cluster randomized design, with the cluster being farm type and an estimated interclass correlation of 0.2, equal group sizes of 15 farms, a normalized effect size for a single lipid of 1 SD, a Bonferroni adjusted α of 2.5×10^{-4} (α 0.05 and 200 independently assessed lipids), 2-sided test, and study power of 80%. The result of this estimation required 6 cows per parity strata per farm to be sampled. The Stata code used was as follows: power twomeans 0, diff(1) k1(15) k2(15) rho(0.2) a(0.00025). A concurrent study design sample size estimation required 9 heifers, 6 second parity cows, 6 third parity cows, and 8 greater than third parity cows; consequently, these numbers were collected and analyzed.

Sample Collection. If insufficient animals were available per parity strata, the eligibility window for days was extended as required, without exceeding the range of 20 to 100 DIM or >80 d from expected calving date. Each farm was visited once or twice, depending on the farm's breeding strategy, with each cohort being completely sampled on a single day. Sampling began November 17, 2022, and concluded June 15, 2023. Breed information was collected from on-farm assessment and cow card information.

Blood samples were collected from the coccygeal vein into a 10-mL lithium heparin Vacutainer tube (Becton Dickinson, Franklin Lakes, NJ). Samples were gently rotated upon collection and immediately cooled on ice,

in darkness, using a polystyrene cooler box. Samples were centrifuged at 1,500 × *g* for 15 min at ambient temperature (DM0412, DLAB Scientific Co. Ltd., Beijing, China) within 1 h of collection, with efforts to reduce light exposure. Aliquots of 1 mL were collected, stored temporarily at −18°C (Engel, Carole Park, Queensland, Australia) during field travel (<4 d), before long-term storage at −80°C (Isotemp, Thermo-Fisher Scientific, Waltham, MA). Hemolysis was visually assessed using a hemolysis palette and samples greater than or equal to 250 mg/dL hemoglobin excluded from analysis (n = 39; Kosecki et al., 2021). No reducing or antioxidant agents were added to the plasma.

Sample Preparation. Lipids were extracted utilizing a modified single-phase method developed by Liu et al. (2016). In brief, plasma was thawed in the dark, and 50 µL was added to 500 µL of a butanol/methanol/chloroform (3:5:4) solvent. Sonication was not performed, with samples vortexed for 60 s and centrifuged for 15 min at 13,300 × *g* at 4°C. Supernatant (1 µL) was transferred to amber HPLC vials and stored at −80°C.

Quality Control and Injection Order

For each cohort, 11 randomly selected cows contributed 50 µL to a pooled plasma sample, and these samples were processed as described previously. External standards of known concentrations for each lipid class were used for calibration (Supplemental Table S1, see Notes). Injection order was stratified by dry and peak milk cohorts and randomized (Microsoft Excel, Redmond, WA). Pooled quality control and external standards were analyzed every 20 samples.

Liquid Chromatography–Mass Spectrometry

The lipid extract of plasma was separated by a Kinetex hydrophilic interaction liquid chromatography column (100 × 2.1 mm, 1.7 µm, Phenomenex, CA) on a Vanquish ultra high performance liquid chromatography system (Thermo Fisher Scientific). The column compartment was maintained at 30°C. The mobile phase was composed of 10 m*M* ammonium formate (A) and acetonitrile containing 0.1% formic acid (B). The gradient elution was performed by a linear increase of mobile phase A from 5% to 25% over 8 min with a flow rate of 0.3 mL/min. The injection volume was 4 µL. A Q Exactive MS instrument (Thermo Fischer Scientific) was used for lipid quantification and a full scan of parent ions (120–1600 *m/z*) at a resolution of 140,000 was conducted simultaneously in positive and negative mode. Lipid species was identified using retention time and accurate mass match and quantified using external calibration. The full list of targeted lipid species with sum structure is reported in Table 1.

Batch Correction and Calibration. External standards were used to correct for injection run-order and batch effects. Injection order correction was via a calibration curve of the external standards for each lipid class. Batch correction was subsequently applied by adjusting for the known external standard concentrations. The effectiveness of the batch and run-order correction was assessed through principal component analysis (**PCA**; Supplemental Figures S1 and S2, see Notes).

Data Exclusion. Cows that were initially enrolled and subsequently excluded were due to the following reasons: an uncertain birth date or parity of enrolled cow (n = 4), DIM <20 or >100 (n = 16), sampling and calving dates >80 d or did not calve (n = 41), or died before calving (n = 2). Cows were grouped according to breed within farm, referred to hereafter as groups. If a group was ≤5 animals, that group was removed from analysis (n = 78 cows). Two cows were removed for having outlying ages (13.6 and 14.7 yr old; Figure 1).

One cow was removed because >25% of lipids were missing. Missing lipid data were imputed as 1/5 of the lowest detected concentration (n = 9 missing data points). Cows that had any lipid >10 SD were removed (n = 27 cows) and cows with a sum of their standardized lipid concentration greater than 4, indicating hemoconcentration or dehydration, were removed (n = 11; Figure 1).

The final dataset included 696 dry cows and 783 peak milk cows, with parity and breed distribution described in Figure 1.

Statistical Analysis

Initial data visualization was performed with PCA in PLS Toolbox (version 9.3.1, Eigenvector Research Inc., Manson, WA), cows with large Q^2 and T^2 Hotelling quotients indicating a relative lack of fit were individually examined; however, no further exclusions were made. A strong separation into dry and peak milk cohorts confirmed our decision to perform all analyses separately for the 2 cohorts (Supplemental Figures S3 and S4, see Notes).

Further visualization included the production of volcano plots of the $\log_2$ fold change concentration between heifers and >3 parity animals ($\log_2(\bar{x}_{heifers}/\bar{x}_{>3parity})$) and the $-\log_{10}$ *P*-value from Welch's unequal unpaired *t*-tests for each lipid (Welch, 1947) using *ttest*, *unequal* in Stata.

Simple Linear Regression

Mixed effect univariable linear regression, with group (breed within farm) as the random effect was performed, with each lipid as the independent variable and age (d) as the dependent variable, using mixed *age lipid* ||group: in Stata. Lipids were centered and normalized on group. The

Table 1. The complete list of plasma lipid classes and species targeted and quantified in the LC-MS analysis

Lipid class	Abbreviation	Species targeted
Phosphatidylcholine	PC	PC(31:1), PC(31:0), PC(30:1), PC(30:0), PC(29:0), PC(28:0), PC(33:1), PC(33:0), PC(32:2), PC(32:1), PC(32:0), PCN1, PC(34:3), PC(34:2), PC(34:1), PC(35:3), PC(35:2), PC(35:1), PC(37:3), PC(37:2), PC(36:5), PC(36:4), PC(36:3), PC(36:2), PC(36:1), PC(40:7), PC(40:6), PC(40:5), PC(40:4), PC(38:6), PC(38:5), PC(38:4), PC(38:3), PC(38:2)
Lysophosphatidylcholine	LPC	LPC(14:0), LPC(16:1), LPC(15:0), LPC(18:3), LPC(18:2), LPC(17:1), LPC(17:0), LPC(16:0), LPC(20:5), LPC(20:4), LPC(20:3), LPC(18:1), LPC(18:0), LPC(22:6), LPC(22:5), LPC(20:0)
Ether-linked Phosphatidylcholine	PC-O	PC(O-34:0), PC(O-33:2), PC(O-32:2), PC(O-32:1), PC(O-30:0), PC(O-34:3), PC(O-34:2), PC(O-34:1), PC(O-38:5), PC(O-38:4), PC(O-37:5), PC(O-36:4), PC(O-36:3), PC(O-36:2), PC(O-36:1)
Phosphatidylethanolamine	PE	PE(35:2), PE(35:1), PE(34:3), PE(34:2), PE(34:1), PE(33:1), PE(36:5), PE(36:4), PE(36:3), PE(36:2), PE(36:1), PE(40:6), PE(40:5), PE(38:6), PE(38:5), PE(38:4), PE(38:3), PE(38:1)
Lysophosphatidylethanolamine	LPE	LPE(18:3), LPE(18:2), LPE(18:1)
Ether-linked Phosphatidylethanolamine	PE-O	PE(O-34:3), PE(O-34:2), PE(O-34:1), PE(O-33:2), PE(O-33:1), PE(O-32:2), PE(O-32:1), PE(O-40:6), PE(O-40:5), PE(O-38:5), PE(O-38:4), PE(O-36:5), PE(O-36:4), PE(O-36:3), PE(O-36:2), PE(O-36:1)
Phosphatidylinositol	PI	PI(33:1), PI(33:0), PI(32:1), PI(32:0), PI(31:0), PI(40:6), PI(40:5), PI(38:6), PI(38:5), PI(38:4), PI(38:3), PI(38:2), PI(37:4), PI(37:3), PI(37:2), PI(36:5), PI(36:4), PI(36:3), PI(36:2), PI(36:1), PI(35:3), PI(35:2), PI(35:1), PI(34:3), PI(34:2), PI(34:1), PI(34:0)
Sphingomyelin	SM	SM(31:1), SM(30:1), SM(28:1), SM(34:4), SM(32:2), SM(32:1), SM(34:2), SM(34:1), SM(33:2), SM(33:1), SM(36:4), SM(36:2), SM(36:1), SM(35:2), SM(35:1), SM(38:2), SM(38:1), SM(37:1), SM(41:3), SM(41:2), SM(41:1), SM(40:3), SM(40:2), SM(40:1), SM(39:2), SM(39:1), SM(44:5), SM(44:4), SM(44:2), SM(43:4), SM(43:3), SM(43:2), SM(43:1), SM(42:3), SM(42:2), SM(42:1)
Triacylglycerol	TG	TG(54:1), TG(54:2), TG(54:3), TG(52:1), TG(52:2), TG(52:3), TG(51:1), TG(51:2), TG(50:1), TG(50:2), TG(50:3), TG(49:0), TG(49:1), TG(49:2), TG(48:0), TG(48:1), TG(48:2), TG(47:0), TG(47:1), TG(46:0), TG(45:0)

false discovery rate was controlled to 5% with the Benjamini–Hochberg procedure (Benjamini and Yekutieli, 2001; Newson and The ALSPAC Study Team, 2003).

ANOVA Simultaneous Component Analysis

To understand the influence of study design and inform future preprocessing of the data, several ANOVA simultaneous component analysis (**ASCA**) models were performed with combinations of farm, breed, feeding system, group (breed and farm), and parity (1, 2, 3, >3) in PLS Toolbox (Smilde et al., 2005; Thiel et al., 2017). As feeding system is invariant within farm or group, these variables could not be included together in multivariable analysis. Autoscaling, which uses the SD as the scaling factor, was applied to the lipid data (Westfall and Young, 1992). The number of principal components for each submodel was the lesser of the maximum number of principal components or 20. Results indicated that group (a feature of the study design) explained ~36% and 41% of the variation in lipid profile for the dry and peak milk cohorts, respectively. As such, efforts were made in subsequent data preprocessing to control for the variation in the lipid profile associated with group, to limit spurious associations (Table 2).

Calibration: Test Set and Cross-Validation

For each dry and peak milk group, the data were split into calibration and test datasets, at an 80:20 split, following the Kennard–Stone method with Euclidean distances (Kennard and Stone, 1969). This method ensures a uniform coverage of samples and incorporation of outliers into the calibration set. The test set was excluded from all model calibration to prevent data leakage.

Unless otherwise stated, cross-validation was performed using the venetian blinds method: each cross-validation set used every 10th sample of the calibration dataset, starting at sample 1 through to 10, resulting in 10 datasets with 10% data left out (Eigenvector Research, 2023a).

Regression Models. Orthogonal-partial least squares (**O-PLS**), multiple linear regression (**MLR**), and locally weighted regression models were performed to explore the associations between age (d) and their lipid profile. The root mean square error (**RMSE**) of calibration of the locally weighted regression models was consistently very poor, and consequently this model will not be discussed further.

Model Optimization. The model optimizer tool in PLS_Toolbox was used to efficiently compare the effect of different combinations of data preprocessing and model parameter options (Eigenvector Research, 2016). To limit over-fitting, specifications that consistently

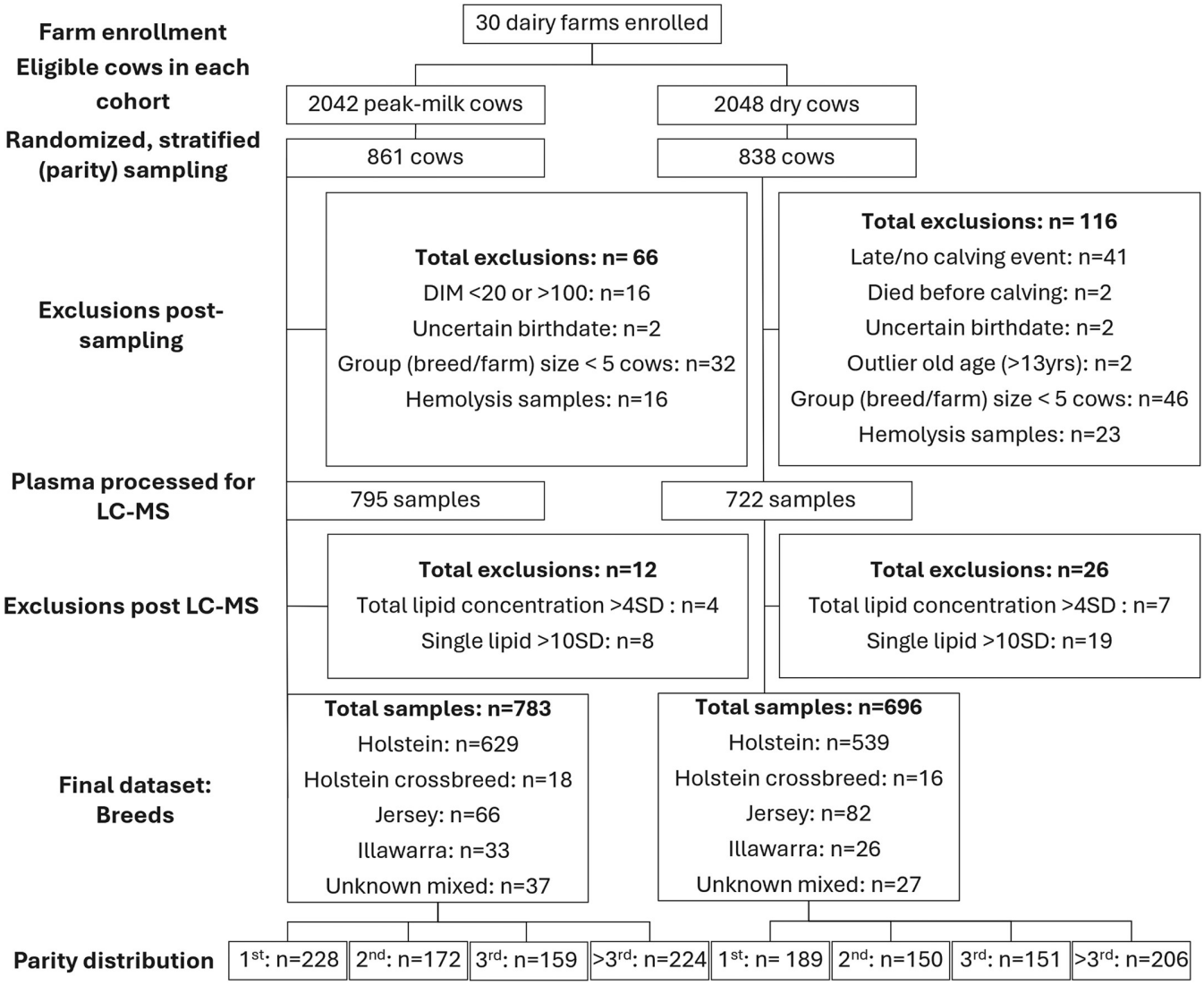

Figure 1. Flow diagram of cow eligibility, enrollment, and subsequent exclusions for the dry and peak milk cohorts. The parity and breed distribution of the final dataset are reported. LC-MS = liquid chromatography-MS.

produced low RMSE of cross-validation were chosen for the final models, and not necessarily the specific model specification that produced the single lowest RMSE of cross-validation.

Model Specification. The MLR algorithms tested included standard least squares, ridge, lasso, and elastic net regularization (Hoerl and Kennard, 1970; Tibshirani, 1996; Zou and Hastie, 2005; Eigenvector Research, 2022). The O-PLS models explored latent variable counts between 1 and 12, with confirmation of an appropriate latent variable count visually assessed by comparison of RMSE of calibration and RMSE of cross-validation.

Preprocessing. To control for the variance in the lipid profile associated with group (breed within farm; Table 2), either group centering or external parameter orthogonalization (**EPO**) with group-mean removal were explored (Roger et al., 2003; Eigenvector Research, 2023b). The EPO filter performs a hard orthogonalization on the differences between groups by performing PCA and removing a specified number of partial components (Roger et al., 2003). A case example of the EPO ability to remove the variation associated with a class variable is provided in Supplemental Figure S4, using cohort as the class variable to be controlled. In the model optimizer, the number of EPO partial components tested were between 1 and 6. Following class centering or EPO, variables were autoscaled, with the expectation that the biological relevance of a lipid is not due to its absolute plasma concentration.

Table 2. Results of ANOVA simultaneous component analysis for the dry cow and peak milk cohorts to determine the proportion of the variation in lipids that were attributable to components of the study design

Item[1]	Principal components	Cumulative Eigenvalue	Effect[2]	Residuals[3]
Dry cow cohort				
Univariable Terms				
Parity	3	9.36	5.01	94.99
Breed	4	18.74	4.34	95.66
Farm	20	66.39	35.61	64.39
System	1	13.13	6.95	93.05
Group	20	68.33	36.35	63.65
Multivariable Terms				
Parity	3	9.42	5.02	
Breed	4	18.38	4.35	90.64
Parity	3	9.23	4.80	
Farm	20	66.23	35.47	59.73
Parity	3	9.46	5.06	
System	1	13.28	7.01	87.93
Parity	3	9.33	4.82	
Group	20	68.25	36.23	58.95
Peak milk cohort				
Univariable Terms				
Parity	3	6.12	3.27	96.73
Breed	4	17.29	4.71	95.29
Farm	20	75.17	40.83	59.62
System	1	21.25	11.35	88.68
Group	20	76.33	40.67	59.33
Multivariable Terms				
Parity	3	5.85	3.11	
Breed	4	16.68	4.54	92.35
Parity	3	5.79	3.01	
Farm	20	74.83	40.22	56.77
Parity	3	5.88	3.11	
System	1	20.99	11.17	85.72
Parity	3	5.83	3.02	
Group	20	76.00	40.51	56.47

[1]Parity is grouped into lactations 1, 2, 3, and >3. System is TMR (15 farms) or pasture-based (15 farms). Group is breed within farm (dry: 34 groups, peak milk: 33 groups).

[2]The effect can be interpreted as the percentage of variation in the lipid profile that is explained by the listed terms.

[3]Residuals are the remaining unexplained variation in the lipid profiles after the univariate term or combined multivariate term effects are removed.

Age (d) was preprocessed as either mean-centered or log-transformed then mean-centered, with the latter being consistently superior according to RMSE of cross-validation.

Variable Selection. To reduce the number of variables and potentially increase the predictive ability of the specified models, variable selection using a genetic algorithm (**GA**) was performed in PLS Toolbox (Lucasius and Kateman, 1993; Zhang et al., 2018). The chosen parameters for GA are included in Supplemental Table S2 (see Notes).

Discriminant Analysis. Lipids associated with the distinct parity groups of heifers, second, third, and greater than third were investigated with orthogonal partial least squares discriminant analysis (**O-PLS-DA**), random forest (**RF**), and support vector machines (**SVM**; Breiman, 2001; Trygg and Wold, 2002; Zhang et al., 2006). Models were unable to clearly discriminant between neighboring parities, particularly sequentially from second, third, and greater than third parity (Supplemental Figure S5, see Notes) and a decision was made to include only heifers and third or greater lactation cows in the discriminant analysis.

Random forest and SVM analysis were performed in the MetaboAnalyst 6.0 online suite (https://www.metaboanalyst.ca; Pang et al., 2024), and O-PLS-DA was performed in PLS Toolbox (Eigenvector Research, 2023c). Groups were mean-centered to control for the

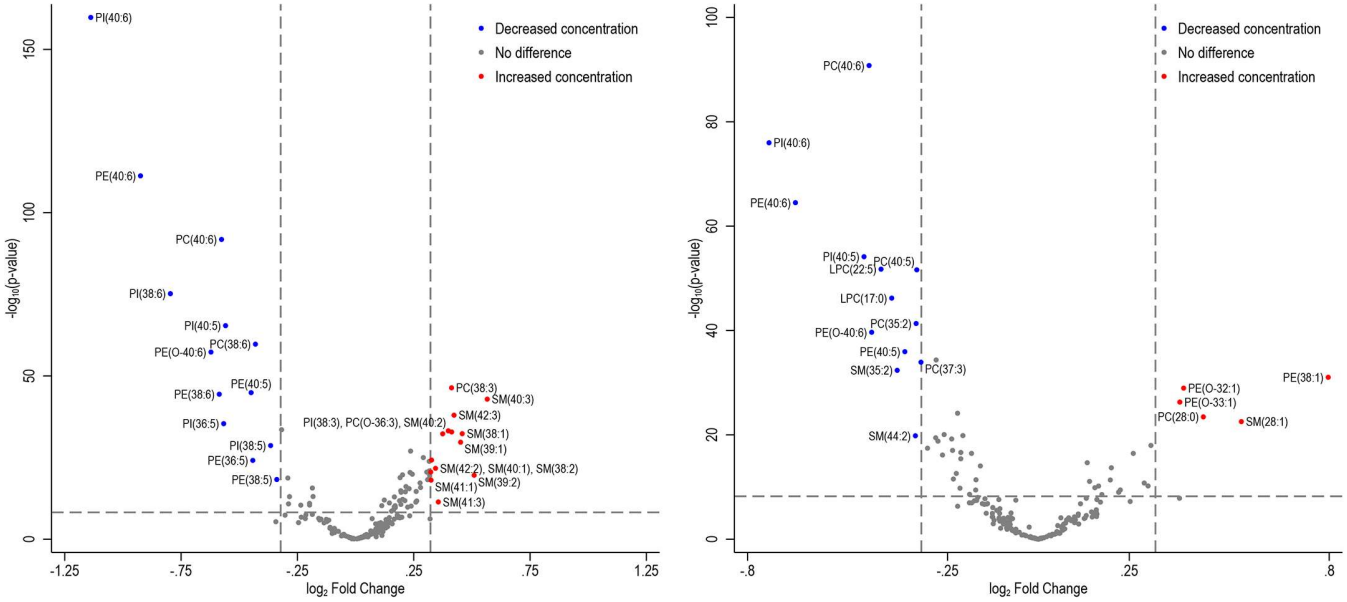

Figure 2. Volcano plots for plasma lipids in the dry cow cohort (left) and the peak milk cohort (right) comparing the fold change between heifers and >3 lactation cows and the *P*-value associated with an unpaired unequal *t*-test. Vertical dashed lines indicate a 1.25-fold change in concentration. The dashed horizontal dashed line is a Bonferroni adjusted *P*-value for 185 individual tests, equivalent to $P = 2.7 \times 10^{-4}$. Lipids that are colored have both large fold changes and are statistically significant.

effect of breed and farm, and auto-scaled. The RF and SVM analysis used 100 Monte Carlo cross-validations with balanced subsampling and one-third sample left out; O-PLS-DA were cross-validated as previously described.

Performance is graphically presented as receiver operator curves (**ROC**) with area under the ROC curve (**AUC**) reported. Figures were produced in each model's respective software.

Important Lipids. For the regression models, the relative importance of specific lipids are reported as their selectivity ratio (Kvalheim, 2020). Selectivity ratio is the ratio of variation attributed to a specific lipid and the unexplained variation. Lipids that consistently have a high selectivity ratio across multiple models are expected to be robustly associated with parity and age of cattle. For the discriminant analysis, feature ranking methods were specific to the classification method used and the magnitude of a feature's average importance cannot be directly compared across models. The top 10 lipids for each model and cohort are presented in tables, with the full list of ordered lipids provided as a supplementary datasheet.

Based upon their consistent high ranking in the regression and discriminant analyses, 8 lipids were empirically chosen for further graphical presentation. These selected lipids were stratified by parity, group-centered, and their plasma concentration distribution was smoothed through an Epanechnikov kernel density estimation function (Epanechnikov, 1969; *kdensity* in Stata). The resulting smoothed functions were plotted as ridgeline figures for each parity group, per lipid.

RESULTS

Volcano plots visually identify lipids that significantly differ between heifers and greater than third lactation cows by comparing the fold differences in plasma concentration and Welch's *t*-tests of significance. The resulting volcano plots (Figure 2) indicate that long-chain polyunsaturated phosphatidylinositol (**PI**), phosphatidylethanolamine (**PE**), and phosphatidylcholine (**PC**) were often found in lower concentrations in the older cows across both dry and peak milk cohorts. Sphingomyelins with long-chain FA (≥20 carbons) appear to be moderately higher in concentration in the older cows in the dry cohort (Figure 2). Note that centering was not performed in the fold change calculations, as this creates negative concentrations that make the direction and magnitude of the fold change uninterpretable (for example, −1:1 and 1:−1 are both equivalently a fold change of −1, despite a different direction of change, and $\log_2(-1)$ is the complex number 1.36i).

The ASCA results indicate that between 3.2% and 5.0% of the lipid profiles are explained by parity (1, 2, 3, >3), whereas farm accounted for the most, at 35% to 40% of the variation (Table 2). The farm systems of TMR or pasture-based explained more of the variation in the peak milk cohort (11.35%) than for the dry cohort (6.95%). As

expected, the difference in the cumulative eigenvalues of the ASCA models between farm and group were similar, as 86% to 89% of enrolled farms had only one breed of cow after exclusions were applied.

The regression results (including effect sizes and SE) from the mixed effect univariable regression models for all lipids species are reported in Supplemental Tables S3 and S4 (see Notes) for the dry and peak milk cohorts, respectively. A subset of the lipids that were significantly ($P < 0.001$) associated with age is reported in Table 3.

Model specifications for the regression and discriminant analysis are described in detail in the final model specifications provided in the supplemental material ("Final model specifications;" see Notes). The GA variable selection procedure removed many collinear lipids producing final models with 63 lipids in the dry cohort and 55 in the peak milk cohort. Though the predicted ability of the GA variable selection (according to RMSE of prediction) was maintained, many highly ranked lipids from the other specified models were excluded. This finding suggests a limited utility to identify associations of biologically important lipids. As such, GA model results are included in the supplemental data ("Supplementary Data—Lipid ranking.xlsx;" see Notes) for completion but are not discussed further. The RMSE of prediction for all models were close to the RMSE of calibration, indicating that over-fitting of data is of low concern for these models.

In all cases, according to the RMSE of prediction, the MLR models were better predictors of age than the O-PLS models. The dry and peak milk cohort RMSE of prediction were 340 and 483 d, respectively (Figure 3), indicating an average error in prediction of ~1 to 1.3 yr. There was a notable skew in the model residuals (Figure 3) starting from ~5 yr of age (~1,825 d), whereby the age of cows was increasingly underestimated.

The DA models showed excellent discriminant ability, with the AUC for cross-validation for O-PLS-DA, RF, and SVM above 95% for both cohorts. The O-PLS-DA ROC is shown in Figure 4, with the RF and SVM ROC in Supplemental Figure S6 (see Notes). The classification error with the test dataset for O-PLS-DA, RF, and SVM was 1.4%, 1.3%, and 6.3% in the dry cohort and 4.7%, 7.0%, and 4.7% in the peak milk cohort, respectively.

There was close agreement for the top ranked lipids in the dry and peak milk cohorts for the MLR, O-PLS, RF, and O-PLS-DA models (Figures 5, 6, 7, and 8), though the order of lipid ranking in the peak milk model was less consistent. This decrease in consistency aligns with the RMSE of the regression models and classification errors from discriminant models being larger for the peak milk cohort compared with the dry cohort. These observations indicate that variations in the lipid profile of peak milk cows are less capable of predicting age or parity than in dry cows.

Table 3. Significant lipid species following univariable mixed effect regression[1]

	Dry cohort		Peak milk cohort	
Lipid class	Increase with age	Decrease with age	Increase with age	Decrease with age
Phosphatidylcholine (PC)	34:2, 36:3, 37:3, 38:3, 38:4, 40:4	36:5, 38:5, 38:6, 40:5, 40:6	28:0, 30:1, 32:1, 34:1, 34:2, 34:3, 36:5	31:1, 32:0, 33:0, 33:1, 35:1, 35:2, 35:3, 36:1, 36:2, 37:2, 37:3, 38:4, 38:5, 38:6, 40:4, 40:5, 40:6, 40:7
Lysophosphatidylcholine (LPC)	18:2, 20:3, 20:4, 20:5, 22:6	17:0, 22:5	16:1, 18:2, 18:3	15:0, 17:0, 17:1, 18:0, 22:5, 22:6
Ether-linked Phosphatidylcholine (PC-O)	36:3	32:1, 36:1, 38:5	34:1	36:1, 36:2, 37:5, 38:4, 38:5
Phosphatidylethanolamine (PE)	38:3	33:1, 35:1, 36:1, 36:5, 38:5, 38:6, 40:5, 40:6	38:1	33:1, 34:2, 35:1, 35:2, 36:2, 36:3, 36:4, 36:5, 38:4, 38:5, 38:6, 40:5, 40:6
Ether-linked Phosphatidylethanolamine (PE-O)	36:3, 38:4	36:5, 40:5, 40:6	32:1, 32:2, 33:1, 33:2, 34:1, 34:2, 34:3, 36:3, 36:4, 36:5	40:5, 40:6
Phosphatidylinositol (PI)	37:3, 38:2, 38:3	34:3, 36:5, 38:5, 38:6, 40:5, 40:6		35:2, 37:4, 38:6, 40:5, 40:6
Sphingomyelin (SM)	36:1, 36:4, 37:1, 38:1, 38:2, 39:1, 39:2, 40:1, 40:2, 40:3, 41:1, 41:2, 41:3, 42:1, 42:2, 42:3, 43:3, 43:4, 44:4, 44:5	30:1	28:1, 30:1, 32:1, 34:4, 38:1, 39:1, 39:2, 40:1, 40:2, 41:1, 43:4	32:2, 33:1, 33:2, 34:2, 35:1, 35:2, 36:2, 40:3, 42:3, 43:1, 43:2, 43:3, 44:2, 44:5
Triacylglycerol (TG)	54:2	54:2	46:0, 48:1, 48:2	51:1, 51:2, 54:2, 54:3

[1]Age was the dependent variable, with breed/farm (group) the random effect. The false discovery rate was controlled at 5% through the Benjamini–Hochberg procedure. Increase and decrease with age indicate that older cows had increased and decreased plasma concentrations of the listed lipid, respectively. The effect size and SE of all lipid species are reported in Supplemental Tables S3 and S4.

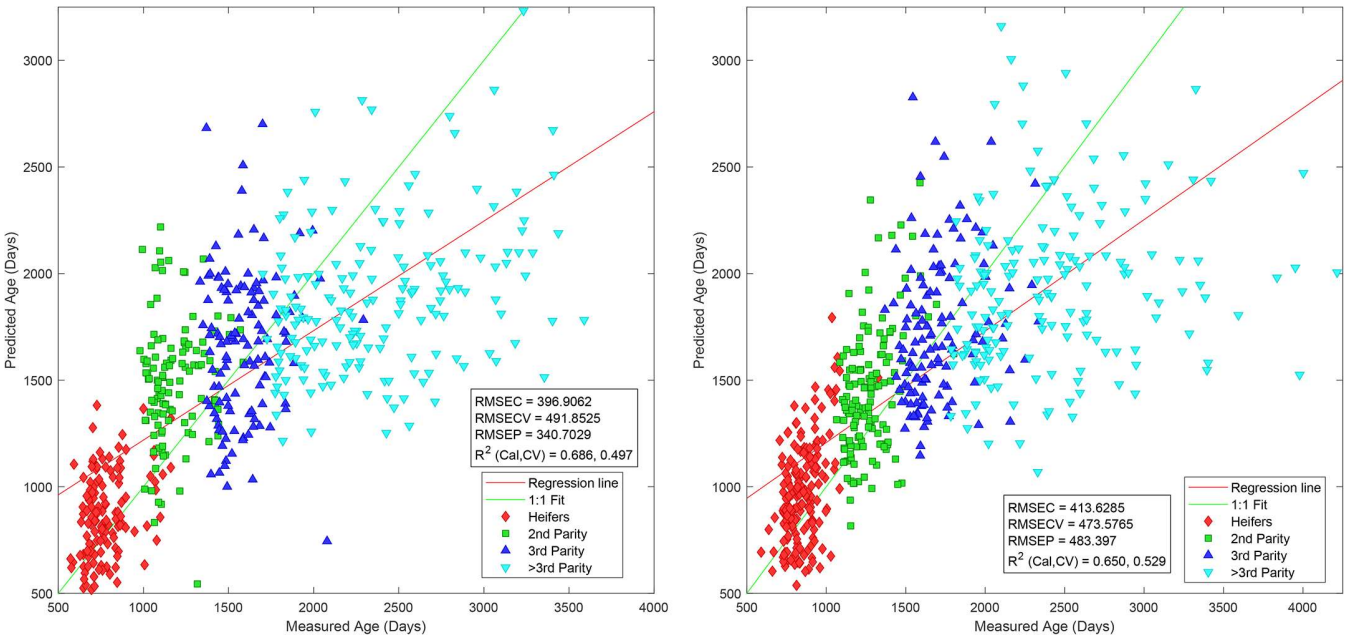

Figure 3. Predicted and measured age following multiple linear regression of lipid data for nonlactating, late pregnant (dry) cows (left) and peak milk cows (right). There is a skew in residuals such that the predicted age consistently underestimates that age of older cows (seen as more observations below the green line; predicted age < measured age). RMSEC = root mean square error of calibration (d); RMSECV = root mean square error of cross-validation (d); RMSEP = root mean square error of prediction (d).

The SVM discriminant analysis models often selected features that were of low ranking in the RF and O-PLS-DA models (Figures 7 and 8), though the SVM models had similar predictive ability (Supplemental Figure S6).

The PL with 40 C atoms and 5 or 6 double bond equivalents (**DBE**) were consistently highly ranked lipids across both cohorts and were lower in older cows (Figures 5, 6, 7, and 8). Phospholipids with 38:6 were decreased and 38:3 were increased and highly ranked in the dry cohort (Figures 5 and 7), whereas LPC(17:0) was decreased and highly ranked in the peak milk cohort (Figures 6 and 8).

We observed little consistency in which specific sphingomyelins were included in the top 10 lipid rankings, though SM(35:1) and SM(35:2) ranked high in the peak milk regression models (Figure 6). None of the 21 triacylglycerol classes quantified appeared in any of the top 10 ranked lipids for the multivariable regression models or discriminant analysis, though the univariable analysis identified TG(54:2) as being increased with age in both cohorts ($P < 0.001$, Table 3).

The ridgeline plots (Figure 9) depict the concentration distribution in each parity group of 8 selected lipids: PI(40:6), PC(40:6), PE(40:6), PI(40:5), PC(40:5), PC(38:6), SM(35:2), and LPC(17:0). The magnitude of difference appeared greatest between heifers and second lactation cows for the phospholipids with 40:5, 40:6, and 38:6, whereas the change appeared more linear for LPC(17:0) and SM(35:2).

DISCUSSION

We explored the association of plasma lipid species with parity and age of dairy cows in a large multisite, cross-sectional study. Multiple statistical models were specified with the objective of identifying lipids with strong and consistent associations among models. Phospholipids (PI, PC, PE) with long-chain PUFA, specifically those with 40 C atoms and 6 DBE were consistently decreased in the plasma concentration of older cows (Figures 2, 4, 5, 6, 7, and 8; Table 3). Additionally, phospholipids with 40:5 and 38:6, LPC(17:0), SM(35:1), and SM(35:2) were commonly identified lipids that decreased in concentration with parity and age (Figures 2, 4, 5, 6, 7, and 8; Table 3).

Though it was beyond the scope of our study to determine the specific fatty acyl or alkyl constituents for each lipid, there is very strong supporting evidence that the phospholipid 40:6 species would mostly comprise stearate (C18:0) or C18:1 in the sn-1 position and docosahexaenoic acid (**DHA**, C22:6 n-3), an n-3 fatty acid, or C22:5, in the sn-2 position. This assumption is supported by polyenoic acids C20 and C22 almost exclusively occupying the sn-2 position (Marai and Kuksis, 1969), whereas the sn-1 position is most frequently occupied by either palmitate (C16:0) or stearate (C18:0) in plasma PL (Douglas et al., 2007; Van et al., 2020). Similarly, we can assume the highly ranked 38:6 lipids are 16:0 and

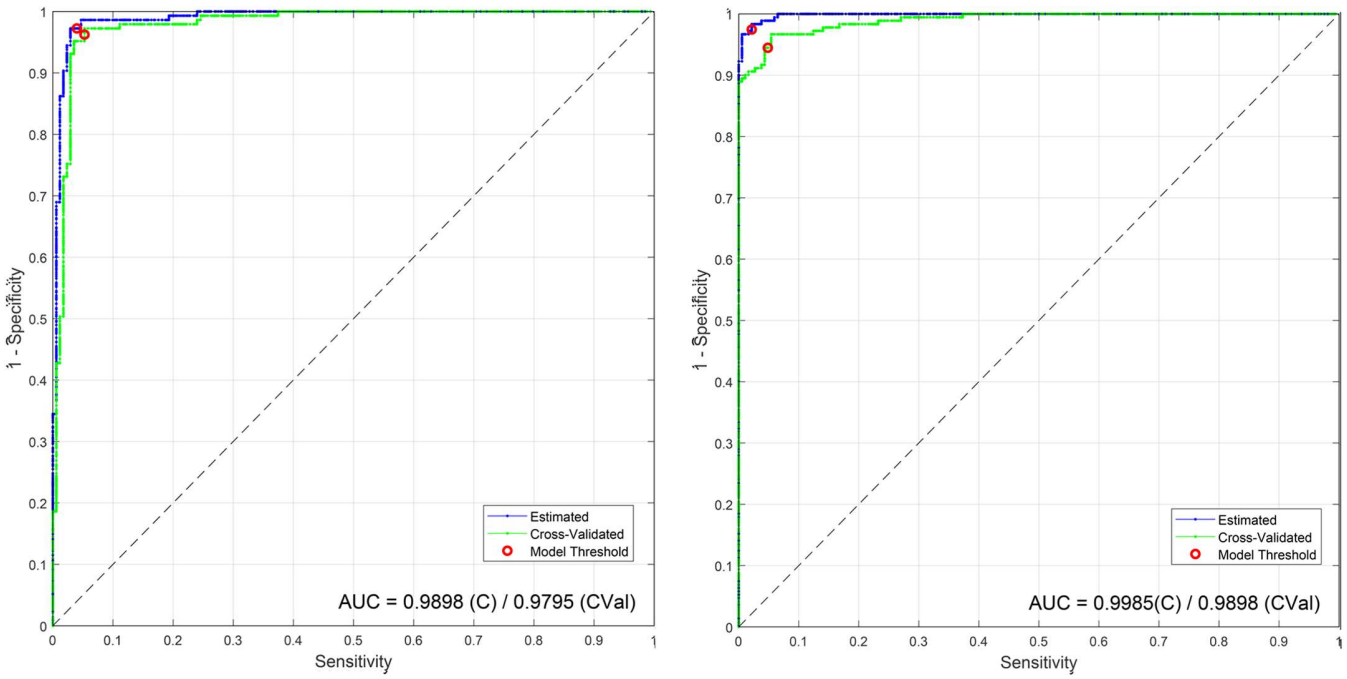

Figure 4. Receiver operator curves for orthogonal partial least squares discriminant analysis between heifers and >3 lactation cows for the dry cohort (left) and the peak milk cohort (right). AUC = area under the ROC curve; C = calibration; CVal = cross-validation.

22:6, whereas the 40:5 phospholipids constitute docosapentaenoic acid (**DPA**) in the sn-2 position (18:0/22:5), with DPA being the last fatty acid along the elongation pathway from dietary α-linolenic acid (**ALA**, C18:3n-3) to DHA (Ponnampalam et al., 2021). Accordingly, our results indicate that older cows have fewer circulating PL containing DHA (and DPA), which may indicate a whole-body depletion of DHA, and that this depletion is consistent during both the dry period and at peak milk. It cannot be determined from our study whether other body tissues similarly reflect lower 40:6 phospholipid concentrations with increasing parity and age. The cause of the observed depletion may be from increased irreversible loss of precursors, especially in milk, reduced consump-

	MLR		O-PLS		
Lipids	Rank	SR	Rank	SR	Direction
PI(40:6)	1	0.89	1	1.37	Down
PC(40:6)	2	0.52	3	0.62	Down
PE(40:6)	3	0.43	2	0.76	Down
PI(40:5)	4	0.35	5	0.43	Down
PI(38:6)	5	0.33	4	0.43	Down
PE(O-40:6)	6	0.30	6	0.34	Down
PC(38:6)	7	0.22	7	0.29	Down
PC(40:5)	8	0.21	10	0.21	Down
PE(40:5)	9	0.16	8	0.23	Down
PE(38:6)	10	0.15	9	0.22	Down

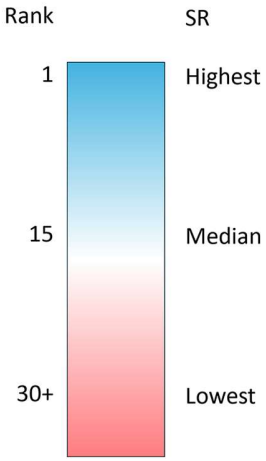

Figure 5. Depicts the rank and selectivity ratio (SR) of the top 10 lipids in the dry cow cohort that were identified following multivariate regressions models on age and lipids. Direction refers to the change in each specific lipid's plasma concentration with increasing age, according to the univariable mixed linear model analysis. MLR = multiple linear regression; O-PLS = orthogonal partial least squares.

Lipids	MLR Rank	MLR SR	O-PLS Rank	O-PLS SR	Direction
PC(40:6)	1	0.35	1	0.43	Down
SM(35:1)	2	0.25	6	0.34	Down
SM(35:2)	3	0.22	2	0.37	Down
LPC(17:0)	4	0.20	5	0.34	Down
PC(40:5)	5	0.18	3	0.37	Down
PI(40:6)	6	0.17	8	0.30	Down
PC(37:3)	7	0.17	12	0.25	Down
PE(40:6)	8	0.15	14	0.23	Down
LPC(22:5)	9	0.15	11	0.26	Down
PE(38:1)	10	0.14	15	0.22	Up
PI(40:5)	11	0.14	4	0.35	Down
PC(35:2)	17	0.12	7	0.32	Down
SM(38:1)	47	0.04	9	0.29	Up
SM(39:1)	44	0.05	10	0.26	Up

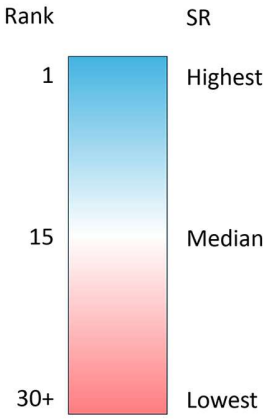

Figure 6. The rank and selectivity ratio (SR) of the top 10 lipids in the peak milk cow cohort that were identified following multivariate regressions models on age and lipids. Direction refers to the change in each specific lipid's plasma concentration with increasing age, according to the univariable mixed linear model analysis. MLR = multiple linear regression; O-PLS = orthogonal partial least squares.

tion of DHA or its precursors, or an impaired ability to produce or absorb DHA and its precursors.

That parity and PL containing DHA are strongly associated may be an important finding, given the association of parity with increased risk of disease, reproductive failure, and removal from the herd (Lean et al., 2023a), as well as the bioactivity of DHA and its association with positive health outcomes (Narayan et al., 2006; Bionaz et al., 2020; Fabjanowska et al., 2023). Interest in DHA from a dairy cow perspective has included both the potential health benefits of increasing DHA content in milk (Parodi, 2004; Huang et al., 2020; Plata-Pérez et al., 2022) and meat for human consumption (Scollan et al., 2001; Dannenberger et al., 2007) and the direct health benefits for the cow (Bradford et al., 2015; Moallem, 2018; Bionaz et al., 2020; Veshkini et al., 2023). Several review papers have focused on the importance of PUFA, including DHA, on dairy cow reproduction, inflammation, oxidative stress, milk production, and transition cow health (Bradford et al., 2015; Moallem, 2018; Bionaz et al., 2020; Fabjanowska et al., 2023; Veshkini et al., 2023). However, we are the first to show a probable association between DHA and age or parity, and this association is plausibly causal, with increased risk of disease and culling in older cows.

In support of our finding of PL(40:6) being lower in older cows, a small study investigating the fatty acid composition of follicular fluid in multiparous and primiparous cows also reported the serum fatty acid concentration for a subset of cattle (n = 4 each), with a 70% reduction in circulating DHA in multiparous cows (Bender et al., 2010). Humer et al. (2016) used a targeted lipidomic platform to investigate early lactation lipolysis and compared the plasma lipid profiles of nulliparous (n = 12) and multiparous (n = 18) cows. Their results showed a significant difference in long-chain PC (C40–C44) in multiparous cows across different levels of saturation (DBE 1–6). Our targeted profile did not include PL with greater than 40 carbons (Table 1) for direct comparison, and it is possible that age or parity is associated with PL with long-chain or very-long-chain FA and not DHA specifically. Although it is also interesting to note a contrasting observation between age and circulating DHA and eicosapentaenoic acid (**EPA**) in human subjects, whereby older people have increased DHA and EPA independent of diet (Crowe et al., 2008; Walker et al., 2014), there is a poor analogy between the modern dairy cow and older humans, stressing the importance of longevity research specific to the dairy industry.

The fatty acid composition of different tissues and milk of cattle can be substantially altered through dietary changes (Scollan et al., 2001; Dannenberger et al., 2007; Sinedino et al., 2017; Plata-Pérez et al., 2022). Enrichment with n-3 PUFA can be achieved through increased pasture feeding, which contains 50% to 75% of its total FA content as ALA (Elgersma, 2015), a common precur-

Lipids	RF Rank	RF MI	O-PLS-DA Rank	O-PLS-DA MI	SVM Rank	SVM MI	Direction
PI(40:6)	1	13.28	1	2.29	1	0.12	Down
PE(40:6)	2	10.95	2	1.86	2	0.09	Down
PI(38:6)	3	10.71	3	1.24	6	0.06	Down
PC(40:6)	4	10.38	4	1.20	4	0.07	Down
PC(38:3)	5	8.49	10	0.60	16	0.03	Up
PC(38:6)	6	8.21	5	0.94	59	0.02	Down
PE(38:6)	7	7.42	6	0.91			Down
PI(40:5)	8	7.25	7	0.67	14	0.03	Down
PE(O-40:6)	9	6.58	11	0.58	36	0.02	Down
SM(40:3)	10	6.48	15	0.43	57	0.02	Up
PI(36:5)	11	6.32	8	0.64	83	0.01	Down
PE(40:5)	16	5.39	9	0.60	62	0.01	Down
PC(O-36:1)	21	4.07	59	0.10	3	0.08	Down
LPC(17:0)	69	1.74	87	0.05	5	0.06	Down
PC(28:0)			134	0.01	7	0.05	Down
PE(O-38:4)	66	1.84	78	0.07	8	0.05	Up
PC(O-36:2)			100	0.03	9	0.04	Down
SM(36:4)	45	2.53	76	0.08	10	0.04	Up

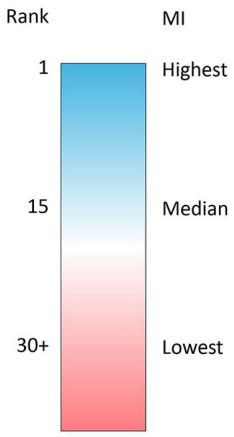

Figure 7. The rank and average importance (MI) of the top 10 lipids in the dry cow cohort that were identified by discriminant analysis between heifers and greater than third parity cows in random forest (RF), orthogonal partial least squares discriminant analysis (O-PLS-DA) and support vector machine (SVM) models. Direction refers to the change in each specific lipid's plasma concentration with increasing age, according to the univariable mixed linear model analysis.

sor for DHA de novo synthesis (Ponnampalam et al., 2021). Farms that incorporate less pasture into their rations have lower blood and milk PUFA concentrations (Moate et al., 2008; La Terra et al., 2010), which may result in an older herd lipid profile and an associated increased risk of disease and removal (Lean et al., 2023a). Enrichment can also be achieved with supplementation of seed oils, for example linseed (flaxseed) is particularly high in ALA, 52.5% of FA, or fish oils or algae, which contain high amounts of EPA and DHA (Stamey et al., 2012; Suksombat et al., 2016; Sinedino et al., 2017; Moallem, 2018). Diets enriched with ALA can provide more efficient fat mobilization and subsequent protein accretion in early lactation (von Soosten et al., 2012), which could support older cows that typically have lower body condition during this critical period (Lean et al., 2022). Given that this was a cross-sectional study, we cannot establish whether the lower circulating 40:6 PL are only correlated with advanced age, or if there is functional significance that increases the risk of disease and removal from the herd with age. However, the combination of n-3 FA bioactivity and the ability to modify PL fatty acid profiles through dietary intervention provides ample justification for further investigation.

The PL(38:6) species, assumed to mostly comprise palmitic (C16:0) and DHA (22:6) or (18:1) and EPA(20:5), were highly correlated with the 40:6 species and were significantly associated with age and parity in the dry cohort (Figures 5 and 7). It is unclear why the strength of association of PL(38:6) with parity and age was lower in the peak milk cohort, though palmitic acid availability may be limited at peak milk production (Douglas et al., 2007; Loften et al., 2014). Both stearate and palmitic acid have similar plasma concentrations at 45 d prepartum and differentially change in the early transition period, with plasma concentration of palmitic acid being reduced at 65 DIM (Douglas et al., 2007; Loften et al., 2014). The dynamic changes in FA metabolism occurring during the transition to peak milk may mask the association of 38:6 phospholipids with parity that was observed in the dry cohort.

Sphingomyelins appeared to be generally positively associated with parity in the dry cows according to the volcano plots (Figure 2) and the univariable regressions (Table 3). The SM with large fold changes all had at least 38 carbons (Figure 2). The multivariable regression and discriminant analysis indicated that the SM were ranked as less important than the long-chain PUFA PL and the specific SM that were ranked were inconsistent among models (Figures 6 and 7, Supplemental Dataset S1, see Notes). It is difficult to assume the FA composition of the important SM without further laboratory analysis. Mammalian SM contain a phosphocholine head, an N-linked

Lipids	RF Rank	RF MI	O-PLS-DA Rank	O-PLS-DA MI	SVM Rank	SVM MI	Direction
PI(40:6)	1	13.96	2	0.84	3	0.09	Down
PE(40:6)	2	11.59	3	0.61	5	0.06	Down
PC(40:6)	3	11.43	1	0.85	6	0.06	Down
PI(40:5)	4	8.84	4	0.51	31	0.02	Down
PE(38:1)	5	8.45	10	0.32	29	0.02	Down
LPC(17:0)	6	8.30	5	0.43	7	0.06	Down
LPC(22:5)	7	7.35	7	0.41	83	0.01	Down
PC(35:2)	8	7.20	9	0.34	17	0.03	Up
PC(40:5)	9	7.18	6	0.42	26	0.02	Down
PE(O-40:6)	10	6.91	15	0.22	16	0.03	Down
PC(35:1)	15	6.07	8	0.39	106	0.01	Down
PC(O-36:1)	42	3.02	57	0.07	1	0.15	Down
PC(28:0)	11	6.90	17	0.21	2	0.11	Down
PE(O-33:1)	14	6.13	26	0.14	4	0.07	Up
PC(34:3)	34	3.40	35	0.11	8	0.05	Down
PE(O-34:3)	19	4.98	42	0.09	9	0.04	Up
LPC(22:6)	56	2.33	34	0.11	10	0.04	Up

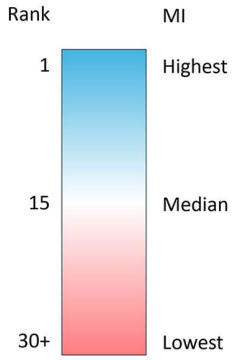

Figure 8. The rank and average importance (MI) of the top 10 lipids in the dry cow cohort that were identified by discriminant analysis between heifers and greater than third parity cows in random forest (RF), orthogonal partial least squares discriminant analysis (O-PLS-DA) and support vector machine (SVM) models. Direction refers to the change in each specific lipid's plasma concentration with increasing age, according to the univariable mixed linear model analysis.

acyl chain, and a common long-chain base of sphingosine, a C18 amino alcohol with an unsaturated hydrocarbon (d18:1). However, variation in the DBE of the base is sufficiently frequent to make difficult strong assumptions about the most common fatty acid chain composition from the summed composition (Ramstedt et al., 1999). Thus, with considerable caution, we can ascribe the SM that were identified as importantly decreased in the older cows at peak milk as potentially SM(d18:2,17:0), and SM(d18:1,17:0) (Figure 6). The SM with increased concentration in >3 parity dry cows (Figure 2) had acyl groups of ≥20 carbons. Previous studies have reported that specific very-long-chain SM and ceramides are associated with lipolysis, and insulin resistance (Rico et al., 2015; Humer et al., 2016; McFadden and Rico, 2019). It is plausible that the significant fold change difference observed in very-long-chain SM in dry cows reflects differences in lipid mobilization between younger and older cattle as they approach parturition and early lactation. Further experimental research is required to test this hypothesis and determine any clinical significance of the observed association.

Lysophosphatidylcholine(17:0) and potentially SM(d18:2,17:0) and SM(d18:1,17:0) were consistently listed as importantly decreased in older cows, particularly the peak milk cohort (Figures 6 and 8). Humer et al. (2016) also reported a decrease in LPC(17:0) in multiparous cows (compared with primiparous) and cows with high levels of lipolysis in early lactation. The proportion of milk FA that are C17:0 is reduced in second, third, and greater than third lactations when compared with first-lactation cows (Sun et al., 2022). It is not clear why C17:0 should be specifically different with parity, and this could indicate a higher degree of lipolysis in old cattle or a reduced pool of C17:0 in tissue for mobilization, with older cattle generally having lower BCS (Lean et al., 2022). The odd-chain FA, including C17:0, are mostly produced de novo in the rumen through bacterial activity (Vlaeminck et al., 2006) and are associated with pastures high in ALA, to the extent that milk C17:0 concentration has been suggested as a biomarker for authenticating that milk is from grass-fed cows (Moate et al., 2008; Paredes et al., 2018; Riuzzi et al., 2021). The associations between C17:0, pasture, and parity present an interesting parallel to the earlier discussion on DHA, pasture, and parity.

Plasma TG are transported in association with various lipoprotein particles and represent either transport of FA from the diet or utilization of FA energy stores (Bauchart, 1993). Only one TG was significant ($P < 0.001$) in both the dry and lactating univariable analysis, TG(54:2), whereas no TG featured in any of the volcano plots (Figure 2) or as important features in the regression or discriminant analysis (Figures 5, 6, 7, and 8). The relation between aging, age-related disease, and circulating TG in human and animal models remains unclear (Spitler and

Lipids	MLR Rank	MLR SR	O-PLS Rank	O-PLS SR	Direction
PI(40:6)	1	0.89	1	1.37	Down
PC(40:6)	2	0.52	3	0.62	Down
PE(40:6)	3	0.43	2	0.76	Down
PI(40:5)	4	0.35	5	0.43	Down
PI(38:6)	5	0.33	4	0.43	Down
PE(O-40:6)	6	0.30	6	0.34	Down
PC(38:6)	7	0.22	7	0.29	Down
PC(40:5)	8	0.21	10	0.21	Down
PE(40:5)	9	0.16	8	0.23	Down
PE(38:6)	10	0.15	9	0.22	Down

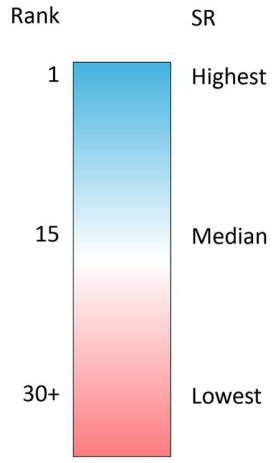

Figure 9. Kernel weighted density plots of specific lipid species concentrations stratified by parity groups. Presented lipids were identified as influential in differentiating parity and age. Data were centered and normalized on breed within farm (group) and cohorts of dry cows and peak milk cows. All figures contain both lactating and dry cow cohorts except PC(38:6) in the dry cohort only and LPC(17:0) and SM(35:2) in the peak milk cohort only.

Davies, 2020), supported by our paucity of significant results in spite of substantial study power.

The multivariable regression models consistently underpredicted the age of older animals. This may simply reflect a nonlinear change in lipid concentrations as cows age, which could be implied by observing the nonlinear change in the distribution of plasma concentration across parity groups of the important lipids depicted in Figure 9. It is also possible that the skewed residuals may reflect a form of selection bias, survival bias. This form of bias is inherent to aging studies, particularly aging studies in production animal species, where the selection pressure on an individual cow includes both production characteristics (for example adequate milk production, successful reproduction), and avoiding illness or death. Consider the scenario where a "young" lipid profile protects cows from removal. Cows with "old" profiles will be removed at higher rates, leaving only older cows with relatively "young" profiles on farm. This would manifest as a nonlinear change in lipid concentration with age. In extreme cases of survival bias, we might erroneously attribute a lipid as being important to survival because we cannot analyze the lipids of cows that failed to survive. Consider an alternative scenario where cows with low concentrations of long-chain PUFA (including DHA), have a lower peroxidation index (Hulbert et al., 2007) and may better adapt to the oxidative stress of lactation (McFadden, 2020). Under this scenario, and without changing our dataset, low PL(40:6) would be considered protective for survival. With the current study design, it is not possible to quantify the effect or direction introduced by survival bias. Options to control for survival bias in longevity studies include longitudinal studies, which can be expensive and time-consuming to perform, or in human longevity studies, investigators have compared offspring of long-lived individuals with age-matched people who did not have long-lived parents (Schoenmaker et al., 2006; Collino et al., 2013). A similar approach in herds with excellent reproductive records could be useful in controlling for survival bias in the study of longevity and for confirming the results of this study.

By design, our targeted LC-MS approach was limited to only the summed composition of the specific lipids listed in Table 1, which notably did not include FFA. The summed compositions necessitated speculation on the most likely FA composition for the highly associated lipid species. Following our results indicating a strong association between age, parity, and PUFA, we recommend that future investigations incorporate the measurement of oxylipins. This class of bioactive lipids, including eicosanoids, resolvins and lipoxins, are produced via the oxidation of PUFA (Camunas-Alberca et al., 2023) and may provide critical insights into the aging processes of dairy cows.

CONCLUSIONS

This large multisite cross-sectional study revealed strong and consistent associations between increasing parity and age and decreasing circulating PL(40:6), PL(40:5), PL(38:6), LPC(17:0), SM(35:1), and SM(35:2), whereas TG were not associated. Importantly, the strongest and most consistent association was with PL(40:6), which most likely comprise stearate (C18:0) and docosahexaenoic acid (C22:6;n-3), an n-3 fatty acid. Previous studies have shown an association with DHA and im-

proved cattle health, reproduction, and milk production and quality. Our study provides a plausible hypothesis that the decreased DHA concentration in older cows is a risk factor for the increased adverse health events, poor reproduction, and removal from the herd in older cows. Further prospective studies are required to determine if providing diets that increase circulating DHA can may increase health and longevity of dairy cows.

NOTES

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Nonstandard abbreviations used: ALA = α-linolenic acid; ASCA = ANOVA simultaneous component analysis; AUC = area under the ROC curve; CVal = cross validation; DBE = double bond equivalent; DHA = docosahexaenoic acid; DPA = docosapentaenoic acid; EPA = eicosapentaenoic acid; EPO = external parameter orthogonalization; FA = fatty acid; GA = genetic algorithm; LC-MS = liquid chromatography-MS; LPC = lysophosphatidylcholine; LPE = lysophosphatidylethanolamine; MLR = multiple linear regression; O-PLS = orthogonal partial least squares; O-PLS-DA = O-PLS discriminant analysis; PC = phosphatidylcholine; PCA = principal component analysis; PC-O = ether-linked phosphatidylcholine; PE = phosphatidylethanolamine; PE-O = ether-linked phosphatidylethanolamine; PI = phosphatidylinositol; PL = phospholipid; RF = random forest; RMSE = root mean square error; RMSECV = root mean square error of cross-validation; RMSEP = root mean square error of prediction; ROC = receiver operator curve; SM = sphingomyelin; SR = selectivity ratio; SVM = support vector machines; TG = triacylglycerol.

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CHAPTER 6

Confinement and pasture-based dairy herds differ in plasma lipid profiles

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OVERVIEW OF CHAPTER 6

There are two broad categories of housing management systems in dairy production, confinement-based housing and pasture-based management. This chapter explored the association between these categories with blood lipids and metabolites. There were 14 pasture-based and 15 confinement-based farms in each of the dry-cow and peak-milk cow cohorts. The *Material and Methods* of this chapter also introduced the variable stabilisation technique to control the false discovery rate in high-dimensional datasets; the technique is also applied in Chapter 7. The results showed glycerophospholipids associated with omega-3 fatty acids were greater in pasture-based farms compared to confinement farms in both the dry-cow and peak-milk cow cohorts. Glycerophospholipids associated with omega-6 fatty acids were decreased in pasture-based farms compared to confinement farms in the peak-milk cohort only. We speculate the difference in lipids profiles were related to the main forage source offered by our enrolled farms; pasture-based farms fed kikuyu or ryegrass while confinement-based farms fed corn silage as part of a total mixed ration. The fatty acids profile of fresh pasture is high in omega-3 and low in omega-6, and corn-silage is conversely low in omega-3 and high in omega-6.

These results may indicate that confinement-based farms provide an unbalanced lipid diet, leading to confinement-based cows having an ‘older’ lipid profile compared to pasture-based cows (Chapter 5), and are at increased risk of culling (Chapter 7).

Note on supplementary material

The published article refers to a supplementary document available at https://doi.org/10.6084/ m9.figshare.28813217. The tables and figures from the supplementary document are also available in the *Appendix.* Supplementary Table S1 is reported as Table A15, Table S2 as Table A1, Table S3 as Table A13, Figure S1 as Figure A1, Figure S2 as Figure A2, and A2, Figure S3 as Figure A7, and Spreadsheet as Tables A8 and A9.

Supplemental Spreadsheets S2, S3, and 4 are available from the above Figshare link and the thesis supplementary repository at https://doi.org/10.6084/m9.figshare.29456534.

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Confinement and pasture-based dairy herds differ in plasma lipid profiles

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ABSTRACT

Dairy cow housing and management can be broadly described as either intensive confinement-based (CONFINE) or extensive pasture-based (PAST) systems. The diets between systems typically differ in their forage base, with CONFINE farms often utilizing maize silage in a TMR. Consequently, the lipid composition of diets differs between systems. The influence of housing system on blood lipidomics is currently unknown, but due to the bioactive role of lipids in influencing overall health and productivity, differences in diet may have consequences for reproduction, health, and aging of cows. The objective of this cross-sectional, multisite study was to investigate blood lipids and metabolites from cows in PAST and CONFINE systems, in the dry period (~27 d prepartum) and at peak milk (~58 DIM). After exclusions, blood samples from 303 PAST and 398 CONFINE dry-period cows and 350 PAST and 431 CONFINE peak-milk cows from 15 PAST and 15 CONFINE farms were analyzed. A total of 185 lipid species (including glycerophospholipids, sphingomyelins, and triacylglycerols) were evaluated using targeted liquid chromatography-MS, as were 11 routinely measured metabolites. Dry and peak-milk cohorts were analyzed separately throughout. Lipids and metabolites associated with housing system were selected using a variable stabilization approach that was achieved by calculating the frequency of inclusion in categorical (housing system) penalized models using bootstrapping. Variables were retained if inclusion frequency exceeded a false-positive threshold. Five different statistical models were used with variable stabilization. Dry cows in CONFINE systems had decreased globulin, urea, and glycerophospholipids associated with n-3 fatty acids. The highest total inclusion rates in the dry cohort were phosphatidylcholine (PC; 36:5), which mostly comprises palmitic acid (C16:0) and eicosapentaenoic acid (EPA; 20:5n-3), then phosphatidylethanolamine (PE; 38:5, 16:0/22:5n-3 or 18:0/EPA) and PC(34:3; 16:0/18:3 α-linolenic acid [ALA]). No lipids were increased in more than one stabilized model in CONFINE dry cows. Peak-milk CONFINE cows had increased glycerophospholipids associated with n-6 fatty acids. The highest total inclusion-rate lipids in the peak-milk cohort were phosphatidylinositol (PI; 38:3; 18:0/20:3n-6 dihomo-γ-linolenic acid), PC(34:2; 16:0/18:2 linoleic acid [LA]), PC(40:7; 18:2/22:5n-6), PC(34:1; 16:0/18:1), and PE(34:2; 16:0/LA). The CONFINE peak-milk cows also had decreased PC(34:3; 16:0/ALA). This study identified specific lipids that were strongly associated with housing systems, findings that have not been reported elsewhere. Given the important biological functions of omega fatty acids, the pattern of glycerophospholipids with increased n-6 and decreased n-3 in CONFINE cows may indicate housing systems create different risk profiles for reproduction, health, and aging.

Key words: lipidomics, omega fatty acid, housing, phospholipids

INTRODUCTION

Dairy cow housing systems can broadly be classified as either intensive confinement-based (**CONFINE**) or extensive pasture-based (**PAST**) systems. Beyond the inherent physical and managerial differences between these systems, diet composition typically differs substantially. Maize silage, a high DM yield forage, is commonly the main forage source in CONFINE systems and is provided as a component of a TMR. The distinct differences in lipid compositions of maize silage and fresh pasture may influence health, reproduction, and longevity of cows.

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-25. Nonstandard abbreviations are available in the Notes.

Fresh pasture contains ~50% to 75% of its total fatty acid (**FA**) content as an n-3 FA, α-linolenic acid (**ALA**; C18:3n-3), while the n-6 FA, linoleic acid (**LA**; C18:2n -6) is much lower at ~11% to 13% of total FA (Glasser et al., 2013; Elgersma, 2015; NASEM, 2021). In contrast, maize silage has a much lower proportion of ALA (~7% total FA) and a higher proportion of LA (~47% total FA; Glasser et al., 2013; NASEM, 2021). Ryegrasses (*Lolium* spp.) also contain relatively higher crude fat content (immature: 5.0% ± 0.4% DM, mid mature: 4.37% ± 0.4% DM) than maize silage (2.9% ± 0.4% DM; NASEM, 2021). Importantly, ALA and LA are both essential FA that serve as precursors to the bioactive very long-chain PUFA, including eicosapentaenoic acid (**EPA**; C20:5n-3) and docosahexaenoic acid (**DHA**; C22:6n-3) in the n-3 pathway, or arachidonic acid (**ARA**; C20:4n-6) in the n-6 pathway. These long-chain PUFA have complex roles associated with immune regulation, intracellular signaling, and oxylipid production (Sordillo, 2016; Moallem, 2018).

Poor human health outcomes, including chronic inflammation, cardiovascular disease, cancer, and other chronic diseases, have been associated with high n-6, low n-3 diets (Simopoulos, 2002; Saini and Keum, 2018; Djuricic and Calder, 2021), leading to studies exploring means to decrease the n-6:n-3 ratio of milk and beef (Moate et al., 2008; Daley et al., 2010; Lanier and Corl, 2015; Moallem, 2018). These studies consistently reported that grass-fed cattle produced milk and meat with lower n-6, higher n-3 content compared with grain- or maize silage–based diets (Moate et al., 2008; Daley et al., 2010; Gebreyowhans et al., 2019). However, with the focus primarily on improving human health, there are comparatively fewer studies that have explored the effect of n-6 and n-3 dietary components on cattle health. Experimental supplementation that increases n-3 (e.g., with flaxseed or marine products) has reported improved health and reproduction (Zachut et al., 2010; Rodney et al., 2015; Sinedino et al., 2017; Moallem, 2018; Moallem et al., 2020).

Lipidomics, the large-scale study of lipids, has become increasingly accessible to researchers (Avela and Sirén, 2020) and provides new opportunities to investigate lipid metabolism in relation to milk composition (Lopez et al., 2008; Liu and Rochfort, 2023) and cattle health (Imhasly et al., 2015; Humer et al., 2016; McFadden, 2020; Sheedy et al., 2025a). Specific lipid classes, such as glycerophospholipids (**PL**), sphingomyelins (**SM**), and triacylglycerols (**TG**), and their FA compositions are increasingly recognized for their diverse biological functions that include regulating cell signaling, inflammation, trafficking, release of bound FA, and as direct sources of energy (Ohanian and Ohanian, 2001; Palmquist, 2009; Lordan et al., 2017). There are no studies to date that have examined the circulating lipid profiles of cows raised under different housing systems using a lipidomics approach. As a result, the specific lipids associated with different housing remain unknown.

The objective of this cross-sectional study was to compare the lipid and metabolic profiles of cattle in CONFINE systems with year-round PAST systems from cows in the dry and peak-milk periods. Our aim was to provide insight into how housing systems influence cow lipid profiles at different stages of production. Our hypothesis was that polar lipids containing n-3 and n-6 FA will be significantly decreased and increased, respectively, in CONFINE systems compared with PAST.

MATERIALS AND METHODS

The study was carried out in accordance with the recommendations of The Australian Code for Care and Use of Animals for Scientific Purposes. Protocols were approved by the *Scibus* Animal Ethics Committee (Scibus # 1022-1024) and The University of Sydney Animal Ethics Committee (Project # 2022/2247).

Farm Enrollment

Farms were purposively selected to obtain an even distribution of 15 PAST farms and 15 CONFINE farms, based completely upon the management system of the lactating herd, and allowing the dry-cow management system to vary, reflecting that many CONFINE herds graze far-off dry cows. Selection criteria for farms were a mean lactating herd size of >200 cows, conventional system (nonorganic), and maintained accessible electronic records (including a minimum of reproduction, birth date, and health treatment details). Most PAST farms had kikuyu (*Cenchrus clandestinus*, previously *Pennisetum clandestinum*) or ryegrasses (*Lolium* spp.) as substantial pasture grasses in their swards for part of the year, and on all PAST farms, lactating cows were offered grain-based supplemental concentrates in the dairy parlor (mean 7.1 kg ± 2.1 SD). The FA profile of kikuyu is provided in Supplemental Table S1, see Notes. All CONFINE farms offered TMR throughout the lactation period. The PAST farms did not house their cattle at any time during the year. The CONFINE farms included freestall barns (n = 4/15, 26%), compost-bedded pack barns (n = 11/15, 73%), and drylots (n = 2/15, 13%), with some farms using more than one housing system. The mean lactating herd size for PAST farms was 330 (range: 140–760) and was 1,720 (range: 360–9,100) in CONFINE farms. Mean daily milk production of the sampled cows at the sampling date was 26.9 L (±5.4 SD) in PAST farms and 39.2 L (±8.7 SD) in CONFINE farms. Individual farm details of breed, herd size, milk production, and sampling dates are included in the Supplemental Table S2, see Notes.

Cow Enrollment

Two cohorts of cattle were selected from each farm: a precalving dry cohort (50–20 d prepartum, based on expected calving dates) and a peak-milk cohort (40–90 DIM). Exclusion criteria included cattle on "to be culled," "do not breed," or "to be sold" lists; cows with <4 functional teats; or cows with >2 lameness scores at the time of visit (Sprecher et al., 1997). For each cohort, a disproportionate, stratified, random sampling procedure (Stata Statistical Software, Release 16, StataCorp, College Station, TX) was used on lists of eligible cows that were provided by the farm managers. Due to a concurrent study, stratification was performed on parity (dry cohort: nonlactating heifers, parity 1, parity 2, and parity >2; peak-milk cohort: parity 1, parity 2, parity 3, and parity >3). There was one PAST farm that contributed only to the dry cohort and one PAST farm only to the peak-milk cohort, such that there were 14 PAST and 15 CONFINE farms analyzed per cohort.

Sample Size Estimate

A sample size estimate was performed for a 2-sample mean test in a cluster randomized design, with the cluster being farm, an estimated interclass correlation of 0.25, 29 cows sampled per farm (stipulated by a concurrent study), a normalized effect size of housing system for a single lipid of 1 SD, a Bonferroni adjusted α of 2.5×10^{-4} ($\alpha = 0.05$ and 200 independently assessed lipids), 2-sided test, and study power of 80%. The result of this estimation required 12 PAST and CONFINE farms each. The STATA code: power twomeans 0 1, m1(29) rho(0.25) alpha(0.00025).

Diets

The peak-milk cow diets are quantitatively described for each farm in the Supplemental Spreadsheet S4 (see Notes) and summarized by housing system in Table 1. Samples of diet were collected on the day of visit, and wet chemistry analysis was performed. The CONFINE diets are direct results of TMR wet chemistry. The PAST farm diet compositions were estimated from fresh pasture wet chemistry, the stated supplemental feed offered in the dairy parlor, and cow milk production, with the total diet composition estimated in NDS Professional (3.13.3.02b CNCPS 6.56, RUM&N Sas, Reggio Emilia, Italy). While the forage base differed between the CONFINE (maize silage) and PAST farms, the concentrate base was more similar, with wheat being the predominant grain fed. Some CONFINE farms fed supplemental fat sources, including whole cottonseed and protected palm fats. For the dry-cow diet, there was such substantial within-farm variation in feed offered and supplementation strategies according to stage of pregnancy, nulliparous versus multiparous, extensive grazing, and pasture availability and variability to negate the possibility of a quantitative analysis of the diet. There were 3 to 4 dry-cow diets per farm, ranging from carefully formulated TMR diets, often maize silage–based, to transition diets containing anionic feeds, minerals, or zeolite, to extremely extensive grazing on mixed swards, including native grasses, kikuyu, ryegrass, couch (*Cynodon dactylon*), and paspalum (*Paspalum dimidiatum*), and were set stocked or rotationally grazed. Some CONFINE farms used pasture in the far-off diets, and some pasture farms used TMR or partial mixed rations with supplements fed with pasture or in the dairy parlor for "close-up" dry cows. Nonlactating heifers were commonly fed separately from nonlactating cows in the far-off dry period. A qualitative summary of the dry diets is available in the Supplemental Spreadsheet S4.

Sample Collection

Each cohort of cows (dry and peak-milk) was sampled on a single day, between November 17, 2022, and June 15, 2023. Breed information was collected from on-farm assessment and cow-card information.

Two blood samples per cow were sequentially collected from the coccygeal vein: first into a 10-mL serum activator Vacutainer tube ("red-top"), second into a 10-mL lithium heparin Vacutainer tube ("green-top"; Becton Dickinson, Franklin Lakes, NJ). Samples were gently rotated upon collection. Serum samples were allowed to clot at room temperature, in darkness, for 45 to 60 min before centrifugation. Plasma samples were immediately cooled on ice, in darkness, using a polystyrene cooler box. Samples were centrifuged (DM0412; DLAB Scientific Co. Ltd., Beijing, China) at 1,500 × *g* for 15 min at ambient temperature within 1 h of collection with efforts to reduce light exposure. Serum and plasma aliquots of 1 mL were collected and stored temporarily at −18°C (Engel, Carole Park, QLD, Australia) during field travel (<4 d) before long-term storage at −80°C (Isotemp, Thermo Fisher Scientific, Waltham, MA). No reducing or antioxidant agents were added. Serum and plasma samples were transported to Agriculture Victoria, AgriBio (Bundoora, VIC, Australia) on dry ice for laboratory analysis. Hemolysis was visually assessed using a hemolysis palette, and samples greater than or equal to 250 mg/dL hemoglobin were excluded from analysis (n = 39; Kosecki et al., 2021).

Note on Terminology

We write "compounds" when referring to both the metabolite and lipidomic panels and either "metabolites" or

Table 1. Comparison of lactation diets from pasture-based, extensive systems (PAST) and confinement systems that were fed TMR (CONFINE)

Variable[1]	PAST[2]	CONFINE[2]	*t*-statistic[3]	*P*-value
ME, MJ/kg DM	11.20 (0.67)	10.73 (0.42)	2.35	0.026*
DM %	24.97 (8.46)	48.86 (5.76)	−9.15	<0.001***
CP % DM	18.24 (2.60)	16.68 (1.68)	1.98	0.058
Soluble protein % CP	40.94 (7.46)	39.81 (5.51)	0.47	0.639
ADICP % DM	1.10 (0.31)	1.07 (0.33)	0.24	0.812
ADICP % CP	5.98 (1.03)	6.43 (1.80)	−0.81	0.422
NDICP % DM	4.08 (0.96)	2.24 (0.67)	6.05	<0.001***
NDICP % CP	22.37 (4.21)	13.22 (3.28)	6.59	<0.001***
NFC % DM	28.56 (6.37)	36.54 (5.40)	−3.72	<0.001***
ESC (simple sugars) % DM	5.65 (3.21)	6.57 (8.92)	−0.37	0.716
Starch % DM	14.79 (5.15)	20.76 (7.15)	−2.59	0.015*
ADF % DM	20.66 (4.04)	23.14 (2.99)	−1.93	0.064
aNDFOM % DM	40.72 (5.72)	32.09 (4.39)	4.67	<0.001***
Lignin % DM	3.37 (0.84)	6.06 (1.90)	−4.57	<0.001***
Crude fat, EE % DM	4.03 (0.51)	5.32 (1.02)	−4.16	<0.001***
Ash % DM	9.92 (0.88)	7.86 (1.10)	5.47	<0.001***

[1]ADICP = acid-degradable insoluble CP; aNDFOM = amylase NDF OM; EE = ether extract; ESC = ethanol-soluble carbohydrates; NDCIP = neutral detergent insoluble protein.

[2]Mean (SD).

[3]Wald 2-sided *t*-test.

*$P < 0.05$, ***$P < 0.001$.

"lipids" when referring to compounds from each specific panel, respectively.

Standard Metabolite Panel

Serum samples were analyzed for bilirubin, glucose, nonesterified fatty acids, total protein, urea, cholesterol, triglycerides, BHB, albumin, calcium, and magnesium concentrations on a ChemWell 2910 Automated EIA and Chemistry Analyzer (Awareness Technology, Inc., Palm City, FL) using Catachem Inc. (Oxford, CT) reagents, controls, and calibrators as per manufacturer's instructions. Globulin concentration was calculated by subtracting albumin concentration from total protein concentration.

Targeted Lipidomics

Sample Preparation. Plasma samples were used to perform targeted lipidomics. Lipids were extracted utilizing a modified single-phase method developed by Liu et al. (2016). In brief, plasma was thawed in the dark, and 50 µL added to 500 µL of a butanol/methanol/chloroform (3:5:4) solvent. Sonication was not performed, with samples vortexed for 60 s and centrifuged for 15 min at 13,300 × *g* at 4°C. Supernatant was transferred to amber HPLC vials and stored at −80°C.

Quality Control and Injection Order. There were 11 randomly selected cows that contributed 50 µL to a pooled plasma sample from each cohort and processed as above. External standards of known concentration for each lipid class were used for calibration (Supplemental Table S3, see Notes). Injection order was randomized (Microsoft Excel, Microsoft Corp., Redmond, WA), with a pooled quality control and external standards analyzed every 20 samples.

Liquid Chromatography-MS. The lipid extract of plasma was separated by a Kinetex HILIC column (100 × 2.1 mm, 1.7 µm, Phenomenex, CA) on a Vanquish UHPLC system (Thermo Fisher Scientific). The column compartment was maintained at 30°C. Mobile phase A was composed of 10 m*M* ammonium formate and phase B of acetonitrile containing 0.1% formic acid. The gradient elution was performed by a linear increase of mobile phase A from 5% to 25% over 8 min with a flow rate of 0.3 mL/min. The injection volume was 4 µL. A Q Exactive MS instrument (Thermo Fisher Scientific) was operated alternatively between positive or negative modes for simultaneous lipid quantification and a full scan of parent ions (120–1,600 *m*/*z*) at a resolution of 140,000. Lipid species were identified using retention time and accurate mass match and quantified using external calibration. The full list of targeted lipid species with summed structure is reported in Table 2.

Batch Correction and Calibration. External standards were used to correct injection run-order and batch effects. A calibration curve was generated via linear regression between 2 neighboring external standards for each lipid class, with run-order as the independent variable and observed abundance of the lipid standard as the dependent variable. The calibration curve was subsequently adjusted for the known concentration of the external standards. The calibration curve for each lipid class provided adjustment for run-order by the samples' relative proximity between 2 neighboring

Table 2. The complete list of plasma lipid classes and species targeted and quantified in the liquid chromatography-MS analysis

Lipid class	Abbreviation	Species targeted
Phosphatidylcholine	PC	PC(31:1), PC(31:0), PC(30:1), PC(30:0), PC(29:0), PC(28:0), PC(33:1), PC(33:0), PC(32:2), PC(32:1), PC(32:0), PC(34:3), PC(34:2), PC(34:1), PC(35:3), PC(35:2), PC(35:1), PC(37:3), PC(37:2), PC(36:5), PC(36:4), PC(36:3), PC(36:2), PC(36:1), PC(40:7), PC(40:6), PC(40:5), PC(40:4), PC(38:6), PC(38:5), PC(38:4), PC(38:3), PC(38:2)
Lyso-phosphatidylcholine	LPC	LPC(14:0), LPC(16:1), LPC(15:0), LPC(18:3), LPC(18:2), LPC(17:1), LPC(17:0), LPC(16:0), LPC(20:5), LPC(20:4), LPC(20:3), LPC(18:1), LPC(18:0), LPC(22:6), LPC(22:5), LPC(20:0)
Ether-linked phosphatidylcholine	PC-O	PC(O-34:0), PC(O-33:2), PC(O-32:2), PC(O-32:1), PC(O-30:0), PC(O-34:3), PC(O-34:2), PC(O-34:1), PC(O-38:5), PC(O-38:4), PC(O-37:5), PC(O-36:4), PC(O-36:3), PC(O-36:2), PC(O-36:1)
Phosphatidylethanolamine	PE	PE(35:2), PE(35:1), PE(34:3), PE(34:2), PE(34:1), PE(33:1), PE(36:5), PE(36:4), PE(36:3), PE(36:2), PE(36:1), PE(40:6), PE(40:5), PE(38:6), PE(38:5), PE(38:4), PE(38:3), PE(38:1)
Lyso-phosphatidylethanolamine	LPE	LPE(18:3), LPE(18:2), LPE(18:1)
Ether-linked phosphatidylethanolamine	PE-O	PE(O-34:3), PE(O-34:2), PE(O-34:1), PE(O-33:2), PE(O-33:1), PE(O-32:2), PE(O-32:1), PE(O-40:6), PE(O-40:5), PE(O-38:5), PE(O-38:4), PE(O-36:5), PE(O-36:4), PE(O-36:3), PE(O-36:2), PE(O-36:1)
Phosphatidylinositol	PI	PI(33:1), PI(33:0), PI(32:1), PI(32:0), PI(31:0), PI(40:6), PI(40:5), PI(38:6), PI(38:5), PI(38:4), PI(38:3), PI(38:2), PI(37:4), PI(37:3), PI(37:2), PI(36:5), PI(36:4), PI(36:3), PI(36:2), PI(36:1), PI(35:3), PI(35:2), PI(35:1), PI(34:3), PI(34:2), PI(34:1), PI(34:0)
Sphingomyelin	SM	SM(31:1), SM(30:1), SM(28:1), SM(34:4), SM(32:2), SM(32:1), SM(34:2), SM(34:1), SM(33:2), SM(33:1), SM(36:4), SM(36:2), SM(36:1), SM(35:2), SM(35:1), SM(38:2), SM(38:1), SM(37:1), SM(41:3), SM(41:2), SM(41:1), SM(40:3), SM(40:2), SM(40:1), SM(39:2), SM(39:1), SM(44:5), SM(44:4), SM(44:2), SM(43:4), SM(43:3), SM(43:2), SM(43:1), SM(42:3), SM(42:2), SM(42:1)
Triacylglycerol	TG	TG(54:1), TG(54:2), TG(54:3), TG(52:1), TG(52:2), TG(52:3), TG(51:1), TG(51:2), TG(50:1), TG(50:2), TG(50:3), TG(49:0), TG(49:1), TG(49:2), TG(48:0), TG(48:1), TG(48:2), TG(47:0), TG(47:1), TG(46:0), TG(45:0)

standards and batch effect correction due to adjustment for the known concentration of the standards. The effectiveness of the batch correction was assessed through principal component analysis (**PCA**; Supplemental Figures S1 and S2, see Notes).

Data Exclusion

Cows that were initially enrolled and subsequently excluded were due to the following reasons: an uncertain birth date or parity of enrolled cow (n = 4/1,699, 0.24%), DIM <20 or >100 (n = 16/861, 1.86%), calving dates >80 d after dry cohort sampling or no calving event (n = 44/838, 5.25%), or death before calving (n = 2/838, 0.24%). Cows were grouped according to breed within-farm. If breed within-farm groups had ≤5 cows, this group was removed from analysis (n = 78/1,699, 4.59%; Figure 1).

One cow was removed, as >25% of lipids were missing. Missing lipid data were imputed as one-fifth of the lowest detected concentration (n = 9 missing data points). Cows that had any lipid with >10 SD were removed (n = 27/1,516, 1.78%), and cows with a sum of their standardized lipid concentration >4, indicating hemoconcentration or dehydration, were also removed (n = 11/1,516, 0.73%). Serum was unavailable to process in 8 cows (0.53%). The final dataset included 689 dry and 781 peak-milk cows (Figure 1).

Statistical Analysis

The mean blood concentration (molar concentration) and mean mass concentration (e.g., µg/L) for all lipids and metabolites by cohort and housing system are reported in the Supplemental Spreadsheet S1 (see Notes).

PCA. Initial data visualization was performed with unsupervised PCA to explore whether variance in lipid and metabolite profiles were strongly associated with housing system and identify potential outlier samples (Figure 2). No further cows were excluded.

Simple Linear Regression. Simple linear regressions were performed with each compound (lipid or metabolite) as the dependent variable, and housing type (PAST or CONFINE) and parity (1, 2, 3, or >3) as fixed effects. Regressions used untransformed molar concentrations (e.g., µmol/L), and autoscaled (mean-centered and normalized) transformed units. Parity was included to prevent bias introduced by the disproportionate, stratified (on parity) sampling frame that was used. Regression was performed in STATA (regress compound i.farm_type i.parity). The false discovery rate (**FDR**) was controlled to 1% using the Benjamini-Hochberg-Yekutieli method (Benjamini and Yekutieli, 2001; Newson and The ALSPAC Study Team, 2003).

The linear regression coefficients for housing type from the untransformed and autoscaled data are reported in Supplemental Spreadsheet S2 (see Notes). The regres-

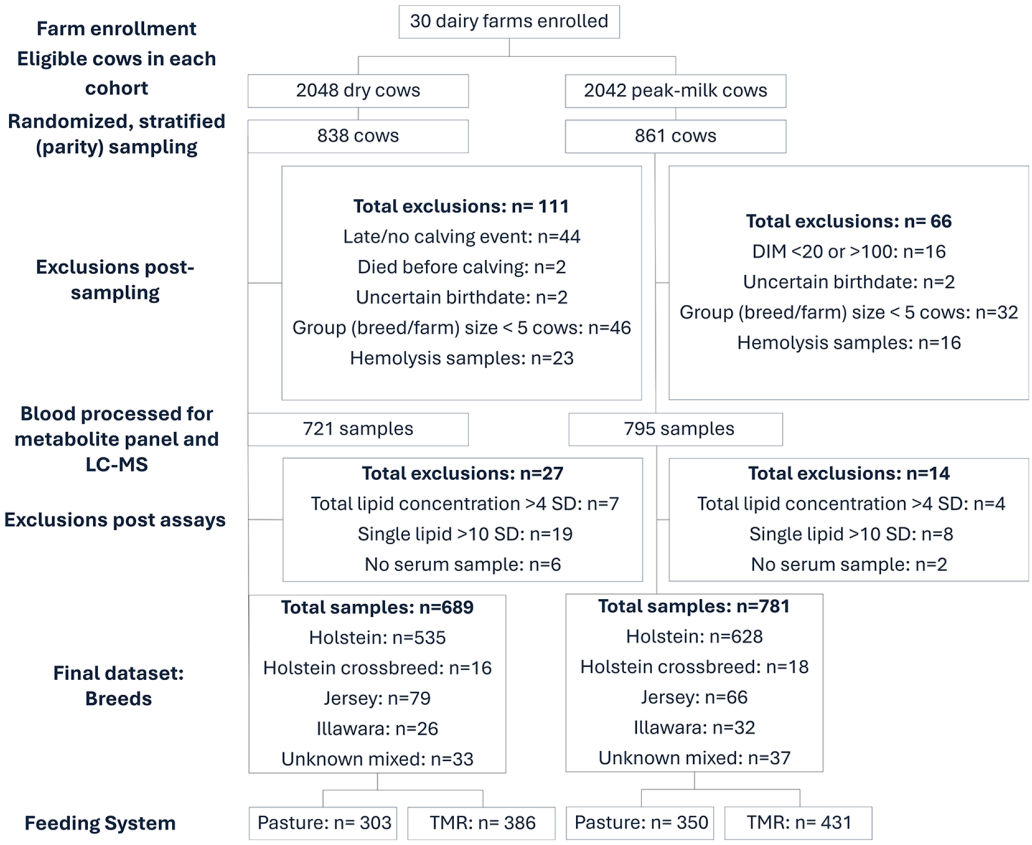

Figure 1. Flow diagram of cow eligibility, enrollment, and subsequent exclusions for the dry and peak-milk cohorts in pasture-based and confinement-based farms. LC-MS = liquid chromatography-MS; CONFINE = confinement-based dairy system, according to lactating herd housing; PAST = pasture-based dairy system, according to lactating herd.

sion results from the autoscaled data are presented as a modified volcano plot, with the measure of effect size (x-axis) being the coefficient associated with housing system (referent: PAST), and the measure of significance (y-axis) being the $\log_{10}$ *P*-value.

Stabilized Variable Selection. The concept of stabilization was used to create a short-list of compounds with strong discriminatory ability for housing system. This involved multiple resampling, with the variables most frequently selected assumed as the most likely to be truly associated with the outcome (Meinshausen and Bühlmann, 2010). For our analysis, the stability selection was performed in 2 steps: first, a variable selection process (e.g., least absolute shrinkage and selection operator [**LASSO**] logistic regression) was bootstrapped to determine the frequency of a specific variable being selected in the final model; second, a model-specific stability-threshold was determined by performing the same variable selection process on a dataset where the outcome variable (housing system) had been randomly reallocated. We refer to these permutated data as "no-information" datasets. Any variable with an inclusion frequency above the stability-threshold was considered stabilized (Lima et al., 2021; Hyde et al., 2022). The no-information dataset maintains the same structure and covariance matrix of the original dataset, but any association with the outcome of interest is removed. Thus, the frequency of variable selection from a no-information dataset provides a plausible cutpoint for false-positive selection and is specific to the dataset and model parameterization.

In this report, the bootstrapping procedure was performed 1,000 times per model, with the no-information datasets permutated 10 times and bootstrapped 100 times per permutation. The stability-threshold was determined by the mean of the highest inclusion frequency of the 10 permutated datasets. In all models, the outcome variable was housing system, and the full model included all lipid and metabolite compounds.

We performed the stabilization variable selection procedure on 5 different models: 3 regularized logistic regression models, a backward variable elimination partial least squares model, and a guided regularized

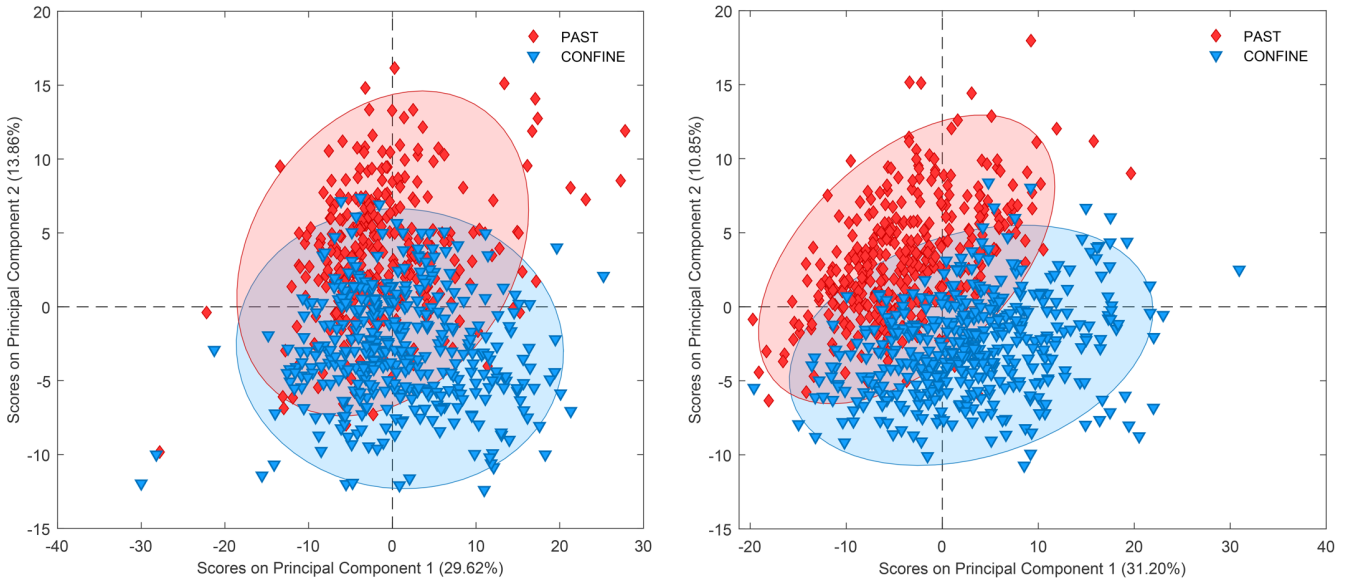

Figure 2. Unsupervised principal component analysis of the dry (left) and peak-milk (right) cohorts. Showing principal component 1 versus principal component 2. In both cohorts, principal component 2 shows reasonable separation between the feeding systems. CONFINE = confinement-based dairy system, according to lactating herd housing; PAST = pasture-based dairy system, according to lactating herd. Shaded ovals are the 95% CI for each respective feeding system.

random forest model. Compounds that stabilized in multiple models were considered most important (Lima et al., 2020).

Regularized Logistic Regression. Logistic models with 3 different regularization penalty methods were conducted: LASSO, smoothly clipped absolute deviation (**SCAD**), and minimax concave penalty (**MCP**). Analysis was performed in R (R Core Team, 2024) using the ncvreg package (Breheny and Huang, 2011). The regularization penalty lambda was determined via 10 k-fold cross validations, and the upper bound for nonzero coefficients was set to 25 compounds.

Backward Variable Elimination Partial Least Squares. Variable selection for an orthogonal partial least squares discriminant analysis (**O-PLS-DA**) model was performed via backward variable elimination. The selectivity ratio, the ratio of the variation attributed to a specific compound and the unexplained variance, was used to iteratively remove variables presented to an O-PLS-DA model. The backward elimination process continued until either all remaining variables were above a selectivity ratio threshold or the number of remaining variables was too few to perform O-PLS-DA. At each elimination step, the classification error was calculated (with a data split of 85 training: 15 test). The procedure was iteratively performed over selectivity ratio thresholds of 0.1 to 1.5 in 0.1 increments. The final model produced the smallest classification error and had ≤25 compounds. In the case of a classification error tie, the model with the least compounds was chosen. The number of partial components was determined by cross-validation (maximum 10 principal components). Analysis was performed in a modification to the R package plsVarSel (Mehmood et al., 2012).

Guided Regularized Random Forest. The final variable selection model was a guided regularized random forest model. First, a full-model random forest procedure was performed to produce variable importance scores that weighted (guided) compounds for the regularized random forest model, using a weighting parameter γ. Greater γ values increase the strength of the regularization penalty. A γ of 0.3 was empirically selected, which limited the number of selected compounds to ~12 (noting that the partial least squares and the logistic models were set to ≤25. This comparatively lower limit was necessitated as the no-information dataset's maximum inclusion frequency was frequently 100% when a lower γ value was used; that is, the threshold for inclusion was 100% with a weaker regularization penalty). Analysis was performed using the R package RRF (Deng, 2013; Deng and Runger, 2013).

Stabilized Variables. Compounds above their respective stability-threshold were graphically presented in a bar chart, showing the summed total inclusion frequency across the 5 stabilized models and the direction of association with housing system. To explore the correlation structure of the stabilized variables, a heat map that included only the stabilized compounds, using pairwise

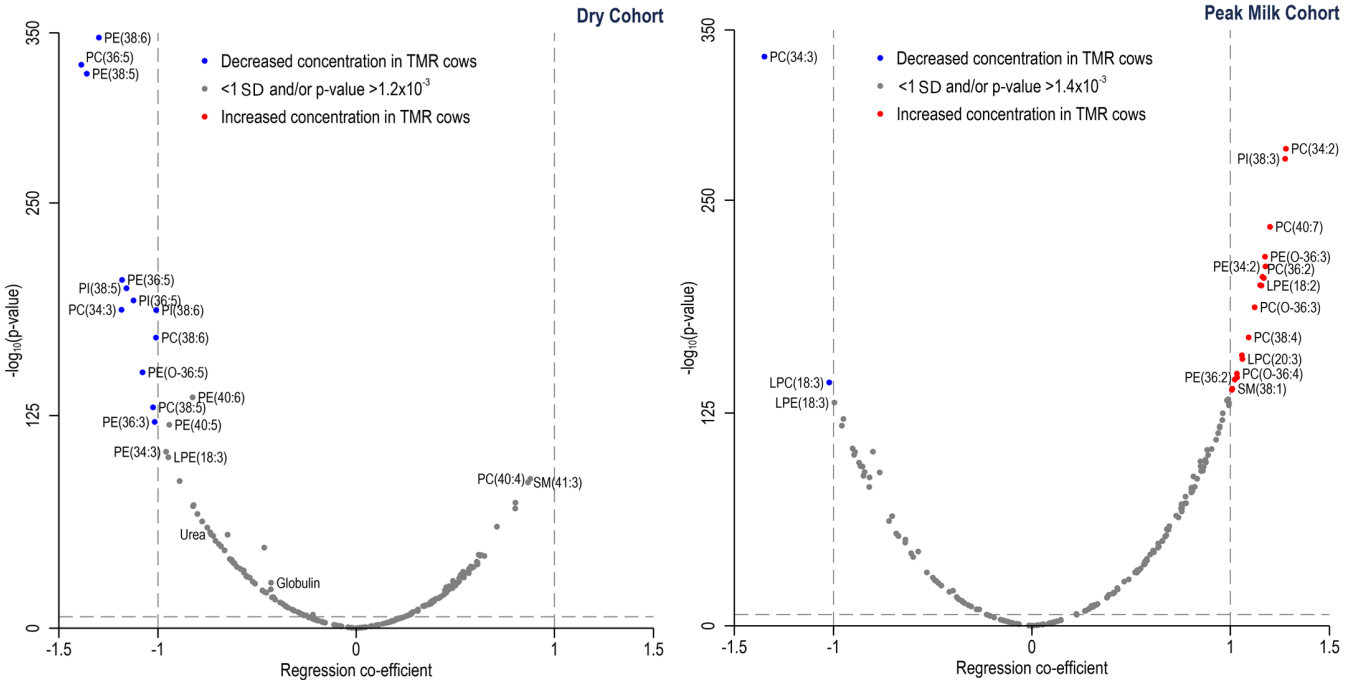

Figure 3. Volcano plots for plasma lipids and metabolites in the dry-cow cohort (left) and peak-milk cohort (right). Simple linear regressions were performed on each compound, with feeding system as a covariate (referent: PAST vs. CONFINE). The regression coefficient measures the strength of association with feeding system. The y-axis is the log-transformed *P*-value corresponding to feeding system. Horizontal dashed lines indicate the 1% FDR-adjusted *P*-value thresholds (dry: $<1.2 \times 10^{-3}$, peak-milk: $<1.4 \times 10^{-3}$). The vertical dashed lines demarcate a regression coefficient with absolute value greater than 1, indicating that feeding system has a larger than 1 SD association with the concentration of the respective blood compound. CONFINE = confinement-based dairy system, according to lactating herd housing; PAST = pasture-based dairy system, according to lactating herd.

Spearman correlations, was created (R package: ComplexHeatmap, Gu et al., 2016; Gu, 2022). To explore the effect size and predictive ability of the stabilized variables, multivariable logistic regression modeling for dry and peak-milk cohorts was performed. The eligibility criteria for inclusion were compounds stabilized in >1 and had <0.6 correlation with another compound (in cases of ≥0.6 correlation, preference was given to the variable with the highest total inclusion rate).

The 2 most stable variables from each cohort, determined by their total inclusion frequency, were visualized using an Epanechnikov kernel density estimation function (Epanechnikov, 1969; STATA: kdensity compound). This function helps to illustrate the distribution and variability of the most important (stable) compounds by housing system and stage of production.

RESULTS

A summary of the lactating cow diets for CONFINE and PAST farms is presented in Table 1. Dry matter content represented the largest statistical difference between the feeding systems (PAST: 24.97% vs. CONFINE: 48.86%, $P < 0.001$). The PAST diet also had significantly lower ($P < 0.001$) NFC, crude fat, and lignin, and higher neutral detergent insoluble CP, amylase NDF OM, and ash than CONFINE farms. The energy density (MJ/kg DM) was slightly higher in the PAST than the CONFINE farms (11.20 vs. 10.73, $P = 0.026$). The vast diversity of the dry cow diets, even within a farm, was an unexpected finding.

The preliminary unsupervised PCA analysis showed reasonable separation between PAST and CONFINE farms (Figure 2). For both cohorts, principal component 2 most visually separated housing systems, suggesting that a supervised classification analysis would likely achieve good discriminative ability.

Linear regression results for each compound, with housing system as the main explanatory variable, and controlling for parity, are presented in the Supplemental Spreadsheet S1 (untransformed molar and mass concentrations) and Figure 3 (autoscaled transformed concentration). The FDR was controlled at 1%, with effective *P*-value threshold of $<1.2 \times 10^{-3}$ and $<1.4 \times 10^{-3}$ for the dry and peak-milk cohorts, respectively.

The volcano plots combine both an FDR-adjusted *P*-value threshold and effect size (±1 SD change associated with farming system) to visualize important compounds

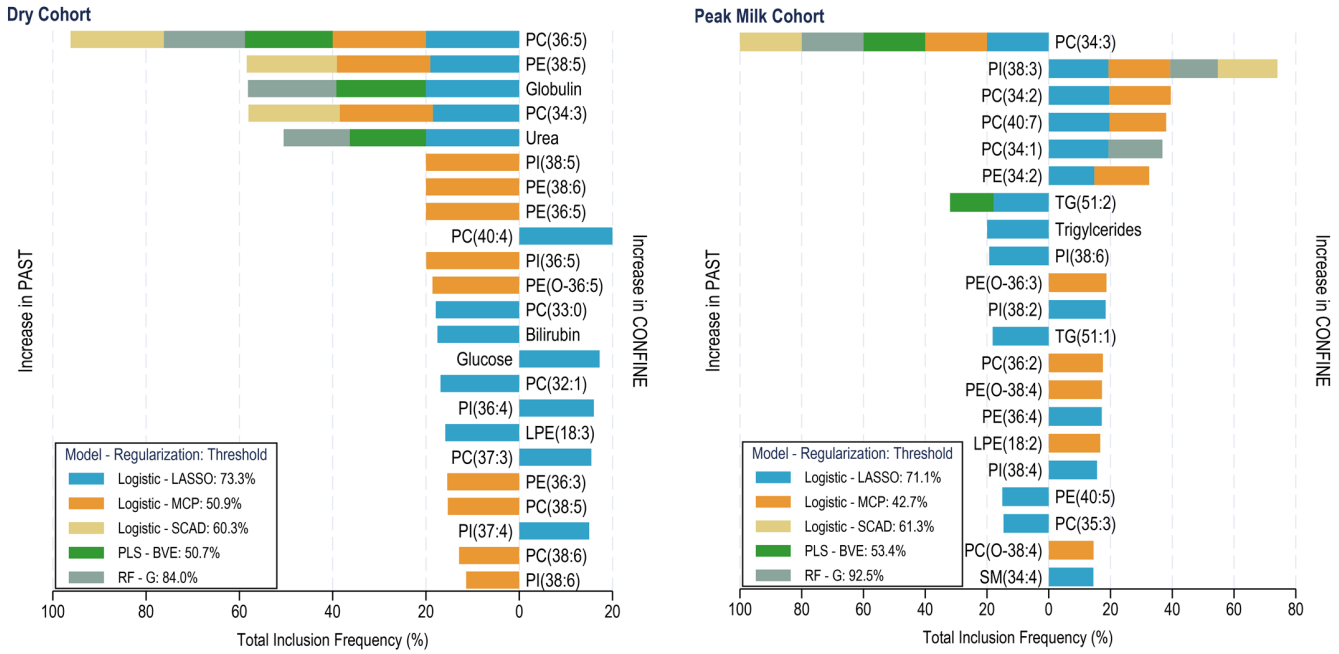

Figure 4. Stabilized compounds selected from both the dry (left) and peak-milk (right) cohorts. A compound was considered stable if its bootstrapped inclusion rate was greater than a stability-threshold inclusion rate determined by bootstrapped permutations of datasets with randomly reallocated feeding system information. Five different models were performed, with the inclusion frequency (x-axis) calculated across all 5 models. Bars extending to the left indicate compounds that were increased in pasture-based, extensive systems (PAST), while extension to the right indicates an increased in confinement, TMR systems (CONFINE). LASSO = least absolute shrinkage and selection operator; MCP = minimax concave penalty; SCAD = smoothly clipped absolute deviation; PLS-BVE = partial least squares-backward variable elimination; RF-G = random forest-guided regularization.

(Figure 3). In the dry cohort, there were no circulating compounds that increased in CONFINE above the combined effect size and significance thresholds. Glycerophospholipids esterified with ALA or its n-3 derivatives were consistently increased in dry, PAST cows. In contrast, PL with LA or its n-6 derivatives were increased in peak-milk CONFINE cows. Only phosphatidylcholine (**PC**; 34:3) and LPC(18:3) were increased above the volcano plot thresholds in PAST, peak-milk cows.

Lipids and metabolites selected with the stabilization selection process are shown in Figure 4. A full list of inclusion frequencies is reported in the Supplemental Spreadsheet S3 (see Notes). In our stabilization selection process, the SCAD, MCP, and random forest models had an apparently higher penalty than partial least squares and LASSO, with the latter 2 methods including more correlated compounds. A total of 23 compounds were identified in the dry cohort, with 5 in more than one model (PC[36:5], phosphatidylethanolamine [**PE**; 38:5], PC[34:3], globulin, and urea), and PC(36:5) was selected in all 5 models. The peak-milk cohort had 21 compounds selected, with 7 being in more than one model (PC[34:3], phosphatidylinositol [**PI**; 38:3], PC[34:2], PC[40:7], PC[34:1], PE[34:2], and TG[51:2]), and PC(34:3) in all 5 models.

The heat map produced from the stabilized blood compounds (Figure 5) shows strong correlations (>0.5) in more than half of the compounds in both the dry and peak-milk cohorts. In the dry cohort, there were 13 correlated compounds with a presumed common link of ALA and its derivatives or metabolism. The remaining compounds do not appear strongly correlated except for 2 pairs of lipids (PI[36:4] with PI[37:4], and PC[37:3] with PC[40:4]). In the peak-milk cohort, there were 13 correlated lipids presumed to be associated with LA and its derivatives, 5 lipids associated with ALA and its derivatives, and a pair of triglycerides (TG[51:2] with TG[51:1]). In both cohorts, there were no stabilized compounds with profoundly strong negative correlations (lowest correlation: dry cohort, −0.42 between PI[36:5] and PC[40:4]; peak-milk cohort, −0.47 between TG[51:2] and PI[38:3]).

Given the strong correlation between many of the selected variables, the final logistic regression models had few variables (Table 3). The dry cohort model included PC(36:5), globulin, and urea, all negatively associated with CONFINE systems. The area under the

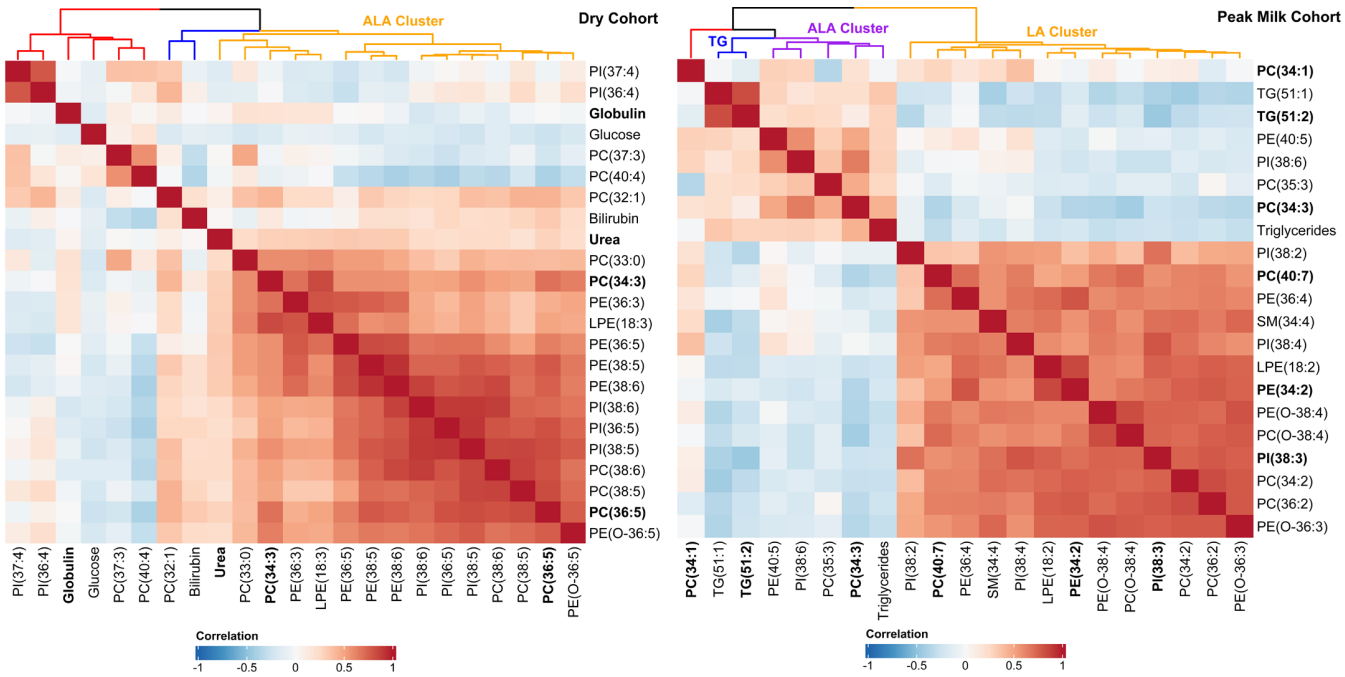

Figure 5. Heat maps of stabilized compounds associated with TMR and pasture-based feeding systems, from the dry-cow (left) and peak-milk (right) cohorts. The dry cohort indicates 13 stabilized compounds correlated with α-linolenic acid (ALA, 18:3n-3) or its derivates, with the remaining compounds not showing strong correlation patterns. The peak-milk cohort's stabilized lipids included 13 with linoleic acid (LA, 18:2n-6) and 5 with ALA. Bolded compounds were stabilized in more than 1 of the 5 models analyzed.

receiver operator curve (**AUC**) for the dry model was 0.955 (95% CI: 0.940, 0.969). The peak-milk cohort model had PC(34:3) and TG(51:2) negatively associated with CONFINE systems, and PI(38:3), PC(40:7), and PC(34:1) positively associated with CONFINE. The AUC was 0.998 (95% CI: 0.997, 1.000). To obtain odds ratios, covariate coefficients must be exponentiated; however, the effect size was so profoundly large for many of the covariates that odds ratios became difficult to interpret (e.g., PC[34:3] has an odds ratio of 8.9×10^{-4} [95% CI: 1.2×10^{-4}, 6.8×10^{-3}]).

The 2 most selected compounds from each cohort are presented in Figure 6. These figures show clear separation between housing system plasma concentrations, with a difference of ~2 SD between peaks for each lipid.

DISCUSSION

This multisite, cross-sectional study demonstrated that cows in different housing systems have substantially different lipid and metabolic profiles. Pasture-based dry cows had higher concentrations of circulating globulin, urea, and PL containing ALA or its n-3 derivatives. At peak milk, CONFINE cows had more PL with LA or its n-6 derivatives and PC(34:1), while peak-milk PAST cows had significantly more ALA and its n-3 derivatives. A comprehensive review of the diverse functions of the omega FA is beyond the scope of this discussion, and we refer to other reviews on the topic (Sordillo, 2016; Moallem, 2018). A broad generalization is that n-3 FA are associated with anti-inflammatory pathways, while the n-6 FA are proinflammatory (Sordillo, 2016). In cattle, the omega FA are importantly associated with reproduction (Silvestre et al., 2011b; Rodney et al., 2015; Sinedino et al., 2017; Moallem et al., 2020), health (Silvestre et al., 2011a; Moallem et al., 2020), and age of dairy cattle (Sheedy et al., 2025a). We speculate that the observed substantial differences in omega FA profiles in circulation between CONFINE and PAST systems may present different health and reproductive risk profiles for cows in each respective system.

This study revealed an intriguing pattern whereby the dry-cow cohort was predominantly differentiated by PL with n-3 PUFA, while the peak-milk cohort was predominantly differentiated by PL with n-6 PUFA (Figures 3 and 5). This pattern is circumstantially supported by experimental work that indicates preferential conservation of PL with long-chain n-3 PUFA over long-chain n-6 PUFA. Cows that were abomasally infused with ALA for 20 d experienced prolonged elevation of plasma PL with EPA (20:5n-3) over the subsequent 20 d period after treatment, compared with cows infused with LA

Table 3. Logistic regression model for feeding system (PAST [Ref] vs. CONFINE) using stabilized compounds[1]

Compound	Coefficient	SE	95% CI	
			Lower	Upper
Dry cohort				
PC(36:5)	−3.50**	0.29	−4.06	−2.94
Globulin	−1.21**	0.16	−1.52	−0.91
Urea	−0.83**	0.14	−1.11	−0.55
Constant	0.28	0.13	0.02	0.54
Peak-milk cohort				
PC(34:3)	−7.02**	1.03	−9.05	−4.99
PI(38:3)	5.35**	0.93	3.53	7.17
PC(40:7)	1.30*	0.46	0.40	2.20
PC(34:1)	2.07**	0.42	1.24	2.90
TG(51:2)	−2.00**	0.42	−2.82	−1.18
Constant	0.79	0.39	0.04	1.55

[1]Eligible compounds were stabilized in >1 of the 5 models and ≤0.6 correlated with another compound. Preference for higher inclusion-rate compound given when correlation >0.6. CONFINE = confinement-based dairy system, according to lactating herd housing; PAST = pasture-based dairy system, according to lactating herd.

*$P < 0.01$, **$P < 0.001$.

that showed no difference in PL with EPA throughout or after treatment (dos Santos Neto et al., 2024). Similarly, Urrutia et al. (2023) reported DHA (22:6n-3) and EPA in plasma PL did not return to baseline in a 7-d washout following a single n-3-enriched abomasal bolus. Stamey et al. (2012) also observed elevated DHA in plasma PL in their 9- to 14-d washout following DHA supplementation. It is unfortunate that we could not readily summarize the dry-cow diets, as within-farm variation was too substantial. However, qualitatively, the dry-cow diets were more similar between housing systems than the peak-milk diets, with CONFINE dry-cow diets containing less or no maize silage in these diets on many farms, and many CONFINE far-off dry cows were held on pasture in extensive stocking situations (Supplemental Spreadsheet S4). Consequently, if we accept that long-chain n-3 PUFA are preferentially conserved over long-chain n-6 PUFA in plasma PL, this effect could explain why the elevated n-6 PUFA observed in peak-milk CONFINE cows (potentially associated with higher maize silage content) was not sustained in the dry period when diets were more similar. In contrast, the deficit of n-3 PUFA in CONFINE cows persisted across both dry and lactating periods as levels of n-3 PUFA were conserved over time. If further studies corroborate these findings, it would indicate that cows in CONFINE have chronically low n-3 PUFA, with the dry-period intakes of dietary n-3 PUFA being insufficient to reach the concentration of n-3 PUFA in PAST cow plasma.

Several PL classes (PC, PE, and PI) were selected in the variable stabilized models for both the dry-cow and peak-milk cohorts, while only 2 TG and 1 SM were selected (Figure 4). In the PAST peak-milk cows, PC(34:3) was the most increased lipid across all 5 models (Figures 3 and 4), with a very large effect size in the final logistic model (odds ratio: 9.0×10^{-4}; 95% CI: 1.2×10^{-4}, 6.8×10^{-3}). The PC(34:3) comprises mostly of palmitic acid (C16:0) and ALA. The PC lipid class is the most abundant phospholipid (including the PL category and SM subclasses) in plasma (63.2% ± 4.1% SD from our data, 63.3%–65.4% calculated from Liu et al., 2025) and is an integral structural component of lipoproteins for transport of FA from the liver (Kleppe et al., 1988; Pullen et al., 1990; Jonas and Phillips, 2008). We note that other PL with the same FA composition, PE(34:3) and PI(34:3), were also significantly increased in PAST peak-milk cows (Supplemental Spreadsheet S2), however, it remains unclear why specifically PC(34:3) had such a profoundly strong effect size without a concurrent increased abundance of other lipids with ALA derivatives. Indeed, PC(36:3), with stearic acid instead of palmitic acid at sn-1, would be expected to be similarly increased in PAST peak-milk cows, however, it was instead significantly increased in CONFINE cows (odds ratio: 1.38; 95% CI: 1.20, 1.59; Supplemental Spreadsheet S2).

In contrast to only one n-3-associated PL being decreased in the peak-milk CONFINE cows, there were many PL with LA or its n-6 derivatives that were increased (Figure 5). The PI(38:3) were the most selected PL increased in peak-milk CONFINE cows, stabilized in 4 out of 5 models. The PI class is less abundant than either PC or PE and is critically involved in many biological processes that include membrane trafficking, signaling pathways, regulation of lipid transport, and precursors for phosphoinositides (Balla, 2013; Dickson and Hille, 2019; Hammond and Burke, 2020). Interestingly, unlike other PL classes that show considerable variation in their composition of FA, the PI class has a relatively homogeneous acyl chain composition within mammals. Cellular PI(38:4) is unusually conserved (>70%) with stearic acid in the sn-1 position and arachidonic acid (C20:4 n-6, ARA) in the sn-2 position, while PI(38:3) is the second most abundant, with mostly dihomo-γ-linolenic acid (**DGLA**; C20:3n-6) in the sn-2 position (Traynor-Kaplan et al., 2017; Barneda et al., 2019; Blunsom and Cockcroft, 2020). In contrast to cellular PI, the concentration of plasma PI(38:4) and PI(38:3) in cows appears to be approximately balanced (6.27 μ*M* ± 1.94 SD and 5.85 μ*M* ± 3.06 SD, respectively; Supplemental Spreadsheet S1). The DGLA in PI(38:3) is notable, as it can either be further desaturated to ARA or actively suppress inflammation through the cyclooxygenase or 15-lipoxygenase pathways to produce prostaglandins or 15-hydroxyeicosatetraenoic acid (also known as 15-HETE), respectively (Mustonen and Nieminen, 2023). Both PI(38:3) and DGLA have been identified as potential biomarkers for

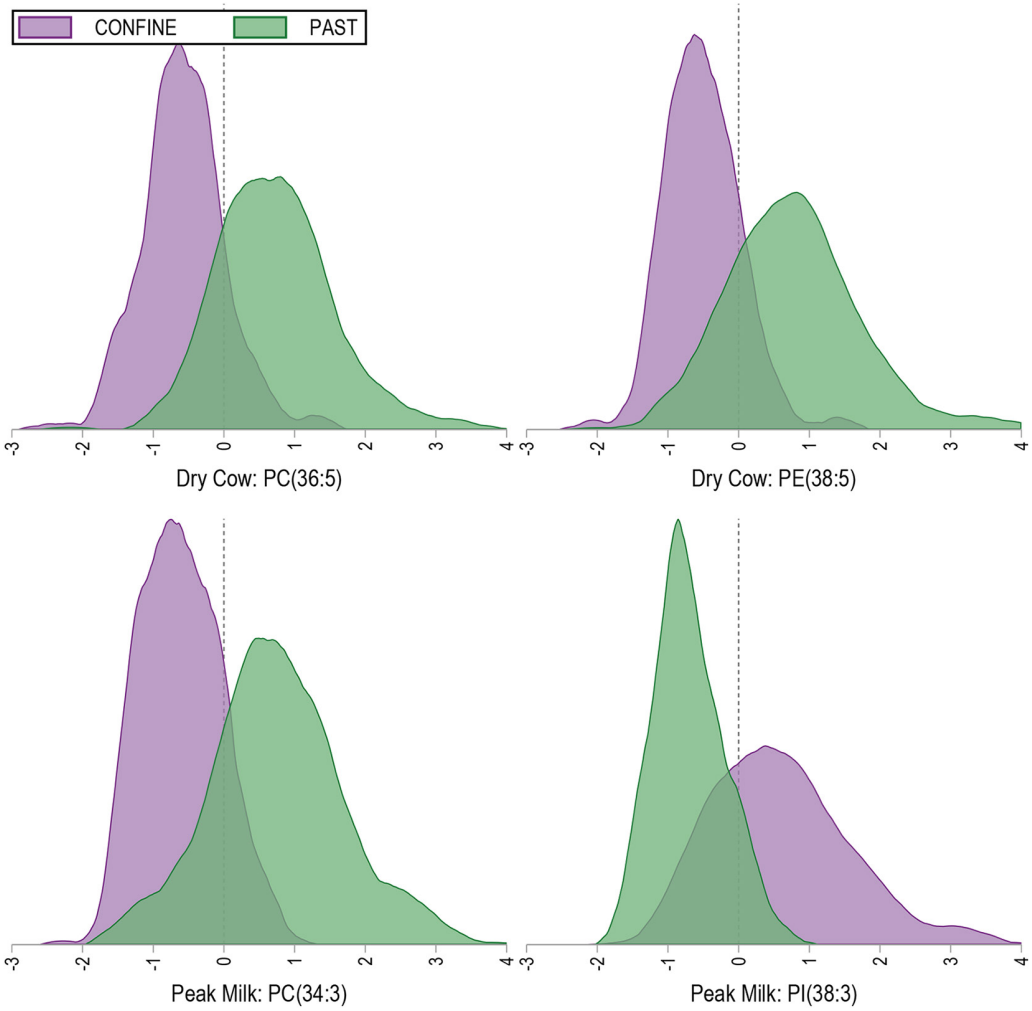

Figure 6. Kernel density estimation functions for the 2 most selected compounds associated with production system, from each cohort. Compounds are standardized with a mean zero and SD of 1. CONFINE = confinement-based dairy system, according to lactating herd housing; PAST = pasture-based dairy system, according to lactating herd. Vertical dashed lines indicate the standardized mean value of zero.

specific diseases, including subclinical ketosis in cows (Sparks et al., 2025) and chronic disease in humans (Mundra et al., 2018; Mustonen and Nieminen, 2023). Bovine red blood cells had increased DGLA content when cows were switched from a mixed grass/maize silage–based diet to a maize silage diet (n = 5 cows, $P < 0.05$; Revskij et al., 2019). We note that PI(38:4) was also increased ($P < 2.4 \times 10^{-3}$) in CONFINE cows, selected in the LASSO model (Figure 4), and highly correlated with PI(38:3; Figure 5). There is little information on plasma PI in cattle, and we are the first to identify significant differences between housing systems. Given the many critical biological functions of PI, the ongoing research interest from human health, and PI(38:3) being the lipid species that most strongly differentiates PAST and CONFINE systems at peak milk, further investigation on the association between PI, health, and reproduction in dairy cattle is justified.

The other PL that increased in CONFINE peak-milk cows that was not correlated with omega FA was PC(34:1), comprising of palmitic acid (C16:0) and most likely oleic acid (C18:1 *cis*-9). The different isomers of C18:1 are of commercial interest due to their different biological activity, health benefits or risks, and availability for consumption in meat and milk (Daley et al., 2010; Ferlay et al., 2017); however, we did not characterize individual FA and cannot distinguish if specific C18:1 isomers were associated with the difference between housing systems. The C18:1 FA can either be provided directly in the diet, result from biohydrogenation of LA and ALA in the rumen, or be desaturated from C18:0 in tissue (Griinari and Bauman, 1999; Ferlay et al., 2017).

Grain from maize is high in both LA and total C18:1 (Khan et al., 2011), and diets with high maize silage content often result in milk and meat higher in total C18:1 (Nielsen et al., 2006; Kalač and Samková, 2010; Łozicki et al., 2012). We speculate that the observed difference in PC(34:1) between housing systems is predominantly from direct C18:1 intake rather than biohydrogenation or desaturation of substrates. Alternatively, oleic acid and palmitic acid are the primary FA that are disproportionately mobilized in the immediate postpartum period during the homeorhetic drive for milk production (Loften et al., 2014). Differences in tissue mobilization between the feeding systems could therefore influence the circulation of PC(34:1). However, the average DIM of sampled cows was after the period of greatest tissue mobilization, and the BCS difference between the dry and peak-milk cohorts was similar across housing systems; ~0.59 and 0.52 BCS lower in peak-milk cows compared with dry cows in PAST and CONFINE farms, respectively (although individual cow BCS change was not monitored, Sheedy et al., 2025b). This indicates that tissue mobilization was unlikely to be the cause for the observed PC(34:1) differences between housing systems.

In the dry period, there were several PL associated with ALA and its derivatives that were low in CONFINE cows, with PC(36:5) the most frequently selected PL (all 5 models) and both PE(38:5) and PC(34:3) selected in 3 models (Figure 4). The FA associated with these PL are expected to predominantly be 16:0/20:5n-3 in PC(36:5), 16:0/22:5n-3 (docosapentaenoic acid) in PE(38:5), and 16:0/ALA n-3 in PC(34:3; Liu et al., 2025). Other bioactive lipid classes, including PI, that were carrying long-chain n-3 PUFA were also selected (Figure 4) and had large effect sizes (Figure 3 and Supplemental Spreadsheet S2). As a whole, this pattern indicates chronic depletion of n-3 PUFA in CONFINE dry cows. Sheedy et al. (2025a) reported that plasma PL with DHA and EPA decreased with increasing age and parity irrespective of farming system. As CONFINE cows have relatively low PL with DHA or EPA, it could be considered that their "lipid biological age" is older than the "lipid biological age" of PAST cows of the same age. This construct requires production of biological-age models (Bafei and Shen, 2023). Parity and age are associated with increased risk of disease and removal from the herd (Lean et al., 2023a,b). Hence CONFINE cows may have lipid profiles associated with higher risk of removal or disease at a younger age than PAST cows.

There is a growing body of evidence that neonatal development and programming is improved with n-3 supplementation in ruminants (Gulliver et al., 2012; Roque-Jiménez et al., 2021). Human neonatal medicine indicates the benefits of n-3 supplementation are increased in those with n-3 deficient diets (Bernardi et al., 2012; Larqué et al., 2012). Accordingly, the intergenerational benefit of n-3 supplementation may be enhanced in CONFINE cows that have chronically low circulating n-3 when compared with PAST cows.

The dry-cow cohort also had lower serum urea and globulin in CONFINE cows. Again, without cow-specific dry-cow dietary information, we are limited to speculation regarding the observed difference. Urea is directly associated with protein intake (Law et al., 2009), with ureagenesis predominantly occurring in the liver to either oxidize surplus AA or to detoxify ammonia from the rumen (Chalupa, 1984; Leng and Nolan, 1984). A higher serum urea concentration in PAST cows may indicate a higher-protein diet. The CP in peak milk diets was moderately higher in the PAST cows (18.24% vs. 16.68%, P-value = 0.058, Table 1) but was not quantified in the dry-cow diets. However, urea was not associated with housing system in the peak-milk cows (5.69 mmol/L PAST vs. 5.62 CONFINE, P-value = 0.549), which could reflect a lack of difference in protein intake between housing systems at peak milk or different clearance rates for urea.

Globulin may also reflect protein intake, catabolism, or immunological responses. Serum globulin increased with increasing parity in late pregnant cows (Cozzi et al., 2011; Ferreira et al., 2021) and in our dry cohort (Supplemental Figure S3, see Notes). Although the disproportionate stratified sampling removed the association between parity and system, an association between system and age (d) remained. This is likely due to the age of first calving being ~6 mo later in our PAST farms. Replacing parity with age (d) in the final logistic regression with housing system decreased the coefficient for globulin by 12.8% to −1.06 (95% CI: −1.39, −0.72), suggesting that some of the association between housing systems and globulin could be confounded by age of cattle. Globulin has been positively associated with heat stress as a consequence of protein catabolism (Cozzi et al., 2011); heat-stress mitigation was limited or absent in our PAST farms. However, it would be speculative to suggest that differences in globulin concentrations were a function of heat stress.

Our analysis used stabilized variable selection to decrease the false-positive rate (Meinshausen and Bühlmann, 2010) at the cost of increasing the false-negative rate. Correlation was very high between many lipid species in our dataset. The penalization algorithms of our models may switch selection between highly correlated lipids, as they will provide similar explanatory power. As such, the overall inclusion frequency of these may decrease. Specifically, the SM were often highly correlated, yet only 1 SM was selected in the stabilized models, despite 27 and 31 SM being significantly associated with housing systems in the dry-cow and peak-milk

cohort linear regression analyses, respectively (Figure 4 and Supplemental Spreadsheet S2). As lipidomics is a burgeoning field that is providing new insights into specific lipid metabolism, we provide the full results from the linear regression and stabilization models in the supplemental documents in case this contains important associations that were not highlighted by our analysis.

CONCLUSIONS

Plasma PL associated with n-3 PUFA were decreased in CONFINE cows compared with PAST cows, while n-6 PUFA were increased in peak-milk CONFINE cows. These results most likely reflect the FA composition of the respective housing system diets, with CONFINE cows consuming more maize silage. As omega FA have been associated with health, reproduction, and aging of cattle, future research should investigate if the diets provided to cattle in CONFINE systems have unintended consequences for longevity of cows. Further research exploring low abundance but biologically active PL, particularly PI, may provide important insights into the mechanisms underlying risk of dairy production.

NOTES

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Nonstandard abbreviations used: ADICP = acid-degradable insoluble CO; ALA = α-linolenic acid; aNDFOM = amylase NDF OM; ARA = arachidonic acid; AUC = area under the receiver operator curve; CONFINE = confinement-based system; DGLA = dihomo-γ-linolenic acid; DHA = docosahexaenoic acid; EE = ether extract; EPA = eicosapentaenoic acid; ESC = ethanol-soluble carbohydrates; FA = fatty acid; FDR = false discovery rate; LA = linoleic acid; LASSO = least absolute shrinkage and selection operator; MCP = minimax concave penalty; NDCIP = neutral detergent insoluble protein; O-PLS-DA = orthogonal partial least squares discriminant analysis; PAST = extensive pasture-based system; PC = phosphatidylcholine; PCA = principal component analysis; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PL = glycerophospholipids; PLS-BVE = partial least squares-backward variable elimination; RF-G = random forest-guided regularization; SCAD = smoothly clipped absolute deviation; SM = sphingomyelins; TG = triacylglycerol.

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CHAPTER 7

A large, multi-site investigation into the lipidomics of survival in dairy cows

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OVERVIEW OF CHAPTER 7

This final chapter explored the association between culling and mortality hazards with lipid and metabolite blood profiles. Our enrolled cows were followed for approximately 700 d with all exit reasons being recorded. Survival analysis was evaluated with a LASSO Cox proportional hazards model with full likelihood, and as also performed in Chapter 6, a variable stabilisation approach was used to help control the false discovery rate. The analysis in this chapter was particularly challenging and is discussed in detail in the *Results and Discussion*. These challenges included; the high-collinearity between lipid species, greater than predicted right censoring of cattle (that is, more cows surviving beyond the follow-up period, reducing study power), survivorship biases, and parity-specific risk. In contrast to Chapters 5 and 6, this chapter was a longitudinal study that ensured the correct temporal association between exposure (metabolite profile) and outcome (removal).

Greater parity was strongly associated with greater hazards of culling and mortality, as was expected. The metabolites assessed from the standard panel were associated with hazards of culling and mortality in alignment with their known biological properties, supporting the analytical approach used. Lipids were generally not associated with the hazards of culling in the dry-cohort, but were robustly associated in the peak-milk cohort. Glycerophospholipids that were associated with very-long chain omega-3 fatty acids, including eicosapentaenoic acid (**EPA**; C20:5n-3) and docosahexaenoic acid (**DHA**; C22:6n-3), reduced the hazards of culling in heifers, while glycerophospholipids associated with the essential omega-3 fatty acid, α-linolenic acid (C18:3n-3) reduced the hazards of culling in parity > 3 cows. Lipids clusters that could be considered pro-inflammatory increased the hazards of culling and mortality, including sphingomyelin with long-chain fatty acids (C > 18), and phosphatidylethanolamine associated with oleic acid. The results identified multiple clusters of lipids associated with cow longevity and these should be a target for future research.

ABSTRACT

Identifying physiological determinants of dairy cow survival, and their potential modulation by parity, may reveal opportunities to improve herd health and longevity. This multi-site, prospective, observational study investigated culling and mortality hazards using targeted lipidomic and standard metabolite assays. Blood samples, stratified by parity, were collected from two cow cohorts (1) dry and (2) peak-milk, from across 29 commercial, Australian farms (14 pasture-based, 15 confinement-based). There were 717 non-lactating, late-pregnant, dry cows (~27 d prepartum) and 794 peak-milk cows (~58 DIM) sampled. A total of 186 lipid species (including glycerophospholipids, sphingomyelin, and triacylglycerols), and 11 routinely measured metabolites were evaluated. Sample cows were followed for an average of 693 d and exit reasons recorded. Competing risk survival models were used to estimate the cumulative incidence of culling and mortality by parity and cohort. Blood analytes were autoscaled within cohort and farm, controlling for farm-level effect on metabolites. Survival analysis was performed using an adaptive LASSO Cox full likelihood model that explored associations among blood analytes and hazards of removal, with a shared frailty of farm (accounting for farm-level baseline hazards), and two removal outcomes considered: culling (censoring: death, cull from farm accident, end of follow-up), and mortality (censoring: cull, death from farm accident, end of follow-up). Separate models were used to estimate survival outcome by cohort, and parity groupings (1st, 2nd and 3rd, and > 3rd), or with parity as a categorical covariate. Due to high correlations, analyte data were reduced to 25 clusters using Ward's hierarchical clustering criterion. Bootstrapping of the LASSO variable selection procedure identified clusters with high selection frequency for use in the final model. The hazards and cumulative incidence of culling and mortality increased with parity. Glycerophospholipids with very long chain n-3 fatty acids were associated with reduced hazards of culling in parity 1 peak-milk cows [0.39 hazards ratio (HR)], while glycerophospholipids with n-3 α-linolenic acid were associated with reduced hazards of culling in parity > 3 peak-milk cows (0.18 HR). Sphingomyelin with > C18 fatty acyl chains were associated with increased the hazards of culling in parity > 3 peak-milk cows (2.02 HR). Clusters containing the routinely evaluated analytes albumin, globulin, urea, magnesium, glucose, triglycerides, β-hydroxybutyric acid, bilirubin and non-esterified fatty acid were associated with culling and mortality, consistent with their roles in health and reproduction. Lipids collected from dry-cows were poor predictors of survival. Many novel plasma lipid

targets for future research into survival of cattle were identified, with pro-inflammatory lipid profiles associated with increased risk of culling and mortality.

Key words

Survival analysis, lipidomics, culling, sphingolipid, omega-3 fatty acids

INTRODUCTION

Longevity of dairy cows is determined by a combination of health, reproduction, production, management practices, and chance events. The most commonly reported reasons for culling are reproductive failure and udder-related disorders (Pinedo et al., 2010; Workie et al., 2021), while mortality is most often attributed to metabolic, calving-associated disorders, mastitis, and on-farm accidents (Stevenson and Lean, 1998; Thomsen and Houe, 2006; Compton et al., 2017). Increased parity is also strongly associated with a greater risk of both culling and mortality events (Stevenson and Lean, 1998; Miller et al., 2008; Pinedo et al., 2010; Lean et al., 2023). Identifying physiological determinants of survival and examining how parity modulates these may reveal opportunities to improve herd longevity and optimize production.

Lipidomics, the large-scale study of lipids, is an appropriate platform to investigate survival given the central role of lipid metabolism in many aspects of dairy production. Examples of the importance of lipid metabolism include the omega fatty acid pathways associations with cattle health (Silvestre et al., 2011; Moallem et al., 2020; Veshkini et al., 2023), reproduction (Rodney et al., 2015; Sinedino et al., 2017; Moallem, 2018; Moallem et al., 2020), inter-generational health (Gulliver et al., 2012; Roque-Jiménez et al., 2021), and aging (Sheedy et al., 2025). The endocannabinoid system, which utilizes phosphatidylethanolamine and phosphatidylinositol precursors, is associated with reproduction, inflammation, and lipolysis (Myers et al., 2021; Zachut et al., 2025). Hepatic glycerophospholipid production and export is also associated with the development of hepatic lipidosis in transition cows (Bobe et al., 2004; McFadden et al., 2020).

Hailemariam et al. (2014) were the first to report use of a metabolomic platform, which included a lipidomic panel, in a longitudinal study to identify biomarkers of disease in 12 transition cows. Subsequent studies used blood metabolomics to predict retained placenta in 22 cows (Zhang et al., 2021), lameness during the transition period with 26 cows (Dervishi et al., 2020), and lipolysis in 16 cows in the transition period (Zhao et al., 2024). These studies demonstrated the potential for metabolomics to identify health-related biomarkers but were limited by study size and hence had limited external validity. These foundational studies focused primarily on disorders within the relatively short transition period, and as such did not consider time-to-event analysis. In contrast, research that investigates survival should also account for the timing of removal. Survival analysis, such as Cox’s proportional hazards, can appropriately incorporate censoring and the timing of a cow’s exit (Kalbfleisch, J.D. and

Prentice, R.L., 2002; Hosmer, D.W. et al., 2008). No studies have utilized a metabolomic or lipidomic platform to investigate time-to-event survival analysis of dairy cows.

The objective of this exploratory, multi-site, prospective, observational study was to investigate the hazards of culling (**HCULL**) and the hazards of mortality (**HMORT**) by utilizing a lipidomic and standard metabolite platform. The null hypothesis was that blood lipids and metabolites are not associated with the HCULL or HMORT. This work has the potential to inform future research and impact nutritional and management strategies aimed at improving cow health, reducing involuntary culling, and optimizing the longevity of dairy herds.

MATERIALS AND METHODS

The study was carried out in accordance with the recommendations of The Australian Code for Care and Use of Animals for Scientific Purposes. Protocols were approved by the Scibus Animal Ethics Committee (Scibus # 1022-1024) and The University of Sydney Animal Ethics Committee (Project # 2022/2247).

Farm enrolment

Farms were purposively selected to obtain an even distribution of 15 extensive, pasture-based farms and 15 TMR, confinement-based farms (**CONFINE**), based upon the management system of the lactating herd. After enrolment, one pasture-based farm relocated and did not contribute to the study. Selection criteria for farms were: a mean lactating herd size of > 200 cows, conventional system (non-organic), and maintained accessible electronic records (including a minimum of reproduction, exit date and reason, birth date, and health treatment details). The pasture-based farms had ryegrasses (*Lolium* spp.), and most had kikuyu (*Cenchrus clandestinus*, previously Pennisetum clandestinum; n = 13/14, 93%) as a substantial pasture for part of the year, and all pasture-based farms offered grain-based supplemental concentrates in the dairy parlour (mean 7.1 kg ± 2.1 SD). The pasture-based farms did not house their cattle at any time during the year. The CONFINE farms included free-stall barns (n = 4/15, 26%), compost-bedded pack barns (n = 11/15, 73%), and dry-lots (n = 2/15, 13%), with some farms using more than one housing system. The mean lactating herd size for pasture-based farms was 330 (range:140-760) and 1,720 (range: 360-9100) in CONFINE farms. Mean daily milk production of the sampled cows at the sampling date was 26.9 L (± 5.4 SD) in pasture-based farms and 39.2 L (± 8.7 SD) in CONFINE farms. Farms were in Australia located between

latitudes 28.6°S and 38.6°S, and longitudes 138°E and 153°E, spanning the states of Victoria, South Australia, and New South Wales, all within temperate climate regions. Individual farm details are provided in Supplementary Table S1. Chemical analysis of peak-milk diets, and details of dry cow feeding are in Supplementary File S1; "Peak-milk cohort diets" worksheet and "Dry cohort diets" worksheet, respectively.

Cow enrolment

Two cohorts of cattle were selected from each farm; a pre-calving dry cohort (selected between 50 and 20 d prepartum, based on expected calving dates) and a peak-milk cohort (selected between 40 and 90 DIM). Exclusion criteria included cattle already on "to be culled", "do not breed", or "to be sold" lists, cows with fewer than 4 functional teats, or cows with > 2 lameness score at the time of blood collection (Sprecher et al., 1997). For each cohort, a disproportionate, stratified, random sampling procedure [Stata Statistical Software (STATA), Release 16, StataCorp, College Station, TX] was used on lists of eligible cows that were provided by the farm managers. Stratification was performed on parity (dry cohort: nonlactating heifers, parity 1, parity 2, and parity > 2; peak-milk cohort: parity 1, parity 2, parity 3, and parity > 3). The parity of dry cows is reported as their upcoming lactation (e.g. a cow that has completed one lactation and will begin her second, is reported as 2nd parity) to provide consistent interpretation across cohorts.

Cow exit recording

Farm managers were provided with standardized cow termination codes that provided sufficient clarity on cow exit reasons. Cows could have up to three reported reasons for exit recorded, and all cows that exited were required to have an exit reason. Farms were regularly contacted to clarify coding and audit anomalous data. The exit-coding scheme is provided in Supplementary Tables S2 and S3.

Sample size estimate

A sample size estimate was performed for a Cox proportional hazards analysis in STATA, with a hazard ratio difference of 1.5 for a simple lipid cluster, a probability of removal within a lactation of 30%, an alpha of 0.05, correlation of 0.7 between lipid clusters, two-sided test, and study power of 80%. The estimated sample size was 531 cows per cohort for 124 cow removals. STATA code: power Cox, hratio(1.5) sd(1) r2(0.7) failprob(0.3) alpha(0.05). A concurrent study design sample size estimation enrolled 29 cows per farm per cohort (heifers

= 9, 2nd parity = 6, 3rd parity = 6, and > 3rd parity = 8), consequently these numbers were deemed sufficient.

Sample collection

Each farm's cow cohorts, dry and peak-milk, were sampled on a single day between November 17, 2022 and June 15, 2023. Two blood samples were sequentially drawn from the coccygeal vein of each cow: first into a 10 mL serum separator tube ("red-top"), followed by a 10 mL lithium heparin tube ("green-top") (Becton Dickinson, Franklin Lakes, NJ, USA). Tubes were gently rotated post-collection. Serum tubes were left to clot at room temperature and protected from light for 45 to 60 min before centrifugation. Plasma tubes were immediately placed on ice in a polystyrene cooler. All samples were centrifuged within 1 h of collection at 1,500 g for 15 min at ambient temperature (DM0412; DLAB Scientific Co. Ltd, Beijing, China), with care taken to minimize light exposure. Serum and plasma were aliquoted into 1 mL volumes and temporarily stored at -18 °C (Engel, Carole Park, QLD, Australia) during field collection (< 4 d), before long-term storage at -80 °C (Isotemp, Thermo Fisher Scientific, Waltham, MA, USA). No antioxidants or reducing agents were added. Samples were later transported to Agriculture Victoria, AgriBio (Bundoora, VIC, Australia) on dry ice for laboratory analysis.

Standard metabolite panel

Serum (1544 samples) was analysed for bilirubin, glucose, non-esterified fatty acids (**NEFA**), urea, cholesterol, triglycerides, beta-hydroxybutyric acid (**BHBA**), albumin, calcium, magnesium, and globulin (calculated by subtracting albumin from total protein) concentrations using a ChemWell 2910 Automated EIA and Chemistry Analyser (Awareness Technology, Inc., Palm City, FL, USA). Catachem Inc. (Oxford, CT, USA) reagents, controls, and calibrators were used as per manufacturer protocols. Quality control checks included repeating the assay on samples yielding null concentrations or values outside the linear ranges for confirmation purposes.

Targeted lipidomics

Sample preparation

Plasma (1552 samples) were subjected to targeted lipidomic analysis. Lipids were extracted utilizing a modified single-phase method developed by Liu et al. (2016). In brief, plasma was thawed in the dark, and 50 µL added to 500 µL of a butanol/methanol/chloroform (3:5:4)

solvent. Samples were vortexed for 60 s, centrifuged at 13,300 g for 15 min at 4 °C, and the supernatant transferred to amber high performance liquid chromatography vials for storage at -80 °C. Sonication was not performed.

Quality control and injection order

A pooled quality control sample was generated from 11 randomly selected cows per cohort (50 μL each) and processed using the same extraction method. Calibration was performed using external standards for each lipid class (Supplementary Table S4). Sample injection order was randomized (Microsoft Excel, Redmond, WA), with pooled quality control and external standards run every 20 samples.

Liquid chromatography – mass spectrometry

Lipid extracts were separated using a Kinetex HILIC column (100 × 2.1 mm, 1.7 μm; Phenomenex, CA) on a Vanquish UHPLC system (Thermo Fisher Scientific), with the column maintained at 30 °C. The mobile phases were 10 mM ammonium formate (A) and acetonitrile + 0.1% formic acid (B). A linear gradient increased phase A from 5% to 25% over 8 min at 0.3 mL/min. Injection volume was 4 μL. Detection was performed using a Q Exactive mass spectrometer (Thermo Fisher Scientific), acquiring full-scan data in both positive and negative ionization modes (120–1600 m/z, resolution 140,000). Lipids were identified based on retention time and accurate mass, and quantified using external calibration. The included lipid classes were phosphatidylcholine (**PC**), lysophosphatidylcholine (LPC); ether-linked phosphatidylcholine [**PC(O)**], phosphatidylethanolamine (**PE**), lysophosphatidylethanolamine, (**LPE**), ether-linked phosphatidylethanolamine [**PE(O)**], phosphatidylinositol (**PI**); sphingomyelin (**SM**), and triacylglycerol (**TG**). A full list of the 186 lipid species and their summed structures by lipid class is provided in the Supplementary Table S5, or by hierarchical clusters in Table 1 (refer to *Statistical analysis* below).

Run-order and batch correction

External standards were used to correct both injection sequence and batch variation. A calibration curve was generated by performing simple linear regression between neighbouring external standards, with run-order as the explanatory variable, and adjusted by the known concentration of the standards. The curve adjusted the sampled lipid concentration based on their relative proximity (run-order) between their two neighbouring external standards, for each lipid class. The efficacy of these corrections was evaluated by principal component analysis (Supplementary Figures S1 and S2). The mean blood concentration of lipids and metabolites are reported in Supplementary File S2.

Data exclusion

After enrolment, cows were excluded from the final analysis for the following reasons: an uncertain birth date or parity of enrolled cow (n = 2/1658, 0.12%), DIM < 20 or > 100 (n = 16/843, 1.90%), calving dates > 80 d after dry cohort sampling or no calving event (n = 44/815, 5.4%), and cows lost to follow-up (n = 4/1658, 0.24%) (Figure 1). Haemolysis was visually scored against a reference palette (Kosecki et al., 2021), and any samples with haemoglobin concentrations ≥ 250 mg/dL were excluded (n = 39/1658, 2.35%). One cow was removed as > 25% of lipid results were missing. Lipid data were imputed at 1/5 of the lowest detected concentration (n = 9 missing data points). Cows that had any lipid with > 10 SD were removed (n = 27/1552, 1.74%) and cows with a sum of their standardised lipid concentration > 4, indicating haemoconcentration or dehydration were also removed (n = 11/1552, 0.71%). Serum was unavailable to be processed in 8 cows (n = 81/1552, 0.48%). The final dataset included 717 dry cows and 794 peak-milk cows (Figure 1).

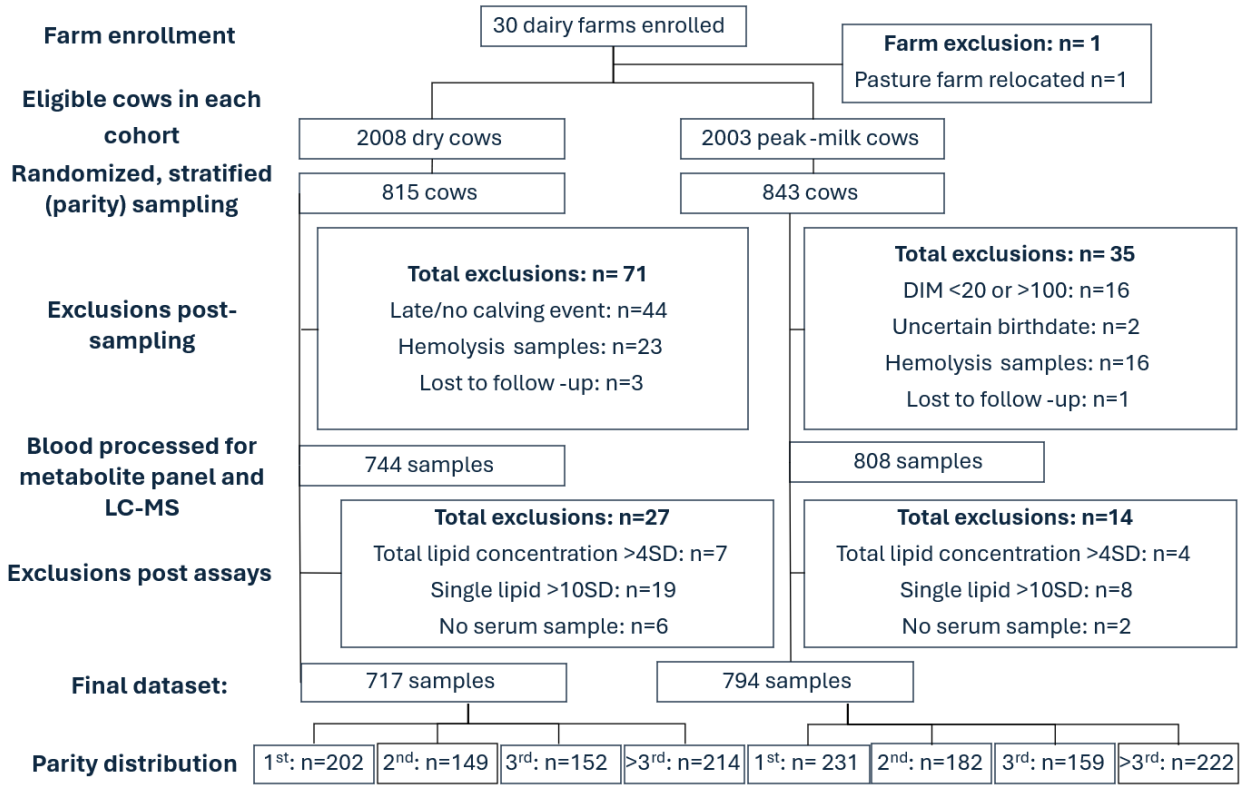

Figure 1: Flow diagram of cow eligibility, enrolment and subsequent exclusions for the dry and peak-milk cohorts.
LC-MS = liquid chromatography-mass spectrometry

Statistical analysis

Survival analysis

Identification of lipids and metabolites associated with HCULL and HMORT followed an analytical pipeline involving data cleaning and transformation, dimension reduction (hierarchical clustering), stabilization of variable selection (frequency of variable selection under bootstrapping), final model selection, and reporting. Each step of the pipeline is addressed individually below and is depicted in Figure 2.

Cox frailty model based on full likelihood

A Cox proportional hazards frailty model based on full likelihood, with farm as a shared frailty, was used to investigate HCULL and HMORT. A culling event was defined as being sold by the producer, excluding sold events related to an accident or if sold to another dairy (that is, cows that would remain milk-producing). A mortality event was defined as any death on farm, including euthanasia but excluded death due to an accident. Accidents were excluded under the assumption of no causal association between bovine physiology and farm accidents. Observation time began at sample collection, was measured in days, and ended either at an event date or censoring date. Right-censoring occurred at the above-described exclusions, mortality date in the culling models, culling date in the mortality models and the end of the follow-up period.

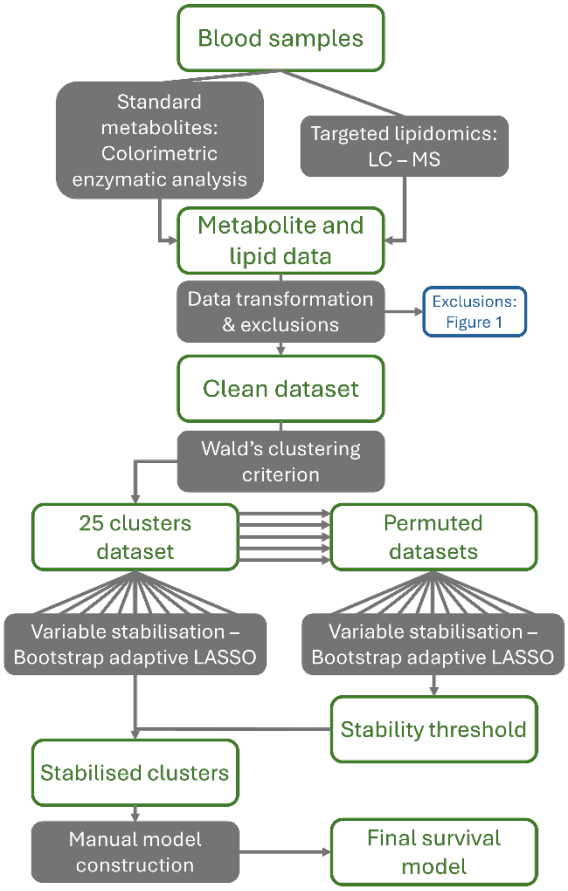

Figure 2: Statistical analysis workflow diagram. Analysis of blood samples produced a dataset of 185 lipids and 11 metabolites. Data was autoscaled and exclusions are reported in Figure 1. High correlations among lipids led to use of Ward's clustering criterion to reduce the dimensions to 25 Clusters. The Clusters robustly associated with survival were selected by calculating their inclusion frequency after bootstrapping of an adaptive LASSO Cox model. The threshold for stability was determined using permuted data. Stabilized Clusters could be interpreted univariably, or in a final, manually constructed multivariable survival model.
LASSO = least absolute shrinkage and selection operator; LC – MS = Liquid chromatography – mass spectrometry

Each farm had a different follow-up period determined by sampling date and final data collection date, with a mean follow-up period of 693 d (SD 84).

The benefits of a full likelihood Cox model compared to a partial likelihood model, which is the standard Cox model in statistical software, include that the baseline hazard function is directly modelled, it performs well in small sized studies where loss of precision in partial likelihood Cox models can be substantial, and it is adaptable to a wide class of frailty distributions (Ren and Zhou, 2011; Hohberg and Groll, 2024). The Cox models were performed in the R package "PenCoxFrail" (Groll, 2024; Hohberg and Groll, 2024; R Core Team, 2024). We utilized the adaptive least absolute shrinkage and selection operator (LASSO) regularization and variable selection feature of this package as appropriate (see *Stabilized variable selection and adaptive LASSO Cox model* below).

The conditional hazard model was of the form:

$$\lambda\left(t\middle|\mathbf{x}_{ij},\mathbf{b}_i\right)=\exp\left(\gamma_0\left(t\right)+\mathbf{x}_{ij}^{\mathrm{T}}\boldsymbol{\beta}+\mathbf{b}_i\right),\qquad i=1,\ldots,n,\qquad j=1,\ldots,N_i,$$

Where $\lambda(t|\mathbf{x}_{ij},\mathbf{b}_i)$ denotes the hazard function for *j*th cow in *i*th farm at time *t* (d), conditional on observed fixed-effect covariates $\mathbf{x}_{ij}$, farm-level random effects (frailty) $\mathbf{b}_i$, *n* is the total farm count and N_i is the total cow count of the *i*th farm. The vector $\boldsymbol{\beta}$ contains the coefficient for the fixed effects. The random effects follow a normal distribution, $\mathbf{b}_i \sim N(0,\theta)$, with variance θ estimating the heterogeneity across farms. The log-baseline hazard function $\gamma_0(t)$ was estimated using B-splines (Hohberg and Groll, 2024).

Dry and peak-milk cohorts were analysed separately throughout. Separate models were fit at the following three parity strata: 1st parity, 2nd and 3rd parity, and > 3rd parity. We refer to these models as parity-stratified; these are not to be confused with "stratified survival models" that estimate different base-line hazards for each strata but use the same set of coefficients (Therneau and Grambsch, 2000). An "all-parity" model that included parity as a categorical covariate was also estimated. These models were also performed univariably for each lipid or metabolite as an independent variable, with parity as a categorical variable as appropriate, and the coefficients are reported in Supplementary File S3, but are not directly presented as results.

Cumulative incidence

The cumulative incidence of culling and mortality, stratified by parity, are graphically presented in competitive risk models. Cumulative incidence will be biased upward if competing risks are not accounted for (Fine and Gray, 1999). Competing risks were any cow exits that

were not culling or mortality events, as specifically defined above, in their respective models. Right-censoring occurred at the end of the follow-up period. Performed in STATA package "stcompet" (Coviello, 2003). The reported culling and mortality reasons for each cohort are included in the Supplementary Tables S6 to S9.

Data cleaning and transformation

Data exclusions and cleaning were reported earlier. Further exploration of the data included principal component analysis, with no further cows excluded. To control the systemic effect of farm and housing system (pasture-based vs. CONFINE) on cow removal, lipid and metabolite data were grouped by farm within cohort (dry or peak-milk) and autoscaled (mean 0, standard deviation 1). Autoscaling allows the comparison of lipids and metabolites based on correlations and not the absolute blood concentration, weighing all blood analytes equally (van den Berg et al., 2006). Data cleaning and transformation was performed in STATA and PLS Toolbox (Ver 9.3.1, Eigenvector Research Inc, Manson, WA). Further control over the farm-level baseline hazards of removal was achieved by incorporating farm as a shared frailty in the Cox model (see *Stabilized variable selection and adaptive LASSO Cox model* below).

Dimension reduction

As many lipid species were very highly correlated (Supplemental Figure S3), presenting all analytes to an adaptive LASSO algorithm caused substantial instability in variable selection. We approached this issue by grouping positively correlated lipids and metabolites to reduce the dimension of our data prior to variable selection. Justification for our chosen approach is provided in the *Results and Discussion* section. The dry and peak-milk cohorts were combined before a hierarchical cluster analysis reduced the variable count from 197 individual blood analytes to 25 clusters. Cluster analysis was performed using the "hclust" command in R (R Core Team, 2024). In brief, the pair-wise Pearson's correlations between all lipids and metabolites were used to create a dissimilarity matrix for Ward's clustering criterion to be applied (method = "ward.D2") (Ward and Hook, 1963; Murtagh and Legendre, 2014). Each compound was assigned to its own cluster, and the cluster analysis algorithm proceeded iteratively to join the two most similar clusters until only one cluster remained. The final number of clusters was empirically decided at a count that controlled the level of correlation between clusters while keeping the number of analytes within a cluster low enough to maintain the biological interpretability of individual clusters. The lipids and metabolites that comprise each cluster are reported in Table 1. The cow-level cluster value was the mean of the respective lipids and metabolites within a cluster. The dimension-reduced dataset was used in all

subsequent analyses, with each variable referred to as a "Cluster". Figure 3 shows the heatmap of the correlation between Clusters, produced in R using the "pheatmap" package (Kolde, 2019; R Core Team, 2024), in addition to the heatmap of the original dataset in the Supplementary Figure S3.

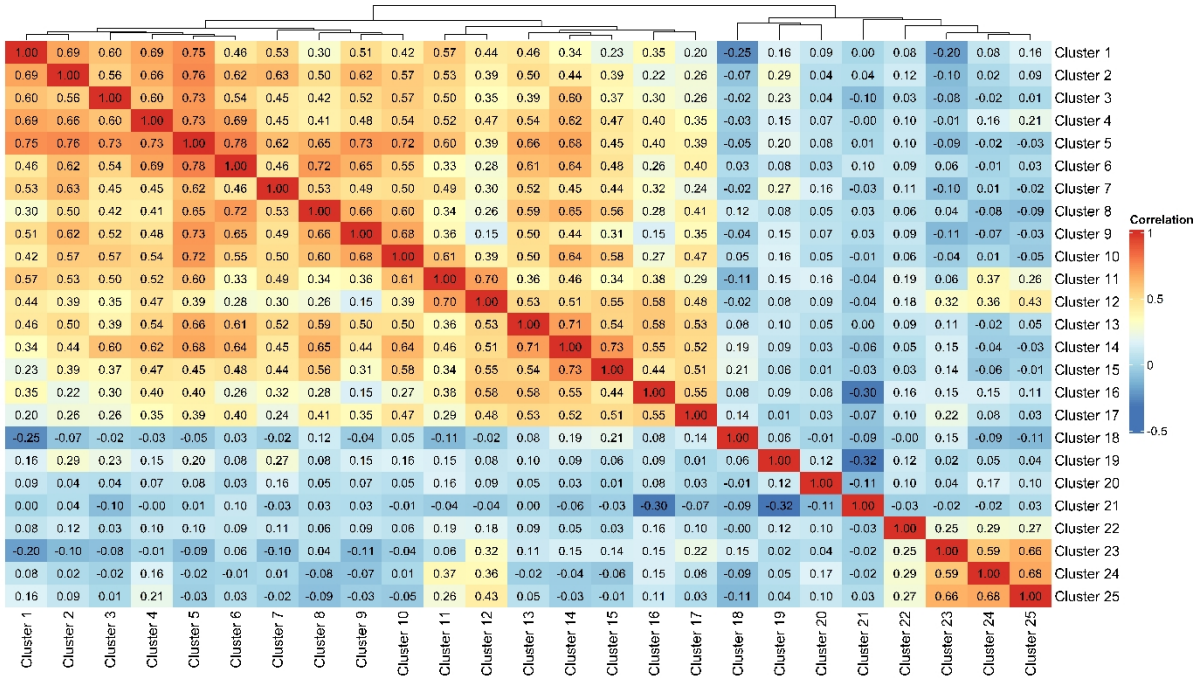

Figure 3: Heatmap showing the Pearson correlations of the 25 metabolite clusters following a hierarchical cluster analysis of 185 lipids and 11 metabolites. Clustering was performed to reduce the dimensionality in our highly correlated dataset, while maintaining the interpretability of the clusters.

Stabilized variable selection and adaptive LASSO Cox model

The concept of variable stabilization was used to generate a list of Clusters that were robustly associated with HCULL and HMORT. Stabilization involves performing multiple resampling of a variable selection process to calculate the frequency of a variable being selected, with the more frequently selected variables assumed to be more robustly associated with the outcome than variables selected infrequently (Meinshausen and Bühlmann, 2010). In our analysis, stability selection of a Cluster was performed in two steps: first an adaptive LASSO Cox frailty model was bootstrapped to determine frequency of Cluster selection, then a model-specific stability-threshold was determined by performing the same variable selection procedure on a dataset where the outcome (event and time-to-event) had been randomly re-allocated. We refer to these permuted datasets as "no-information" datasets as they maintain

the same structure and covariance matrix of the original dataset, but any association with the outcome of interest is removed. The maximum inclusion frequency of a variable in the no-information datasets provides a plausible cut-point for false-positive selection and determines the stability-threshold. A Cluster above the stability-threshold was considered stabilized (Lima et al., 2021; Hyde et al., 2022).

In our analysis, bootstrapping was performed 250 times per model specified, with the no-information datasets permuted 10 times, and bootstrapped 50 times per permutation. The LASSO regularization parameter was determined by 10-fold cross-validation. The stability-threshold was determined by the mean of the highest inclusion frequency of the 10 permuted datasets.

Right-censoring was prevalent in our data, with a mean of 70.1% in the all-parity culling models, and 92.1% in the all-parity mortality models. Subsequently, permutation of the full dataset did not remove associations with the hazards of removal, as random re-allocation of outcomes was primarily between cattle that had no events (right-censored cows). To overcome this, permutation was performed on balanced event data: all cows with model-specific events and an equal number of randomly selected right-censored cows were included. As this reduced the cow count of the permuted datasets, it was not possible to determine stability-thresholds for HMORT in dry 1st parity cows, and peak-milk 1st parity and 2nd, 3rd parity cows.

Final model building

Utilizing only Clusters that were above the stability-threshold for each respective HCULL and HMORT model, final survival models were constructed. Interaction and quadratic transformations were explored. Clusters required a *P*-value > 0.1 to remain in the model. As right-censoring was high, the final models were bootstrapped (n = 1,000) to improve the stability of estimates with changes to the data. The bootstrapped hazards ratio (HR) and confidence intervals are reported. Model discriminatory power was assessed using Harrell's concordance index (C-index), calculated from the fixed effects of the final models (Harrell et al., 1982). The C-index is a generalization of the area under the receiver operating characteristic curve for censored survival data. It measures the agreement between predicted and observed event orderings: a pair is concordant if a cow predicted to be removed earlier than another cow was observed to be removed earlier. The C-index is the proportion of concordant pairs among all comparable pairs (i.e., those cows where ordering was possible, such as between two events, or an event and a later censoring). A value of 0.5 indicates no better event ordering than chance while a value of 1.0 indicates perfect order prediction. The proportional hazards assumption

was formally tested on each Cluster using Schoenfeld residuals (STATA code: estat phtest, rank detail).

RESULTS & DISCUSSION

This study is the first to evaluate survival using a multi-herd lipidomic and metabolite dataset in dairy cattle. The primary goal of our analysis was largely exploratory, as most of our plasma lipids had not been investigated in relation to survival of dairy cows. Although we were able to identify Clusters with robust associations with survival, these Clusters were not consistent across parity groups. This finding should stimulate further scientific enquiry into survival and the effects of parity and age in dairy cattle.

Cumulative incidence

The cumulative incidence of culling and mortality under competing risks are presented in Figure 4. Increasing parity increased the cumulative incidence of both culling and mortality events by 400 d since sampling, regardless of dry or peak-milk cohorts. By 400 d since sampling, the cumulative incidence of culling in parity 1 was 7.3% and 9.7% in the dry and peak-milk cohorts, respectively. For parity > 3, the cumulative incidence of culling was 26.0% and 22.4% in the dry and peak-milk cohorts, respectively. The cumulative incidence of mortality by 400 d since sampling of parity 1 cows was 4.3% and 2.4% in the dry and peak-milk cohorts, respectively. For parity > 3, it was 10.1% and 12.1% of cows in the dry and peak-milk cohorts, respectively. The incidence of mortality was greater soon after sampling in the dry cohort compared to the peak-milk cohort, indicating relatively high mortality in the transition period. The most frequently reported culling reasons were udder related problems (40.5% dry cohort, 31.0% peak-milk cohort), infertility (32.1%, 36.3%), and low production (14.4%, 17.7%). The most frequently reported mortality reasons were acute mastitis (24.3% dry cohort, 21.2% peak-milk cohort), accidents (16.2%, 18.2%), and unknown (9.5%, 15.2%; Supplementary Tables S6 to S9).

Table 1: Composition of clusters used in the survival analysis. Clusters were determined by the Ward's error sum of squares hierarchical clustering. Lipids show their summed compositions.

Cluster	Simple interpretation	Lipids and metabolites
Cluster 1	PC and LPC with odd-chain FA	LPC(15:0), LPC(17:1), LPC(17:0), LPC(18:0), PC(31:1), PC(31:0), PC(33:1), PC(33:0), PC(35:3), PC(35:2), PC(35:1), PC(37:3), PC(37:2)
Cluster 2	Mixed PL with 18:3n-3	LPC(20:0), PC(40:4), PC(38:3), PC(38:2), PE(38:3), PE(38:1), PI(38:3), PI(37:3)
Cluster 3	PC and SM with SFA and monounsaturated FA	LPC(14:0), SM(30:1), SM(28:1), PC(30:0), PC(29:0), PC(28:0), PC(32:2)
Cluster 4	PI with SFA and monounsaturated FA	PI(33:1), PI(33:0), PI(32:0), PI(31:0), PI(38:2), PI(37:2), PI(36:1), PI(35:1), PI(34:0)
Cluster 5	SM with ≤ 18C, PC and PI with 18:3 and 18:2	Cholesterol, SM(31:1), SM(34:4), SM(32:2), SM(32:1), SM(34:2), SM(33:2), SM(33:1), SM(36:2), SM(35:2), SM(35:1), PC(36:3), PC(36:2), PI(36:3), PI(36:2), PI(35:3), PI(35:2)
Cluster 6	SM with > 18C FA	SM(34:1), SM(36:1), SM(38:2), SM(38:1), SM(37:1), SM(41:3), SM(41:2), SM(41:1), SM(40:3), SM(40:2), SM(40:1), SM(39:2), SM(39:1), SM(44:4), SM(44:2), SM(43:4), SM(43:3), SM(43:2), SM(43:1), SM(42:3), SM(42:2), SM(42:1), PC(32:0)
Cluster 7	LPC with mixed FA	LPC(16:1), LPC(18:3), LPC(18:2), LPC(16:0), LPC(20:5), LPC(20:4), LPC(20:3), LPC(18:1), LPC(22:6)
Cluster 8	SM and PC with n-6 FA, and PC(O) with mono- and unsaturated FA	SM(36:4), SM(44:5), PC(34:4), PC(34:2), PC(O-34:0), PC(O-34:1), PC(O-36:1)
Cluster 9	PC(O) with PUFA	PC(O-33:2), PC(O-32:2), PC(O-32:1), PC(O-30:0), PC(O-34:3), PC(O-34:2), PC(O-38:5), PC(O-38:4), PC(O-37:5), PC(O-36:4), PC(O-36:3), PC(O-36:2)
Cluster 10	PE(O) with PUFA	PE(O-34:3), PE(O-34:2), PE(O-33:2), PE(O-32:2), PE(O-36:4), PE(O-36:3), PE(O-36:2)
Cluster 11	PE and LPE with18:3n-3 and 18:2n-6	LPE(18:3), LPE(18:2), PE(35:2), PE(34:3), PE(34:2), PE(36:3), PE(36:2)
Cluster 12	PE with 18:1 oleic acid	PE(35:1), PE(34:1), PE(33:1), PE(36:4), PE(36:1), PE(38:4)
Cluster 13	PC and PI with long chain n-6	PC(36:4), PC(40:7), PC(38:4), PI(38:4), PI(37:4)
Cluster 14	PC and PI with mostly mono-saturated FA	PC(30:1), PC(32:1), PC(34:3), PI(32:1), PI(36:4), PI(34:3), PI(34:2), PI(34:1)
Cluster 15	Mixed PL with mono-unsaturated FA	PC(34:1), PC(36:1), LPE(18:1), PE(O-34:1), PE(O-33:1), PE(O-32:1)
Cluster 16	Mixed PL with very long-chain n-3	LPC(22:5), PC(36:5), PC(40:6), PC(40:5), PC(38:6), PC(38:5), PE(36:5), PE(40:6), PE(40:5), PE(38:6), PE(38:5), PE(O-36:5), PI(40:6), PI(40:5), PI(38:6), PI(38:5), PI(36:5)
Cluster 17	PE(O) with very long-chain n-3	PE(O-40:6), PE(O-40:5), PE(O-38:5), PE(O-38:4), PE(O-36:1)
Cluster 18	Energy and lipid associated metabolites	BHBA, Bilirubin, NEFA
Cluster 19	Mixed metabolites	Albumin, Urea, Magnesium
Cluster 20	Calcium	Calcium
Cluster 21	Globulin	Globulin
Cluster 22	Energy and lipid associated metabolites	Glucose, Triglycerides
Cluster 23	TG with medium-chain FA	TG(52:2), TG(52:3), TG(50:1), TG(50:2), TG(50:3), TG(48:1), TG(48:2)
Cluster 24	TG with long-chain FA	TG(54:1), TG(54:2), TG(54:3), TG(52:1)
Cluster 25	TG with short-chain and SFA	TG(51:1), TG(51:2), TG(49:0), TG(49:1), TG(49:2), TG(48:0), TG(47:0), TG(47:1), TG(46:0), TG(45:0)

BHBA = beta-hydroxybutyric acid; FA = fatty acyls; LPC = lysophosphatidylcholine; LPE = lysophosphatidylethanolamine; NEFA = non-esterified fatty acids; PC = phosphatidylcholine; PC(O) = ether-linked phosphatidylcholine; PE = phosphatidylethanolamine; PE(O) = ether-linked phosphatidylethanolamine; PI = phosphatidylinositol; PL = glycerophospholipids; SFA = saturated fatty acyls; SM = sphingomyelin; TG = triacylglycerol

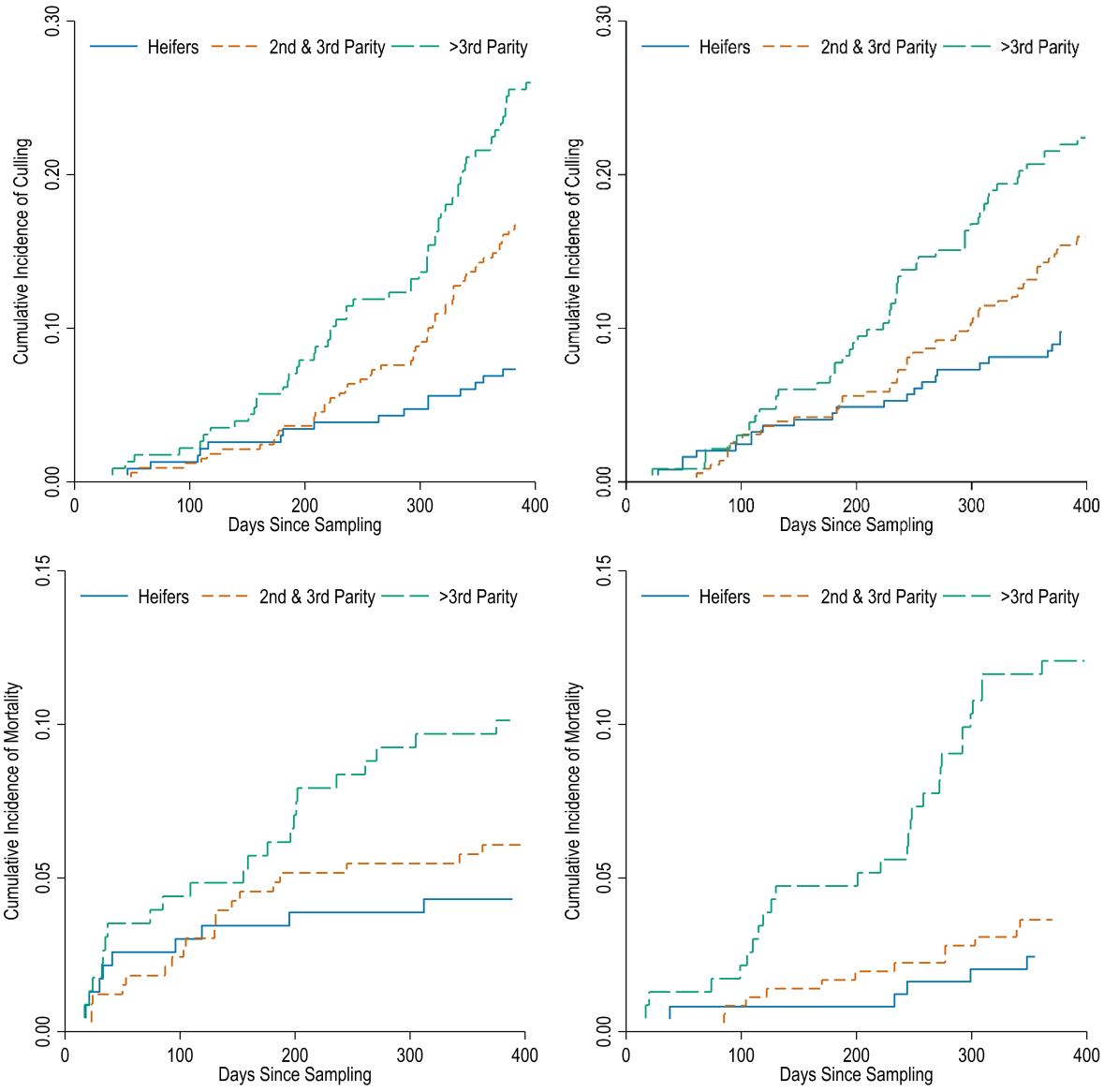

Figure 4: The cumulative incidence of culling in the dry cohort (top left) and peak-milk cohort (top right) and the cumulative incidence of mortality in the dry cohort (bottom left) and lactating cohort (bottom right), stratified by parity groupings. The time scale is days since blood sampling. Analysis assumed the competing events of mortality, culling and exits due to farm accidents as appropriate.

Dimension reduction & stabilization

The dimension reduction using Ward's clustering criterion produced 25 Clusters for analysis. A simple interpretation of the Clusters' lipid and metabolite composition is provided in Table 1 and is referred to throughout the *Results and Discussion*. Liu et al. (2025) was used to aid in the interpretation of the probable fatty acid composition of our lipids, as we analysed the summed fatty acid compositions.

Results from the stabilization procedure for variable selection are presented in Table 2 for culling events, and Table 3 for mortality events. Clusters above the stability-threshold were available for use in the final conditional hazards models. The HCULL models identified fewer stabilized Clusters in the dry-cohort than the peak-milk cohort (Table 2). Though the parity 2nd and 3rd stratified peak-milk HMORT model had no stability-threshold determined, Cluster 19 was empirically considered stabilized for final model inclusion due to its relatively high inclusion frequency (62.9%; Table 3).

Dry cohort survival analysis

Increasing parity increased HCULL ($P < 0.001$), with parity > 3 cows having 4.09 (95%CI: 2.30, 7.38) greater hazards than parity 1 cows (Table 4) and supported the cumulative incidence results (Figure 4). The dry-cohort HMORT model indicated a numerical but non-significant increase in hazards with increasing parity (Table 5). As hazards are a measure of time-to-event, the relatively high mortality of parity 1 cows in the first 50 d after sampling (Figure 4) may explain the non-significant parity results in the HMORT model, despite cumulative incidence of mortality being clearly different between parity groups by 400 d after sampling (Figure 4). The dry-cohort model with the greatest concordance, a non-parametric measure of model fit, was the HMORT parity > 3 model at 71.6% (Table 5).

To limit the scope of our discussion, we discuss only a subset of the Clusters that had relatively large effect sizes, and that could be more readily interpreted based on existing biological knowledge. Although individual Clusters are discussed, we acknowledge that multivariable models are conditional on all included variables.

Table 2: Frequency of a Cluster being selected after 250 bootstrap iterations of an adaptive LASSO Cox proportional hazards model for sold cows (HCULL). The threshold frequency was determined by permuting the dataset to remove any association with outcome and performing the same analysis. There were 10 permuted datasets per model, with 50 bootstraps performed on each. The mean of the maximum inclusion frequency of the permuted datasets represented a logical threshold for false positive inclusion.

	Dry Cohort (Parity)				Peak-milk cohort (Parity)			
Cluster	All	1st	2nd, 3rd	> 3rd	All	1st	2nd, 3rd	> 3rd
Cluster 1	42.8	**20.2***	28.8	35.2	35.8	14.8	31.2	**47.6***
Cluster 2	34.0	5.3	32.0	4.8	**68.7***	**41.4***	10.8	**92.4***
Cluster 3	18.4	9.7	12.4	2.8	39.8	36.9	50.4	**44.0***
Cluster 4	25.2	**29.1***	**53.6***	14.4	56.9	12.3	**70.0***	22.8
Cluster 5	21.6	17.8	20.4	6.0	32.9	25.4	22.0	20.0
Cluster 6	18.4	10.9	10.4	6.8	**76.8***	16.0	43.2	**36.8***
Cluster 7	23.2	7.3	14.4	15.2	32.9	22.1	16.4	19.6
Cluster 8	17.2	**23.1***	13.2	18.4	**83.3***	18.4	12.0	**79.2***
Cluster 9	40.8	11.3	48.4	8.8	43.9	31.6	13.6	18.4
Cluster 10	**53.2***	17.0	45.6	6.8	30.9	12.7	10.4	22.4
Cluster 11	44.4	8.5	38.8	11.2	**82.9***	**46.7***	24.0	19.2
Cluster 12	20.0	8.5	17.6	7.2	**90.7***	**50.0***	24.4	**44.8***
Cluster 13	23.6	14.6	20.8	4.4	**61.0***	16.4	34.4	15.6
Cluster 14	26.0	8.9	14.4	8.0	**61.4***	17.6	11.6	**44.0***
Cluster 15	44.8	9.3	44.8	14.0	45.5	38.9	10.8	26.0
Cluster 16	36.0	**27.1***	15.6	12.0	**90.2***	**73.8***	16.8	18.4
Cluster 17	16.8	10.1	10.8	9.6	48.4	**63.5***	15.6	8.4
Cluster 18	14.0	6.5	10.4	6.4	23.2	20.1	16.0	**38.0***
Cluster 19	22.4	**23.5***	12.8	7.6	44.7	9.8	10.4	13.2
Cluster 20	38.8	9.7	6.8	22.4	26.4	24.2	14.0	22.0
Cluster 21	**71.2***	12.1	22.8	**40.0***	30.5	11.5	31.2	10.4
Cluster 22	**54.8***	11.3	**64.8***	1.6	37.8	13.9	10.8	14.8
Cluster 23	30.8	9.7	12.0	16.0	42.3	13.1	15.6	16.8
Cluster 24	36.4	**21.1***	16.4	7.6	30.1	13.1	30.8	**38.4***
Cluster 25	20.0	18.6	20.8	10.0	35.8	20.9	13.2	24.0
Threshold	51.6	19.1	48.8	38.4	59.6	41.3	53.2	35.2

Clusters above the threshold are indicated by bolded text and *.
Cluster composition and simple interpretation provided in Table 1.

Table 3: Frequency of a Cluster being selected after 250 bootstrap iterations of an adaptive LASSO Cox proportional hazards model for cows with mortality events (HMORT). The threshold frequency was determined by permuting the dataset to remove any association with outcome and performing the same analysis. There were 10 permuted datasets per model, with 50 bootstraps performed on each. The mean of the maximum inclusion frequency of the permuted datasets represented a logical threshold for false positive inclusion.

	Dry cohort (Parity)				Peak-milk cohort (Parity)			
Cluster	All	1st	2nd, 3rd	> 3rd	All	1st	2nd, 3rd	> 3rd
Cluster 1	**46.5***	1.7	36.0	19.2	40.3	17.7	44.5	14.8
Cluster 2	30.5	5.2	15.6	21.6	38.1	10.4	27.3	**26.8***
Cluster 3	40.3	0.0	24.0	16.4	**45.3***	32.9	28.6	14.0
Cluster 4	**65.4***	0.0	24.8	**38.8***	**54.7***	21.2	15.1	**36.0***
Cluster 5	37.0	0.0	22.8	15.2	**70.4***	16.9	22.4	**48.8***
Cluster 6	38.3	0.0	16.8	12.4	**46.7***	15.6	13.5	**30.4***
Cluster 7	35.4	0.0	9.6	9.6	34.4	9.1	29.4	16.4
Cluster 8	31.3	10.3	27.6	10.8	37.4	9.5	31.8	15.2
Cluster 9	29.6	0.0	30.4	7.6	**43.4***	13.9	16.3	19.2
Cluster 10	39.9	0.0	11.6	13.2	41.4	23.4	12.7	8.0
Cluster 11	35.4	0.0	20.0	21.6	**43.2***	9.5	23.3	10.8
Cluster 12	39.5	8.6	14.4	14.4	39.7	37.2	29.0	16.8
Cluster 13	25.1	0.0	16.4	11.6	36.8	15.2	24.5	19.2
Cluster 14	34.2	5.2	23.6	10.8	**50.4***	24.2	13.1	**25.2***
Cluster 15	30.5	5.2	24.0	14.0	36.2	16.5	36.3	11.6
Cluster 16	32.1	0.0	12.4	18.8	**44.9***	28.1	31.0	17.2
Cluster 17	27.2	5.2	15.6	11.6	34.4	11.3	13.9	12.8
Cluster 18	**62.1***	0.0	**47.6***	14.8	**46.1***	15.2	32.2	**48.0***
Cluster 19	37.4	0.0	14.0	12.8	**78.2***	7.4	**62.9**	17.2
Cluster 20	32.5	1.7	9.2	8.8	**49.0***	5.2	10.2	**32.8***
Cluster 21	**85.6***	3.4	**44.0***	14.8	**43.4***	9.1	15.5	**23.2***
Cluster 22	**46.9***	1.7	21.6	22.0	39.7	11.3	35.1	**51.2***
Cluster 23	**42.8***	5.2	13.2	**39.2***	**49.4***	28.1	20.8	12.4
Cluster 24	33.3	3.4	11.2	8.8	43.0*	13.0	16.7	17.6
Cluster 25	32.5	0.0	16.0	14.4	37.9	29.4	31.8	20.0
Threshold	41.6	-	40.0	27.9	42.3	-	-	21.3

Clusters above the threshold are indicated by bolded text and *.
Blank stability-thresholds occurred when there were too few events for model conversion in the permuted data. We considered Cluster 19 stable in peak-milk cohort 2nd and 3rd parity, despite no estimated threshold.
Cluster composition and simple interpretation provided in Table 1.

The association between lipid-containing Clusters and survival of dry-cohort cows was generally low. Of the eight dry-cohort models only three included lipid-containing Clusters. These were: Cluster 9, containing PUFA PC plasmalogens, in the 2nd and 3rd parity HCULL model (0.75 HR; 95%CI: 0.59, 0.98; Table 4); Cluster 1, containing odd-chain fatty acid PC and LPC, in the all-parity HMORT model (0.62 HR; 95%CI: 0.47, 0.89; Table 5); and Cluster 4, containing mono-saturated and saturated PI, in the parity > 3 HMORT model (0.49 HR; 95%CI: 0.32, 0.78; Table 5).

In contrast, Clusters that contained commonly evaluated metabolites were often significant in the dry-cohort HCULL and HMORT models (Tables 4 and 5). Cluster 19, containing albumin, urea, magnesium, significantly reduced the HCULL for parity 1 (0.66 HR; 95%CI: 0.40, 1.03; Table 4) and Cluster 21, containing only globulin, had a quadratic relation with HCULL in the parity > 3 and all-parity models, and increased the HMORT in the all-parity model (1.37 HR; 95%CI: 1.00, 1.87; Table 5). Ruprechter et al. (2018) reported that low blood albumin at 2 wk before calving increased the odds of metritis and retained placenta and Cattaneo et al. (2021) found that high blood albumin:globulin at dry-off protected against culling. However, albumin:globulin was also strongly associated with parity and stratification by parity was not evaluated in that study. Regardless of analytical differences, the direction of the effect from these studies was consistent with our finding that higher albumin reduced hazards (Ruprechter et al., 2018; Cattaneo et al., 2021).

Cluster 22, containing glucose and triglycerides, significantly increased HCULL at 2nd and 3rd parity (1.45 HR; 95%CI: 1.06, 1.95) and in the all-parity model (1.21 HR; 95%CI: 1.02, 1.40), and Cluster 18, containing BHBA, bilirubin and NEFA, significantly increased HCULL in the 2nd and 3rd parity model (1.89 HR; 95%CI: 0.87, 3.29). Both Clusters (18 and 22) contained metabolites associated with energy and lipid metabolism, and increased hazards of removal. Greater NEFA and BHBA concentrations in the dry period indicate mobilization of adipose tissue, and greater post-partum concentrations are associated with poor health and increased culling (Lean et al., 1994; Ospina et al., 2010; Seifi et al., 2011). Associations for these metabolites measured in the dry period and health outcomes are less reported (Chapinal et al., 2011; Jackson et al., 2011), though greater glucose concentrations in dry cows was associated with peri-parturient disease (Bicalho et al., 2017).

Table 4: Hazards of being sold (HCULL) estimated from Cox full likelihood survival models with frailty (farm) in the dry cohort. The effect of parity was controlled through covariate inclusion (All Parity model) or by model stratification. Models were manually constructed using Clusters above the stability threshold of an adaptive LASSO stabilization procedure.

Model	Covariate	HR[1]	95% CI[2]	Sign test[3]	*P*-value	Concord[4]	Event count
All Parity	Cluster 22	1.21	1.02, 1.40	0.026	0.017	62.4%	211/717 (29.4%)
	Cluster 21 (Q)	1.13	1.02, 1.22	0.014	0.007		
	Parity 1	Ref.					
	Parity 2,3	2.98	1.75, 5.17	0.000	< 0.001		
	Parity > 3	4.09	2.30, 7.38	0.000	< 0.001		
Parity 1	Cluster 19	0.66	0.40, 1.03	0.074	0.094	61.7%	32/202 (15.8%)
Parity 2,3	Cluster 9	0.75	0.59, 0.98	0.032	0.022	59.7%	99/301 (32.9%)
	Cluster 22[5]	1.45	1.06, 1.95	0.032	0.015		
Parity > 3	Cluster 21 (Q)	1.26	1.11, 1.45	0.000	0.001	52.8%	80/214 (37.4%)

Bootstrapping (n = 1000) performed. Clusters with *P*-value < 0.1 retained. Interactions were explored. (Q) indicates a quadratic association. Cluster composition and simple interpretation provided in Table 1.

[1]Hazards ratio.

[2]Bootstrap 95% confidence interval.

[3]Non-parametric sign test indicates the proportion of bootstrap estimates above or below zero.

[4]Concord = Harrell's concordance index (C-index), a non-parametric test of model accuracy.

[5]Cluster 22 failed the assumption of proportional hazards based on Schoenfeld residuals in this model (*P* = 0.018). Parametric (Weibull) regression coefficient was similar: HR = 1.49 (*P* = 0.004).

Table 5: Hazards of a mortality event (HMORT) estimated from Cox full likelihood survival models with frailty (farm) in the dry cohort. The effect of parity was controlled through covariate inclusion (All Parity model) or by model stratification. Models were manually constructed using Clusters above the stability threshold of an adaptive LASSO stabilization procedure.

Model	Covariate	HR[1]	95% CI[2]	Sign test[3]	*P*-value	Concord[4]	Event count
All Parity	Cluster 1	0.62	0.47, 0.89	0.004	0.004	68.5%	63/717 (8.8%)
	Cluster 21	1.37	1.00, 1.87	0.052	0.054		
	Parity 1	Ref.					
	Parity 2,3	1.43	0.57, 3.72	0.450	0.461		
	Parity > 3	2.43	0.89, 7.53	0.088	0.098		
Parity 2,3	Cluster 18	1.89	0.87, 3.29	0.116	0.084	61.1%	23/301 (7.6%)
Parity > 3	Cluster 4	0.49	0.32, 0.78	0.000	0.001	71.6%	30/214 (14.0%)
	Cluster 23	1.53	1.08, 2.29	0.020	0.026		

Bootstrapping (n = 1000) performed. Clusters with P-value < 0.1 retained. Interactions explored. Stratification by parity 1 cows did not identify any significant Clusters; event count 10/202 (4.95%). Cluster composition and simple interpretation provided in Table 1.

[1]Hazards ratio.

[2]Bootstrap 95% confidence interval.

[3]Non-parametric sign test indicates the proportion of bootstrap estimates above or below zero.

[4]Concord = Harrell's concordance index (C-index), a non-parametric test of model accuracy.

Commonly evaluated metabolites reflect selection for use based on years of clinical research; their association with survival was unsurprising, but their presence and direction of effect in our models supports the robustness of our analytical approach.

Peak-milk cohort survival analysis

The HCULL increased with increased parity in the peak-milk cows; parity > 3 cows had 2.95 greater hazards of culling than parity 1 cows (95%CI: 1.84, 5.42; Table 6). The peak-milk HMORT model indicated that parity > 3 had greater HMORT compared to parity 1 cows (4.97 HR; 95%CI: 2.72, 11.13; Table 7). The peak-milk model with the greatest concordance was the HCULL parity > 3 model, with 71.8%.

In contrast to the dry-cow cohort, there were many lipid-containing Clusters associated with the hazards of removal in the peak-milk cohort (Tables 6 and 7). Cluster 16 contained mixed glycerophospholipid classes (PC, PE, PI) attached to very-long chain PUFA, most likely the n-3 fatty acids docosahexaenoic acid (C22:6n-3, DHA) and eicosapentaenoic acid (C20:5n-3, EPA) (Liu et al., 2025). Cluster 16 reduced HCULL in the all-parity (0.67 HR; 95%CI: 0.67, 1.00) and parity 1 peak-milk models (0.39 HR; 95%CI 0.23, 0.65; Table 6). The n-3 fatty acids are positively associated with cattle health (Silvestre et al., 2011; Moallem et al., 2020; Veshkini et al., 2023), and reproduction (Rodney et al., 2015; Sinedino et al., 2017; Moallem, 2018; Moallem et al., 2020. Unexpectedly, Cluster 16 was not associated with HCULL in the 2nd and 3rd parity, and parity > 3 stratified models (Table 2 and Table 6), considering that EPA and DHA associated PC, PE, and PI were the lipids that had the largest relative decrease in concentration with increasing age (Sheedy et al., 2025). There is a potential influence of survivorship bias for this result, discussed later in *Study Challenges and limitations*.

Table 6: Hazards of being sold (HCULL) estimated from Cox full likelihood survival models with frailty (farm) in the peak-milk cohort. The effect of parity was controlled through covariate inclusion (All Parity model) or by model stratification. Models were manually constructed using Clusters above the stability threshold of an adaptive LASSO stabilization procedure.

Model	Covariate	HR[1]	95% CI[2]	Sign test[3]	*P*-value	Concord[4]	Event count
All Parity	Cluster 2	0.69	0.52, 0.95	0.022	0.019	64.3%	241/794 (30.4%)
	Cluster 6	1.23	0.96, 1.55	0.098	0.091		
	Cluster 8	1.49	1.16, 1.96	0.000	0.003		
	Cluster 11	0.72	0.56, 0.91	0.008	0.008		
	Cluster 12	1.73	1.31, 2.33	0.000	< 0.001		
	Cluster 13	1.36	1.01, 1.85	0.040	0.051		
	Cluster 14	0.64	0.48, 0.86	0.002	0.003		
	Cluster 16	0.67	0.44, 1.00	0.056	0.052		
	Cluster 11#16	1.42	1.13, 1.76	0.010	0.002		
	Cluster 13#12	0.72	0.56, 0.89	0.002	0.005		
	Parity 1	Ref.					
	Parity 2,3	2.13	1.31, 3.81	0.002	0.006		
	Parity > 3	2.95	1.84, 5.42	0.000	< 0.001		
Parity 1	Cluster 16	0.39	0.23, 0.65	0.002	< 0.001	68.2%	46/231 (19.9%)
	Cluster 17	2.01	1.46, 2.91	0.000	< 0.001		
Parity 2,3	Cluster 4	0.69	0.52, 0.93	0.006	0.010	57.5%	114/341 (33.4%)
Parity > 3	Cluster 1	2.03	1.12, 3.99	0.024	0.033	71.8%	81/222 (36.5%)
	Cluster 2	0.18	0.09, 0.31	0.000	< 0.001		
	Cluster 6	2.02	1.12, 3.81	0.020	0.023		
	Cluster 8	2.63	1.52, 4.89	0.000	0.001		
	Cluster 12	2.53	1.51, 4.93	0.000	0.002		
	Cluster 14	0.34	0.16, 0.63	0.000	0.002		
	Cluster 18	1.83	1.13, 3.08	0.010	0.018		
	Cluster 24	0.61	0.38, 0.94	0.026	0.034		

Bootstrapping (n = 1000) performed. Clusters with *P*-value < 0.1 retained. Significant interactions included, indicated by #. Cluster composition and simple interpretation provided in Table 1.
[1]Hazards ratio.
[2]Bootstrap 95% confidence interval.
[3]Non-parametric sign test indicates the proportion of bootstrap estimates above or below zero.
[4]Concord = Harrell's concordance index (C-index), a non-parametric test of model accuracy.

Table 7: Hazards of a mortality event (HMORT) estimated from Cox full likelihood survival models with frailty (farm) in the peak-milk cohort. The effect of parity was controlled through covariate inclusion (All Parity model) or by model stratification. Models were manually constructed using Clusters above the stability threshold of an adaptive LASSO stabilization procedure.

Model	Covariate	HR[1]	95% CI[2]	Sign test[3]	*P*-value	Concord[4]	Event count
All Parity	Cluster 19	0.63	0.40, 1.01	0.060	0.051	70.8%	56/794 (7.05%)
	Parity 1	Ref.					
	Parity 2,3	1.43	0.60, 3.49	0.412	0.425		
	Parity > 3	4.97	2.72, 11.13	0.000	< 0.001		
Parity 2,3	Cluster 19	0.37	0.18, 0.75	0.012	0.008	64.2%	16/341 (4.7%)
Parity > 3	Cluster 20	0.74	0.55, 0.97	0.036	0.039	65.9%	31/222 (14.0%)
	Cluster 22	0.61	0.39, 0.90	0.010	0.026		

Bootstrapping (n=1000) performed. Clusters with *P*-value < 0.1 retained. Interactions explored. Stratification by parity 1 cows did not identify any significant Clusters. event count 9/231 (3.9%). Cluster composition and simple interpretation provided in Table 1.

[1]Hazards ratio.

[2]Bootstrap 95% confidence interval.

[3]Non-parametric sign test indicates the proportion of bootstrap estimates above or below zero.

[4]Concord = Harrell's concordance index (C-index), a non-parametric test of model accuracy.

The most protective cluster for HCULL, according to effect size, was Cluster 2 in the parity > 3 peak-milk cows (0.18 HR; 95%CI: 0.09, 0.31; Table 6). Cluster 2 contains several glycerophospholipid classes (LPC, PC, PE, PI) that were mostly attached to PUFA. Univariable analysis (Supplementary File S3) indicated that the 38:3 phospholipids from this Cluster significantly reduced culling [PC(38:3): 0.72 HR; 95%CI: 0.57, 0.91. PE(38:3): 0.76 HR; 95%CI: 0.60, 0.96. PI(38:3): 0.76 HR; 95%CI: 0.61, 0.96]. The 38:3 fatty acid composition is likely either 20:0/α-linolenic acid (18:3n-3, ALA) or 18:0/20:3 (Liu et al., 2025), though inclusion of the single fatty-acyl containing LPC(20:0) in Cluster 2 suggests that the former predominates. The ALA is an essential n-3 fatty acid only obtained from diet. Desaturation and elongation of ALA can produce other bioactive n-3 fatty acids, including 20:3, DHA, and EPA. When combined with the findings on Cluster 16 in parity 1 cows (0.39 HR), the association of Cluster 2 at parity > 3 (0.18 HR) further supports a beneficial role for n-3 fatty acids in dairy cattle (Rodney et al., 2015; Moallem, 2018).

Plasma phospholipids attached to DHA and EPA are markedly reduced in older cattle (Sheedy et al., 2025), and cows naturally conserve concentrations of plasma DHA and EPA over time (Stamey et al., 2012; Urrutia et al., 2023; dos Santos Neto et al., 2024) as a possible adaptation to the low conversion of ALA to very-long chain n-3 fatty acids (Domenichiello et al., 2015). That Cluster 2 (18:3n-3) was associated with reduced culling in parity > 3 cows and Cluster 16 (20:5n-3 and 22:6n-3) was not, may reflect a greater dependence on the n-3 precursors, or a higher utilization rate of the derivative very long chain n-3 fatty acids in greater parity cows compared to lower parity cows. However, without data on metabolic flux or utilization rates, this hypothesis remains speculative.

Cluster 12 substantially increased the HCULL in both the all-parity (1.73 HR; 95%CI: 1.31, 2.33) and parity > 3 (2.63 HR; 95%CI: 1.51, 4.93) peak-milk cohort models (Table 6). Cluster 12 predominantly contains PE attached to oleic acid (18:1) (Liu et al., 2025). An important biological derivative of PE attached to oleic acid that was not measured is oleoylethanolamide (**OEA**), which belongs to the endocannabinoid-like class of N-acylethanolamines. The OEA, and more broadly the endocannabinoid system, are considered anorexigenic and enhance adipose tissue lipolysis (Bowen et al., 2017; Myers et al., 2021; Zachut et al., 2025). Periparturient cattle with greater bodyweight-loss across the transition period had elevated adipose tissue OEA ($P < 0.05$) compared to lower body weight-loss cattle (Zachut et al., 2018), and excessive lipolysis during the transition period is associated with poor health (Bobe et al., 2004; Chapinal et al., 2011), and adverse reproductive outcomes (Jorritsma et al., 2000; Ospina

et al., 2010; Mann, 2022). The OEA may also contribute to inflammatory regulation, as induced inflammatory lipolysis in adipocytes (lipopolysaccharide-treated adipocytes) significantly increased OEA release compared to canonical lipolysis in adipocytes (ISO-treated adipocytes) (Myers et al., 2024). Activation of the endocannabinoid could also impact survival through a negative association with reproductive efficiency (Zachut et al., 2025). Whether it is possible to modulate OEA with diet is unclear; supplemented dietary flaxseed lowered plasma OEA and numerically lowered adipose OEA in one study (Kra et al., 2023), while flaxseed supplementation did not affect the concentration of OEA in plasma, follicular fluid, or the endometrium in another study (dos S. Silva et al., 2025). Feeding soybean that contains oleic acid content of approximately 70% of fatty acids is reported to improve milk production and quality and may protect from culling. Whether circulating PE attached to oleic acid found in Cluster 12 is directly associated with OEA metabolism and the effect of diet is worthy of further investigation to evaluate how mechanistically the lipids in Cluster 12 could increase HCULL.

The SM have structural roles in blood lipoproteins and serve as reservoirs for derivation into other sphingolipids classes, including the bioactive sphingolipid ceramide. Ceramides are involved in a range of cellular processes including apoptosis, senescence, inflammation, insulin sensitivity, oxidative stress, and are broadly pro-inflammatory mediators (McFadden and Rico, 2019; Zhao et al., 2024b). Given the complexity of sphingolipid metabolism, this discussion was limited to plasma SM, and their potential associations with cow removal. Cluster 6 consisted of PC(32:0) and 22 SM, of which 20 SM contained > 36 carbon atoms. Cluster 6 was associated with increased HCULL in the all-parity model (1.23 HR; 95%CI: 0.96, 1.55) and parity > 3 peak-milk cows (2.02 HR; 95%CI: 1.12, 3.81; Table 6), and was also above the stability threshold in HMORT all-parity model (46.7% inclusion frequency; threshold 42.3%) and parity > 3 (30.4% inclusion frequency; threshold 21.3%) peak-milk cows (Table 2). According to the univariable analysis (Supplementary File S3), 21 of the 23 lipids in Cluster 6 were associated with numerically greater HCULL, although only SM(42:2) was statistically significant (1.27 HR; 95%CI: 1.02, 1.60). In contrast, Cluster 5 contained 10 SM, all with ≤ 36 carbon atoms and had no significant association with HCULL or HMORT in any model. Assuming that the majority of SM were of the typical structure, with a d18:1 amino alcohol sphingosine attached, then Cluster 6 SM contained mostly > 18C fatty acyl chains and Cluster 5 with ≤ 18C chains. Further studies are required to investigate why specific SM fatty acyl compositions were differentially associated with increased HCULL.

The SM can be converted to ceramide by secretory acid-sphingomyelinases in the extracellular space (Schissel et al., 1996; Goñi and Alonso, 2002) and are activated by pro-inflammatory cytokines including tumour necrosis factor-α, platelet activating factor and lipopolysaccharide (Maceyka and Spiegel, 2014). Activation of sphingomyelinases should consume SM to create ceramide and decrease SM plasma concentration during inflammatory states (Kornhuber et al., 2015). However this expected relationship is only observed sporadically in *in-vivo* studies (Kornhuber et al., 2015), and ruminant studies of inflammatory states often report increased plasma SM (Humer et al., 2016; Zhang et al., 2020; Zhao et al., 2024a). The secretory acid-sphingomyelinases are slow to decrease after an initial stimulus, which may cause a compensatory increase in SM in chronic inflammatory conditions. An increased reservoir of circulating SM would allow rapid ceramide accrual under inflammatory stimulus and may reflect a maladjustment to chronic inflammation. This may explain why Cluster 6 (high in very-long chain SM) increased the HCULL.

Humer et al. (2016) explored the relationship between a lipid panel and NEFA concentrations at 21 d postpartum in 30 dairy cows and Zhao et al. (2024a) reported multiomic comparisons between high and low NEFA during the transition period from 7 d prepartum to 21 d postpartum in 16 cows. The SM from both studies were increased in the high NEFA cohorts and were consistently the same SM contained in Cluster 6 (Table 1), indicating a potential link between SM and energy metabolism. However, the correlation between Cluster 6 and the NEFA containing Cluster 18 was low in our study (r = 0.10, Figure 3), which may suggest either a dissociation between SM and energy metabolism at the greater DIM of our study, or a spurious correlation in the former studies. The SM in Cluster 6 were also associated with increased age and parity in dairy cows (Sheedy et al., 2025), and in non-ruminant studies (Mielke et al., 2015; Slade et al., 2021).

Cluster 8 also contained two SM species, with two PC and three plasmalogens, and was associated with increased HCULL in the all-parity (1.49 HR; 95%CI: 1.16, 1.96) and parity > 3 models (2.63 HR; 95%CI: 1.52, 4.89; Table 6). Though all Cluster 8 lipids numerically increased the HCULL, only SM(44:5) was statistically significant in the univariable models (all-parity: 1.19 HR; 95%CI: 1.03, 1.36; parity > 3: 1.58 HR; 95%CI: 1.20, 2.08; Supplementary File S3). Cluster 8 could also be putatively associated with n-6 fatty acids, with the Cluster 8 PC species associated with fatty acyls of 18:2 and 20:4, and the SM with 26:4 (Liu et al., 2025). However, the plasmalogens included in Cluster 8 were associated with mono-unsaturated or saturated fatty acids. The n-6 fatty acids are generally considered pro-

inflammatory, with negative consequences for health and reproduction (Sordillo, 2016; Moallem, 2018), and support the result of Cluster 8 increasing HCULL. However, the uncertainty regarding the fatty acyl composition of Cluster 8 lipids limits further speculation.

Cluster 4 was associated with reduced HCULL for parity 2nd and 3rd peak-milk cows (0.69 HR; 95%CI: 0.52, 0.93; Table 6) and HMORT in parity > 3 dry cohort cows (0.49 HR; 95%CI: 0.32, 0.78; Table 5). Cluster 4 contains only PI, which were mostly attached to monounsaturated or saturated fatty acids. The PI are highly regulated lipids that are involved in intracellular signalling pathways, membrane trafficking, regulating lipid transport, and are the precursors for the phosphoinositides (Balla, 2013; Dickson and Hille, 2019; Hammond and Burke, 2020). In contrast to most other phospholipids, the fatty acid composition of PI are highly conserved, with ~70% of cellular PI composed of PI(38:4) (C18:0/C20:4n-6 arachidonic acid, found in Cluster 13) (Barneda et al., 2019). However, there appears to be considerably more variability in bovine plasma PI fatty acyl composition, with PI(38:4) 22.7% and PI(38:3) 22.1% of total plasma PI in our cows (Supplementary File S2). We are unaware of any studies reporting an association between the plasma lipids contained in Cluster 4 and the risk of mortality, health or reproduction, making these findings novel but mechanistically unclear.

Study challenges and limitations

It is important to address several challenges in the evaluation and analysis, including a high degree of correlation within the dataset, substantial risk of false positives (Type I errors), composite endpoints, and survivorship bias.

The lipids of our dataset were very highly correlated (Supplemental Figure S3), a finding similarly reported by Liu et al. (2025). Highly correlated variables provide challenges for multivariable analysis, as correlated variables provide little additional explanatory power, and cause unstable coefficient values and standard errors (Belsley et al., 1980). Methods used to evaluate correlated variables include manual selection of specific variables, regularization techniques such as LASSO, or to create groups of variables. We elected not to remove specific variables, as there was insufficient knowledge regarding associations of specific lipids with survival of cows. Regularization techniques penalize variable coefficients to improve model stability and prediction, with some (including LASSO) able to penalize coefficient values to zero; this function provides the utility of variable selection (Tibshirani, 1996; Zou and Hastie, 2005). However, penalization is usually a data-driven process and, in the presence of highly correlated variables, the specific variable that is selected is unstable (Zou and Hastie, 2005).

Developments from the initial LASSO regularization (Tibshirani, 1996) have improved variable selection in the presence of collinearity, including elastic net regularization (Zou and Hastie, 2005) and the adaptive LASSO used in our analysis (Zhang and Lu, 2007; Hohberg and Groll, 2024), but do not completely resolve the issue. Unstable variable selection is a particular concern for a stabilized variable selection approach, which rely on frequency of inclusion. Ultimately, we decided to group positively correlated variables through hierarchical clustering, while acknowledging this is an imperfect but pragmatic solution. There is a risk of masking or suppressing important variables when combined in a cluster, and ease of interpretation may be diminished when compared to individual blood analytes. However, it has also been suggested that the ubiquitous nature and constant modification of lipids in biological systems support the use of panel combinations to improve the robustness of analysis (Zhao et al., 2024b). For completeness, we also report the univariable results in the Supplementary File S3.

The risk of false positive association (Type I error) increases with multiple comparisons and is a serious concern with "omics" data (Storey and Tibshirani, 2003; Wacholder et al., 2004; Lay et al., 2006). Options available to reduce Type I error include adjustments to the family-wise error rate or false-discovery rate (Holm, 1979; Benjamini and Hochberg, 1995; Storey and Tibshirani, 2003; Stevens et al., 2017). The stabilization approach used in our analysis is appropriate for finite sample, family-wise error control in LASSO regularization (Meinshausen and Bühlmann, 2010; Lima et al., 2021). Our results show that under bootstrap resampling, every Cluster was selected multiple times in all but one of the 16 models evaluated (parity 1 dry-cow HMORT model; Tables 2 and 3). This demonstrates that adaptive LASSO regularization, without stabilization, would have contributed to an unacceptably high Type I error rate in our analysis.

To maintain sufficient study power, it was necessary to combine several culling and mortality reasons into composite outcomes. However, composite outcomes may increase non-differential misclassification bias, particularly if outcomes within the composite do not share causal biological mechanisms (Freemantle et al., 2003; Cordoba et al., 2010). This bias can increase the false negative rate (Type II error) by increasing noise and diluting true associations. Conversely, for a Cluster to be selected it must either be very strongly associated with a specific removal reason or associated with multiple reasons within the composite. We attempted to limit misclassification bias by not combining mortality and culling into "all-cause" exit models, and right-censored exits attributed to farm accidents, under the assumption that no causal pathway

existed between blood analytes and farm accidents. There are plausible shared biologicalmechanisms among exit reasons, including the association of peripartum inflammation (Horst et al., 2021; LeBlanc, 2023) or n fatty acid metabolism (Rodney et al., 2015; Sinedino et al., 2017; Moallem, 2018; Moallem et al., 2020) with health, production, and reproduction, supporting the use of composite measures.

Selection bias, specifically survivorship bias is a form of bias occurring when analysis is restricted to cows that have survived prior selection pressures and excludes those that did not. Survivorship bias may occur from loss or selection of cows in earlier lactations associated with lipid profiles and other metabolic processes, confounding factors or chance events. Subsequently, observed associations between lipids and survival outcomes in later lactations reflect a combination of metabolic responses from earlier lactations, confounding factors and chance events. For example, Cluster 16, which was composed largely of glycerophospholipids containing very-long chain polyunsaturated n-3 fatty acids (Table 1), was strongly protective for HCULL at peak-milk for parity 1 cows (0.39 HR; Table 6). This suggests that cows surviving beyond parity 1 would represent a selected subset with relatively high Cluster 16 levels compared to cows that were removed at parity 1. However, the protective association between Cluster 16 and survival did not persist in later parities (Table 6). This lack of association could reflect either a true absence of effect in later parities, or the influence of survivorship bias, whereby older cows represent a subset with similar very-long chain polyunsaturated n-3 fatty acid metabolism, reducing detectable associations with removal. Therefore, weak or absent associations in later parities should be interpreted cautiously. Although the precise role of survivorship bias is uncertain, the general observation that different Clusters were associated with removal across parity-stratified models supports the possibility of either parity-specific metabolic effects, or the presence of survivorship bias. Stratifying models by parity provided unbiased estimates within each group, but at the cost of reduced statistical power.

CONCLUSION

This study was the first to use a lipidomic and metabolite platform to investigate time-to-event survival analysis in dairy cows. Plasma lipids assessed in non-lactating, late-pregnant cows were generally not robustly associated with survival. In contrast, many phospholipids in peak-milk cows were associated with HCULL. Glycerophospholipids containing n-3 fatty acids, including ALA, EPA and DHA protected against removal, whereas PE associated with

oleic acid, SM with > 18C, and mono-unsaturated or saturated PI increased the hazards of culling and mortality. Metabolites including BHBA, NEFA, albumin, calcium, and glucose were associated with the HCULL and HMORT. This study addressed some of the challenges in utilizing lipidomic data for cattle health research and took a relatively novel statistical approach, stabilized variable selection, to limit the false positive discovery rate. Novel plasma lipid targets for future research into survival of cattle were identified.

NOTES

Supplemental items are available at https://doi.org/10.6084/m9.figshare.29456534.

NON-STANDARD ABBREVIATIONS

ALA = α-linolenic acid; BHBA = beta-hydroxybutyric acid; CONFINE = confinement-based farms; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; HMORT = hazards of mortality; HR = hazards ratio; HCULL = hazards of culling; LASSO = least absolute shrinkage and selection operator; LPC = lysophosphatidylcholine; LPE = lysophosphatidylethanolamine; NEFA = non-esterified fatty acids; OEA = oleoylethanolamide; PC = phosphatidylcholine; PC(O) = ether-linked phosphatidylcholine; PE = phosphatidylethanolamine; PE(O) = ether-linked phosphatidylethanolamine; PI = phosphatidylinositol; SM = sphingomyelin; TG = triacylglycerol

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GENERAL DISCUSSION

GENERAL DISCUSSION

Increasing age of dairy cows is associated with increased risk of adverse health events, reproductive inefficiencies, culling, and mortality. While considerable research has focused on specific health and reproductive processes, there has been no comparable effort to understand the broader biologic changes that occur in cows with age. This thesis aimed to address that gap by exploring 1) body tissue reserves and association with housing systems, parity and blood metabolites; 2) association between age, housing system, reproduction and health events; and 3) lipidomic and metabolite profile associations with cow age, housing systems, and survival. the lipidomic and metabolic changes associated with aging in dairy cows. An ancillary goal was to establish a robust database for current and future research that included audited data from five different herd management software programs, weather data, herd test data, and bulk-tank milk data.

Chapter 1 was a literature review on the role that glycerophospholipids may have on longevity of cattle. Chapters 2 and 3 introduced a novel body composition scoring system that combined both body condition score (**BCS**) and body weight (**BW**) to augment assessment of body tissue reserves. Chapter 4 was a descriptive study that analysed prospectively collected herd records from 29 farms to investigate parity and housing related risks for reproduction, mastitis, and lameness. The final three chapters utilised a lipidomics dataset to examine associations between blood lipid profiles and cow age or parity (Chapter 5), housing systems (Chapter 6), and survival (Chapter 7). A consistent lipid group that was important across these final three chapters were glycerophospholipids that contained omega-3 fatty acids. These lipids had lower blood concentrations in older or higher-parity cows than younger cows, lower concentrations in confinement systems compared to pasture-based systems, and lower concentrations were associated with increased risk of culling.

Lipidomics

The literature review on glycerophospholipids (Chapter 1) highlighted the broad and expanding evidence for the role of lipid metabolism in dairy cattle health, reproduction, and production. This review provided a strong rationale for using a lipidomics approach to investigate metabolic aging and longevity of cows. Key topics that were discussed in the review included the role of glycerophospholipids in aging, lipid transportation during the transition period, particularly the export of very-low density lipoproteins from the liver and its impact on

hepatic steatosis, and emerging understandings of the relevance of the endocannabinoid system. The endocannabinoid system is associated with lipid metabolism, immune function, and reproduction in mammals (Silvestri and Di Marzo, 2013; Lu and Mackie, 2016). However, early studies suggest that the system may function very differently in ruminants than in monogastric species, emphasizing the need for ruminant specific studies (Myers et al., 2021; Zachut et al., 2025). Although the targeted lipidomics approach used in this thesis did not directly measure endocannabinoids, phosphatidylethanolamines containing oleic acid were associated with an increased risk of culling in parity > 3 cows (2.63 relative hazards, 95%CI: 1.51 – 4.93, Chapter 7). These lipids are potential precursors for oleoylethanolamide, a member of the endocannabinoid-like N-acylethanolamines.

Chapter 5 investigated lipidomic associations with age and parity of cows, with results indicating that older cows had significantly lower circulating concentrations of glycerophospholipids containing very-long chain poly-unsaturated omega-3 fatty acids, including docosahexaenoic acid (C22:6n-3; **DHA**), docosapentaenoic acid (C22:5n-3; **DPA**), and eicosapentaenoic acid (C20:5n-3; **EPA**). The most marked decrease in these lipids occurred between parity 1 and parity 2 cows, though the decline continued monotonically with increasing parity. The publication of Chapter 5 in the Journal of Dairy Science represents the first peer-reviewed article to report this association (Sheedy et al., 2025). This finding may be of considerable importance given the established positive associations between omega-3 fatty acids and longevity-related outcomes, including improved reproduction (Rodney et al., 2015; Sinedino et al., 2017; Moallem, 2018; Moallem et al., 2020) and reduced incidence of adverse health events (Silvestre et al., 2011; Moallem et al., 2020; Veshkini et al., 2023). However, the cross-sectional design of Chapter 5 limited interpretation to speculation about whether reduced concentrations of omega-3 glycerophospholipids in older cattle contribute causally to their declining reproductive efficiency, and increased health risks, as reported in Chapter 4.

To begin addressing the limitations of the cross-sectional study-design, a longitudinal design was used in Chapter 7 to perform an adaptive LASSO Cox full-likelihood survival model that investigated lipids and standard metabolites associated with the hazards of culling or mortality. The longitudinal design allowed for the correct temporal alignment between lipid measurements and outcome, partially overcoming a limitation of cross-sectional studies. The statistical analysis in Chapter 7 faced several challenges, including a higher than predicted right-censoring, the high collinearity of the lipidomic dataset, and potential survival bias. These analytical challenges, and their management are detailed in Chapter 7.

The known relationship between increasing parity and increased hazards of culling or mortality was consistent with Chapter 7 results. Parity > 3 cows had between 2.95 (peak-milk) and 4.09 (dry cohort) relative hazards of culling, and between 2.43 (dry cohort) and 4.97 (peak-milk) relative hazards of mortality, compared to parity 1 cows. Peak-milk parity 1 cows with greater concentrations of glycerophospholipids containing very-long chain omega-3 fatty acids had reduced hazards of culling (0.39 relative hazards, 95% CI: 0.23, 0.65). However, these lipids were not significantly associated with culling in parity > 1 cows. Instead, glycerophospholipids containing the essential omega-3 fatty acid, α-linolenic acid (C18:3, n-3), which can be desaturated and elongated into very-long chain omega-3 fatty acids, were associated with reduced culling in parity > 3 cows (0.18 relative hazards, 95%CI: 0.09, 0.31). The reason for this apparent difference in protection based on the omega-3 fatty acid chain length remains unclear. Potential explanations of survival bias, and differences in metabolic flux rates that may lead to irreversible losses of essential lipids were discussed in Chapter 7.

Chapter 6 examined lipidomic and metabolite differences between pasture-based and confinement-based housing systems. Although Chapters 5 and 7 investigated different outcomes, Chapter 6 similarly found that glycerophospholipids containing omega fatty acids were the lipids most strongly associated with the outcome of interest. Cows housed in confinement-based systems had lower plasma concentrations of glycerophospholipids containing omega-3 fatty acids during both the dry and peak-milk periods. Confinement-based cows from the peak-milk period also had higher concentrations of glycerophospholipids containing omega-6 fatty acids, which are generally associated with pro-inflammatory pathways. A factor that may have caused the lipid profiles of cows to differ between housing systems was the forage type provided. Confinement-based farms fed corn-silage, whereas pasture-based farms provided fresh pasture. Corn-silage has low α-linolenic acid (C18:3, n-3, ~7% of total fatty acid) and high linoleic acid (C18:2, n-6, ~47% total fatty acid) compared to fresh pasture that has between 50-70% of its total fatty acid as α-linolenic acid and 11-13% as linoleic acid (Glasser et al., 2013; Elgersma, 2015; NASEM, 2021). The biohydrogenation of unsaturated fatty acids by rumen microbes is substantial, with around a 90% reduction between the intake amount and the flow to the duodenum (Scollan et al., 2001). However, despite the considerable modification of the long-chain unsaturated fatty acids passing through the rumen, different dietary fatty acid profiles are consistently reported to influence the fatty acid profile of meat (Daley et al., 2010) and milk (Moate et al., 2008). It is reasonable to assume that the

different fatty acid profiles between corn-silage based TMR and pasture-based diets significantly influence the blood lipid profile, and potentially modify survival risk.

When considered together, the results from Chapters 5, 6, and 7 give rise to a compelling thought experiment. Glycerophospholipids containing omega-3 fatty acids were found to decrease with age and parity (Chapter 5), were lower in confinement-based compared to pasture-based cows (Chapter 6), and reduced culling hazards (Chapter 7). This raises the hypothesis that the "lipid-age" of cows in confinement-based systems may be metabolically older than that of the same true-age cows from pasture-based systems, potentially contributing to reduced longevity in confinement systems. Although this hypothesis was not formally tested in this thesis, it raises an important possibility; if the forage base is a modifiable risk factor for accelerated lipid aging, and reduced cow longevity, then altering or supplementing the feed-base could form part of the solution to improve longevity. As discussed in the literature review of Chapter 1, increasing α-linolenic acid through seed-oils such as flaxseed (Daley et al., 2010; Rodney et al., 2015) or direct supplementation of EPA and DHA from marine-derived oils or meal (Moallem, 2018; Huang et al., 2020) can alter the omega fatty acid profile of a cow. Our research is the first to comprehensively associate blood lipid profiles with age, housing system and survival. The cumulation of these results strongly suggests that to optimise cow longevity we must adequately address the lipid requirements of cows, particularly the imbalance in glycerophospholipids with omega-3 and omega-6 fatty in confinement housing systems and as parity increases.

There were several other lipid groups besides glycerophospholipids containing omega fatty acids that demonstrated significant associations with the outcomes explored. Sphingomyelins, particularly those containing long-chain fatty acids (> C18), were greater in older cattle (Chapter 5), and were associated with increased hazards of culling (Chapter 7). Sphingolipid metabolism, which includes both the sphingomyelins measured in this thesis and ceramides, is broadly associated with pro-inflammatory pathways (McFadden and Rico, 2019; Zhao et al., 2024). Together with the omega fatty acid findings, these results suggest that older dairy cows exhibit a more pro-inflammatory or chronic inflammatory profile than younger cattle. A pro-inflammatory state, especially during the peripartum period, is associated with poorer health, reduced reproductive efficiency, lower production (Moallem, 2018; Horst et al., 2021; LeBlanc, 2023), and increased hazards of culling (Chapter 7). These results provide a conceptual link between age-related lipid changes and the increased risk of adverse outcomes for older cattle.

Phospholipids containing odd-chain fatty acids were decreased with age (Chapter 5) and were associated with reduced hazards of mortality in the dry cohort (Chapter 7). The odd-chain fatty acids are associated with de novo synthesis in the rumen through microbial activity (Vlaeminck et al., 2006), and are associated with pasture feeding (Moate et al., 2008; Paredes et al., 2018; Riuzzi et al., 2021). However, our Chapter 6 results did not identify consistent differences between housing systems and phospholipids containing odd-chained fatty acids. The association between age and odd-chain fatty acids in blood (Chapter 5) was also reported for odd-chain fatty acids in milk (Sun et al., 2022). This association with age complicates the proposed use of odd-chain fatty acids as biomarkers for pasture-derived milk (Paredes et al., 2018; Riuzzi et al., 2021), as confounding with herd parity structure could occur.

Body composition

An exploration of the different measurements of body tissue reserves, including BCS, BW, and a novel classification system that combined BCS and BW was presented in Chapters 2 and 3. These body metrics were analysed in relation to parity, housing system, and a standard metabolite panel. The development of the novel classification systems was motivated by the low-to-moderate correlation observed between BW and BCS (Berry et al., 2006a; Roche et al., 2007). The new metric was an unordered 6-level categorical variable, defined by whether a cow's BCS was greater than, equal to, or less than the farm median, and whether BW was greater than or less than the farm median. While the novel metric in Chapter 2 did not include the median BCS category, the introduction of the median category in Chapter 3 can be considered a developmental advance.

Chapter 2 utilised a large, multi-national, retrospective dataset, whereas Chapter 3 used a prospective dataset. Despite the different data sources, both chapters reported consistent associations between the body tissue metrics and parity. Increasing parity was associated with higher BW and lower BCS, except for parity 2 cattle, which often had low BW and low BCS, as reported elsewhere (Kertz et al., 1997; Berry et al., 2006b; Roche et al., 2007). This finding suggests that first-parity cows may not efficiently regain labile tissue reserves that were mobilised in the peripartum period, possibly due to the ongoing demands of skeletal growth through their first lactation, or restricted feed access from the lower social hierarchy of primiparous cows compared to multiparous cows (Phillips and Rind, 2002; Berry et al., 2005; Deniz et al., 2021).

Albumin was the most consistently associated metabolite across all body tissue metrics (Chapter 3). High blood albumin concentrations were positively associated with BW, BCS, and associated with an increased probability of having a high BCS and high BW, and a decreased probability of having a low BCS and low BW. Though there is a well-established positive association between crude protein intake and blood albumin (Payne et al., 1970; Lee et al., 1978; Bobbo et al., 2017), our study design and analysis attempted to remove any association related to farm-level influences, including diet. As such, we speculated that the observed BW, BCS and albumin associations could be attributed to differences specific to the cow-level. The efficiency of microbial protein production, which contributes 40 to 90% of absorbed protein (Castillo-Lopez and Domínguez-Ordóñez, 2019; NASEM, 2021; Lima et al., 2023), may differ among cows and explain the different albumin concentrations. Indeed, cow-level differences in rumen microbiomes and efficiency of protein production may be reflected by the moderate heritability of serum albumin (h2 = 0.19, Peterson et al., 1982; h2 = 0.13, Cecchinato et al., 2018; h2 = 0.27, Luke et al., 2019). Albumin is also a negative acute phase protein that decreases in concentration during periods of inflammation. However, this aspect of albumin metabolism did not appear to importantly influence body tissue metrics, with neither globulin (an acute phase protein) nor the albumin:globulin ratio significantly associated with any body tissue model.

While discussions on body tissue change during the transition period are typically framed solely in the context of lipid mobilisation, these results suggest that protein metabolism, as reflected by albumin levels, play a significant role deserving of greater attention. The finding that blood urea had a positive association with dry-cohort BCS and that low urea was associated with increased probability of low BCS and low BW further supported the importance of protein metabolism body tissue reserves. Similarly to albumin, blood urea is positively associated with protein intake (Staples et al., 1992; Law et al., 2009), and variation in the urea cycle may also be influenced at the cow-level through differences in the rumen microbiome (Prahl et al., 2022).

Future studies are warranted to determine how the novel body composition classification provides additional insight into health and reproductive outcomes beyond that achieved using BCS or BW alone.

Study design and statistical analysis

A core strength of this thesis was the study design, which enabled multiple lines of inquiry to be addressed from a single blood-analysis dataset (Chapters 3, 5, 6, and 7). The sampling

frame used a balanced design for housing system (supporting the investigation in Chapters 4 and 5), while parity was disproportionately stratified to allow analyses of age, parity, and survival that were independent of herd parity structure (Chapter 6 and 7). Parity 1 cows were deliberately oversampled based on the assumption that first-lactation cows have less risk of removal and therefore would have a higher risk of being right-censored, compared to parity > 1 cows (Chapter 7). Sample size estimates accounting for farm-level clustering were performed at thesis onset to ensure sufficient study power across all Chapters. By integrating multiple study designs into a single sampling framework, the total number of animals required was reduced compared to conducting each study in isolation. This reduction in animals required supports a key tenet of the 3Rs (Replacement, Reduction, and Refinement) of animal research (NHMRC, 2019).

A range of advanced and novel statistical approaches were used across the thesis to address the specific objectives of each study. These included standard frequentist models (Chapters 2, 3, and 4), polytomous logistic regression to model multinomial outcomes (Chapter 3), machine learning techniques for variable selection, prediction and inference (Chapters 5, 6, and 7), time-to-event models such as Cox proportional hazards and competing risks frameworks (Chapters 4 and 7), and stability selection was implemented to control Type I errors (Chapters 6 and 7). These modelling approaches were used to address the dataset complexity and study-design structure, and to provide robust, interpretable results. This thesis is an example of the power that can be obtained through careful metabolic epidemiological investigation.

All “omics”-type datasets are at high risk of Type I errors (false positives), due to the large number of multiple comparisons made with the outcomes of interest (Storey and Tibshirani, 2003; Wacholder et al., 2004; Lay et al., 2006), and the potential to overfit in multivariable models (van der Schaaf et al., 2012; Aliferis and Simon, 2024). The approach that was used to limit Type I error varied between Chapter 5 and the later analyses of Chapters 6 and 7. In Chapter 5, lipidomic associations with age and parity were explored using several models in parallel (e.g. random forest, support vector machines, and orthogonal partial least squares discriminant analysis for the classification of parity 1 and parity ≥ 3 cows). The top-ranking lipids from each model were manually compared to identify lipids robustly associated with age and parity. This approach was performed with cross-validation during model construction and an 80:20 training-to-test data split to mitigate overfitting and increase generalisability.

In contrast, Chapters 6 and 7 employed a variable stability approach to reduce the family-wise error rate and improve the robustness of variable selection (Meinshausen and Bühlmann, 2010; Hyde et al., 2022). This method involves bootstrapping (resampling with replacement) the variable selection procedure to assess the frequency that each variable is selected under small perturbations of the data. The underlying assumption is that variables consistently selected across bootstrap iterations are more likely to be robustly associated with the outcome than those infrequently selected. Full details of this method and its implementation are provided in the respective *Materials and Methods* and *Discussion* sections of Chapters 6 and 7. The variable stabilisation procedure is extremely generalisable, is relatively easy to interpret results, and reduces the Type I error regardless of the variable selection procedure chosen (Meinshausen and Bühlmann, 2010). In Chapter 6, the variable stabilisation procedure was applied to multiple models in parallel, whereas Chapter 7 applied it to only one model. The decision to use only one model in Chapter 7 was made to preserve parsimony across the multiple regressions that were reported, and due to limited availability of survival models that simultaneously accommodated frailty terms and variable selection.

The variable stabilisation procedure did present some challenges. In highly correlated datasets, it may increase the risk of Type II error (false negatives), as selection frequencies may be split across correlated variables that reduce the individual inclusion rates of otherwise important variables. Additionally, the computational power required can be substantial, as each model is re-estimated across the many bootstrap iterations.

Survival bias presents a major challenge in longevity research, particularly in cross-sectional observational studies (Chapter 2, 3, 5, and 6). It occurs when the study population has already undergone an unobserved selection process, resulting in a cohort that may not represent the original population. A simple example was discussed in Chapter 5, where older cows had lower concentrations of glycerophospholipids containing very-long chain omega-3 fatty acids. While this may reflect a true age-associated decline, it could also be a consequence of survival bias, where cows with greater concentration of these lipids were removed earlier, leaving only those with lower concentrations in the observed cohort. Unfortunately, there are no statistical tests available to detect survival bias and its identification relies on subject-matter expertise, and contextual understanding of study design. In the example above, the likelihood of a true age-associated decline in glycerophospholipids containing omega-3 fatty acids is supported by converging evidence: previous studies have demonstrated positive associations between omega-3 supplementation, health, and reproduction (Rodney et al., 2015; Moallem, 2018),

Chapter 4 reported negative associations between parity, health, and reproduction, and Chapter 7 showed that higher concentrations of these lipids were protective against culling in parity 1 cows.

While longitudinal studies account for the correct temporal relationship between exposure and outcome, they are not immune to survival bias if a selective process occurred before study enrolment. In Chapter 7, to minimise the influence of survival bias, separate survival models were constructed for each parity group. This stratified approached ensured that the results within each parity level were not confounded by prior survival-related selection. However, this method reduced study power due to the smaller sample sizes within each parity group.

Well-structured randomised controlled feeding trials control bias by randomly distributing both known and unknown confounders across treatment groups. A feeding trial is also our recommended follow-up to this thesis, to test the hypotheses generated from our observational studies. A logical progression would be to investigate whether omega-3 supplementation strategies can alter the lipid profile of older cows to more closely resemble that of younger cows, and to re-balance the lipid profile of confinement-based cows towards that of pasture-based cows. Ultimately, these feeding trials could help determine whether dietary lipid manipulation can contribute to improved cow longevity, with potentially profound positive impacts on the sustainability of the dairy industry.

CONCLUSION

The associations between increasing cattle age or parity, and increased risk of adverse health events, reduced reproduction efficiency, and ultimately increased risk of culling or mortality are well established. These associations are a major impediment to optimising the longevity of cows. Surprisingly, the underlying metabolic changes that accompany aging in cattle have received relatively little attention. This thesis sought to address this knowledge gap, and represents the first comprehensive body of work to investigate lipid and metabolic associations with body tissue metrics, survival, differences between confinement- and pasture-based housing systems, age, and parity.

Several novel statistical approaches were required to address the complexity of the multi-site, parity-stratified, and high-dimensional dataset, including machine learning, survival analysis and variable stability selection. These methods were selected to robustly address each study objective, and to present interpretable and actionable results.

The two chapters that examined associations between metrics of body tissue reserves with parity, housing systems, and a standard panel of serum metabolites indicated that greater parity cows had higher BW, but lower BCS compared to lower parity cows. Among the metabolites analysed, albumin was consistently associated with all metrics of body tissue reserve; positively associated with both BW and BCS, and cows with high albumin concentration had a greater probability of having both a high BCS and high BW, and a lower probability of having a low BCS and low BW. These results indicated that protein metabolism is an important determinant of body tissue reserves, and efficiency of protein metabolism may differ between cows and parity.

A finding from the lipidomic analysis was that older cattle had reduced plasma concentrations of glycerophospholipids containing omega-3 fatty acids compared to younger cattle. These lipids also had lower concentrations in confinement-based housing systems compared to pasture-based systems, and lower concentrations were associated with increased hazards of culling. When considered collectively, these associations indicate that the omega fatty acid pathways may be differentially regulated with age and may increase the risk of adverse outcomes in older cattle. It was speculated that diet was the major contributing factor for the observed difference in the lipid profiles between housing systems; the corn-silage based diets provided by the confinement-based farms contained greater amounts of omega-6 fatty acids and pasture-based diets contained greater amounts of omega-3 fatty acids.

This thesis aimed to contribute to the understanding of longevity of cows by taking an exploratory, hypothesis-generating approach. Although the discussion has focused heavily on glycerophospholipids containing omega fatty acids, due to their consistent relevance in multiple chapters, other lipids classes were also identified as important and their potential importance to improving cow longevity should not be understated. These include lipids involved in sphingolipid metabolism, odd-chain fatty acids, and the endocannabinoid system. This thesis provides a substantial online repository of supplementary data to support future research, which may help inform further lipid longevity investigations.

This body of work has meaningfully advanced our scientific understanding by producing foundational information regarding metabolic signatures of aging and survival in dairy cows, identifying the influence of housing systems on blood metabolite profiles, and introducing novel statistical approaches to high-dimensional agricultural data. A logical follow-up to this thesis would be to conduct long-term, randomised controlled feeding trials of omega-3 fatty

acids to evaluate whether targeted lipid interventions can 1) improve the lipid profile of older cows to become closer to younger cows 2) improve the lipid balance of confinement-based cows to become closer to a pasture-based lipid profile, and 3) ultimately reduce the risk of adverse health events, reproduction failure, and removal risk. Developing practical strategies to improve the dietary lipid balance to mitigate changes associated with aging, and age-related diseases could have profoundly positive impacts on optimising cow longevity and the sustainability of the dairy industry.

NON-STANDARD ABBREVIATIONS

BCS = body condition score; BW = body weight; DHA = docosahexaenoic acid; DPA = docosapentaenoic acid; EPA = eicosapentaenoic acid

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APPENDIX

Supplementary files that were not suitable for print are accessible at:

https://doi.org/10.6084/m9.figshare.29456534

Table A1: Individual farm characteristics

Farm ID	System[1]	Housing	Breed	Herd Size	Milk (L/cow)
1	Pasture	-	Holstein	380	23.4
2	Pasture	-	Red	400	24.2
3	Pasture	-	Holstein/ Jersey	420	25.2
4	Pasture	-	Holstein	320	28.6
5	Pasture	-	Holstein	200	-
6	Pasture	-	Holstein	380	28.4
7	Pasture	-	Holstein/ Jersey	760	19.6
8	Pasture	-	Jersey	210	15.3
9	Pasture	-	Holstein	340	22.9
10	Pasture	-	Holstein	380	26.7
11	Pasture	-	Holstein/ Jersey	350	17.1
12	Pasture	-	Jersey	140	18.0
13	Pasture	-	Holstein/ Jersey	180	19.5
14	Pasture	-	Holstein/ Jersey	280	23.7
15	Pasture	-	Holstein/ Cross	200	21.8
16	Confinement	CBP	Holstein	1260	32.9
17	Confinement	CBP	Holstein	940	31.7
18	Confinement	CBP	Jersey	650	-
19	Confinement	CBP	Holstein/Red	470	35.3
20	Confinement	CBP	Holstein	710	32.2
21	Confinement	Free-stall	Holstein	360	41.4
22	Confinement	CBP	Holstein	490	34.7
23	Confinement	Free-stall	Holstein	2650	38.1
24	Confinement	Free-stall/ CBP	Holstein	4300	37.1
25	Confinement	Dry-lot	Holstein	810	29.3
26	Confinement	CBP	Holstein	1040	-
27	Confinement	Free-stall/ CBP	Holstein	9140	41.8
28	Confinement	Dry-lot	Holstein	1110	31.6
29	Confinement	Free-stall/ CBP	Holstein	890	34.7
30	Confinement	CBP	Holstein	1000	32.0

1: Farming system during lactation period; CBP = compost bedded pack barn

Table A2: Farm and cohort specific body weight (BW) and body condition score (BCS)

Farm ID	Breed	Peak-milk cohort		Dry cow cohort	
		BW (kg±SD)	BCS (1-5±SD)	BW (kg±SD)	BCS (1-5±SD)
1	Holstein	538 ± 92	2.53 ± 0.23	655 ± 102	3.32 ± 0.36
2	Red	548 ± 74	3.13 ± 0.36	675 ± 101	3.56 ± 0.34
3	Holstein-Jersey	498 ± 60	2.82 ± 0.35	588 ± 83	3.36 ± 0.40
4	Holstein	648 ± 76	2.45 ± 0.40	704 ± 86	2.84 ± 0.25
5	Holstein	622 ± 100	2.57 ± 0.39	-	-
6	Holstein	606 ± 72	2.49 ± 0.32	695 ± 90	3.09 ± 0.53
7	Holstein	517 ± 82	2.97 ± 0.30	657 ± 85	3.59 ± 0.31
7	Holstein-Jersey	505 ± 77	2.89 ± 0.20	-	-
8	Jersey	344 ± 51	2.92 ± 0.34	434 ± 32	3.51 ± 0.24
9	Holstein	568 ± 66	2.68 ± 0.39	666 ± 88	3.50 ± 0.38
10	Holstein	591 ± 82	3.04 ± 0.31	698 ± 109	3.56 ± 0.29
11	Holstein	540 ± 38	2.82 ± 0.30	631 ± 80	3.25 ± 0.35
11	Holstein-Jersey	555 ± 46	3.05 ± 0.25	549 ± 41	3.36 ± 0.33
12	Jersey	-	-	469 ± 39	3.55 ± 0.49
13	Holstein	-	-	747 ± 106	3.21 ± 0.43
13	Jersey	-	-	449 ± 73	3.47 ± 0.49
14	Holstein	-	-	700 ± 97	3.08 ± 0.40
14	Holstein-Jersey	-	-	601 ± 38	3.28 ± 0.31
15	Holstein	635 ± 62	2.44 ± 0.16	646 ± 78	2.86 ± 0.44
15	Cross	-	-	608 ± 72	3.17 ± 0.30
16	Holstein	637 ± 85	3.44 ± 0.59	715 ± 115	3.70 ± 0.46
17	Holstein	673 ± 83	2.97 ± 0.36	709 ± 74	3.43 ± 0.53
18	Jersey	-	-	497 ± 53	3.44 ± 0.34
19	Holstein	-	2.69 ± 0.49	-	3.44 ± 0.41
19	Red	-	3.50 ± 0.35	-	4.04 ± 0.57
20	Holstein	590 ± 60	2.60 ± 0.38	678 ± 103	3.36 ± 0.48
21	Holstein	677 ± 82	2.95 ± 0.46	723 ± 93	3.46 ± 0.42
22	Holstein	718 ± 98	3.28 ± 0.41	757 ± 75	3.17 ± 0.35
23	Holstein	670 ± 78	2.98 ± 0.47	708 ± 114	3.47 ± 0.31
24	Holstein	601 ± 83	2.50 ± 0.42	744 ± 126	3.34 ± 0.42
25	Holstein	667 ± 89	2.89 ± 0.42	765 ± 87	3.54 ± 0.29
26	Holstein	600 ± 76	2.94 ± 0.51	712 ± 96	3.46 ± 0.29
27	Holstein	672 ± 102	3.16 ± 0.45	710 ± 122	3.86 ± 0.40
28	Holstein	612 ± 83	2.65 ± 0.46	635 ± 109	3.05 ± 0.35
29	Holstein	654 ± 71	2.72 ± 0.33	746 ± 110	3.34 ± 0.32
30	Holstein	-	-	709 ± 86	3.31 ± 0.46

Table A3: Count of cows sampled within breed and farm, split by parity groups. Eligible cows were based on the reported inclusion and exclusion criterium in Chapter 2.

		Peak-milk cohort						**Dry cohort**					
Farm		Parity						Parity					
ID	Breed	1	2	3	> 3	Total	Eligible	1	2	3	> 3	Total	Eligible
1	Holstein	11	5	3	8	27	28	8	6	3	9	26	58
2	Red	9	6	3	8	26	57	6	4	6	6	22	57
3	Holstein -Jersey	8	3	8	11	30	39	6	5	9	8	28	86
4	Holstein	9	6	6	8	29	58	8	6	5	8	27	55
5	Holstein	8	6	6	6	26	39	-	-	-	-	-	-
6	Holstein	8	6	6	7	27	36	2	2	9	10	23	42
7	Holstein	6	4	2	7	19	52	4	3	5	4	16	40
7	Holstein -Jersey	2	1	3	1	7	52	-	-	-	-	-	-
8	Jersey	16	1	0	8	25	31	10	7	4	7	28	27
9	Holstein	6	6	6	8	26	45	6	6	5	6	23	36
10	Holstein	10	8	4	8	30	79	10	3	3	10	26	39
11	Holstein	2	5	1	3	11	24	4	4	1	4	13	26
11	Holstein -Jersey	2	2	3	4	11	24	5	2	0	2	9	26
12	Jersey	-	-	-	-	-	-	1	8	5	8	22	53
13	Holstein	-	-	-	-	-	-	0	2	2	2	6	26
13	Jersey	-	-	-	-	-	-	6	0	2	1	9	26
14	Holstein	-	-	-	-	-	-	4	4	4	7	19	78
14	Holstein -Jersey	-	-	-	-	-	-	3	3	2	0	8	78
15	Holstein		3	2	7	12	34	7	3	5	8	23	45
15	Cross	-	-	-	-	-	-	0	4	1	1	6	45
16	Holstein	9	6	8	8	31	158	8	8	7	9	32	45
17	Holstein	9	6	6	7	28	46	5	7	6	6	24	141
18	Jersey	-	-	-	-	-	-	8	5	6	9	28	58
19	Holstein	7	6	6	5	24	60	4	0	5	7	16	50
19	Red	2	1	1	2	6	60	3	0	1	3	7	50
20	Holstein	9	8	6	8	31	85	9	8	8	8	33	101
21	Holstein	10	7	6	8	31	36	9	5	6	8	28	52
22	Holstein	9	7	6	8	30	52	1	6	6	8	21	54
23	Holstein	10	6	5	9	30	160	9	7	8	8	32	160
24	Holstein	8	6	6	8	28	160	10	6	6	9	31	180
25	Holstein	6	6	7	9	28	60	5	4	5	4	18	40
26	Holstein	6	5	4	6	21	99	6	7	5	7	25	51
27	Holstein	9	7	6	8	30	231	9	4	7	7	27	104
28	Holstein	10	7	6	8	31	92	8	6	6	6	26	55
29	Holstein	11	8	7	9	35	76	5	10	5	9	29	63
30	Holstein	-	-	-	-	-	-	8	6	7	7	28	226
	Total	212	148	133	197	690	1973	197	161	165	216	739	2273

Table A4: Lactating cow cohort distribution of Body Condition Group (BCG) and parity. Specification for BCG is <, > or median group Body Condition Score (BCS) and < or > median group Body Weight (BW), with group being breed with farm.

Body Condition Group	Parity				Total
	Heifer	2	3	> 3	
Low BCS, Low BW	43	37	18	20	118
Low BCS, High BW	3	19	34	71	127
Median BCS, Low BW	63	23	6	9	101
Median BCS, High BW	3	20	22	43	85
High BCS, Low BW	77	16	4	6	103
High BCS, High BW	11	26	39	41	117
Total	200	141	123	187	651

Table A5: Dry cow cohort distribution of Body Condition Group (BCG) and parity. Specification for BCG is <, > or median group Body Condition Score (BCS) and < or > median group Body Weight (BW), with group being breed with farm.

Body Condition Group	Parity				Total
	Heifer	2	3	> 3	
Low BCS, Low BW	40	56	32	13	141
Low BCS, High BW	2	12	38	59	111
Median BCS, Low BW	64	33	8	11	116
Median BCS, High BW	4	17	29	38	88
High BCS, Low BW	66	21	8	4	99
High BCS, High BW	14	20	42	79	155
Total	190	159	157	204	710

Table A6: Multinomial logistic regression of Body Condition Group for the dry cow cohort. The constant term estimates baseline relative risk for each outcome compared to low BCS, low BW cows. Albumin, urea and glucose are normal standardized within breed and herd such that the co-efficient is the relative risk change following a 1 SD change in the respective metabolite serum concentration. Robust standard errors are provided with clustering around breed and herd.

	Relative Risk Ratio	Robust Standard Error	95% Confidence Interval	
Low BCS, Low BW	(base outcome)			
Low BCS, High BW				
Albumin	1.31	0.17	1.01	1.70
Urea	1.27	0.21	0.92	1.75
Glucose	0.63	0.11	0.45	0.90
Parity: 1	(ref)	-	-	-
2	4.08	3.08	0.93	17.93
3	24.77	18.40	5.78	106.24
> 3	114.10	86.84	25.67	507.16
Days pre-calving	0.97	0.01	0.94	1.00
Constant	0.09	0.07	0.02	0.43
Median BCS, Low BW				
Albumin	1.23	0.18	0.93	1.63
Urea	1.46	0.17	1.16	1.84
Glucose	1.05	0.15	0.79	1.39
Parity: 1	(ref)	-	-	-
2	0.33	0.13	0.15	0.71
3	0.15	0.08	0.05	0.42
> 3	0.51	0.26	0.18	1.40
Days pre-calving	1.01	0.01	0.99	1.04
Constant	1.21	0.42	0.61	2.39
Median BCS, High BW				
Albumin	1.40	0.21	1.04	1.89
Urea	1.21	0.19	0.89	1.64
Glucose	0.99	0.18	0.69	1.43
Parity: 1	(ref)	-	-	-
2	3.22	2.43	0.73	14.13
3	10.25	8.30	2.09	50.16
> 3	35.96	29.03	7.39	175.01
Days pre-calving	0.99	0.01	0.97	1.01
Constant	0.12	0.08	0.03	0.47
High BCS, Low BW				
Albumin	1.33	0.23	0.95	1.87
	1.39	0.20	1.05	1.84
Glucose	1.13	0.16	0.86	1.49
Parity: 1	(ref)	-	-	-
2	0.20	0.09	0.08	0.48
3	0.13	0.07	0.05	0.40
> 3	0.16	0.10	0.05	0.54
Days pre-calving	1.03	0.01	1.01	1.06
Constant	0.78	0.31	0.36	1.71
High BCS, High BW				
Albumin	1.86	0.26	1.41	2.46
Urea	1.29	0.16	1.01	1.65
Glucose	1.10	0.15	0.85	1.43
Parity: 1	(ref)	-	-	-
2	1.06	0.60	0.35	3.19
3	4.13	2.31	1.38	12.37
> 3	20.88	10.80	7.57	57.57
Days pre-calving	1.00	0.01	0.98	1.03
Constant	0.30	0.15	0.11	0.79

Table A7: Multinomial logistic regression of Body Condition Group for the lactating cow cohort. The constant term estimates baseline relative risk for each outcome compared to low BCS, low BW cows. Albumin, BHBA and milk volume are normal standardized within breed and herd such that the reported co-efficient is the relative risk ratio change following a 1 SD change in the respective variable. Robust standard errors are provided with clustering around breed and herd.

	Relative Risk Ratio	Robust Standard Error	95% Confidence Interval	
Low BCS, Low BW	(base outcome)			
Low BCS, High BW				
Albumin	1.03	0.16	0.76	1.40
BHBA	0.97	0.13	0.74	1.27
Parity: 1	(ref)	-	-	-
2	5.27	4.12	1.14	24.39
3	18.11	12.29	4.79	68.51
> 3	33.09	23.04	8.45	129.56
Milk volume	1.40	0.32	0.90	2.19
Constant	0.09	0.06	0.03	0.30
Median BCS, Low BW				
Albumin	1.29	0.23	0.91	1.83
BHBA	0.85	0.13	0.63	1.16
Parity: 1	(ref)	-	-	-
2	0.36	0.17	0.14	0.90
3	0.19	0.09	0.07	0.49
> 3	0.27	0.17	0.08	0.91
Milk volume	1.04	0.26	0.64	1.70
Constant	1.61	0.48	0.90	2.88
Median BCS, High BW				
Albumin	1.46	0.23	1.07	1.99
BHBA	1.14	0.19	0.83	1.57
Parity: 1	(ref)	-	-	-
2	5.56	4.32	1.21	25.50
3	12.67	9.23	3.04	52.79
> 3	20.16	17.21	3.78	107.49
Milk volume	1.13	0.24	0.74	1.72
Constant	0.09	0.06	0.03	0.31
High BCS, Low BW				
Albumin	1.29	0.17	1.00	1.66
BHBA	1.29	0.22	0.93	1.80
Parity: 1	(ref)	-	-	-
2	0.36	0.17	0.14	0.91
3	0.21	0.13	0.06	0.71
> 3	0.30	0.17	0.10	0.92
Milk volume	0.52	0.13	0.32	0.85
Constant	1.12	0.38	0.58	2.18
High BCS, High BW				
Albumin	1.48	0.22	1.11	1.98
BHBA	1.26	0.20	0.92	1.72
Parity: 1	(ref)	-	-	-
2	2.67	1.50	0.89	8.00
3	8.70	4.61	3.08	24.55
> 3	8.23	6.02	1.96	34.51
Milk volume	0.81	0.21	0.49	1.33
Constant	0.25	0.10	0.11	0.57

Table A8 (1): Peak milk cohort wet chemistry dietary data

Farm ID	Sample date	DM %	Moisture %	ME, MJ/kg	CP %	SP %CP	ADICP %	ADICP %CP	NDICP %	NDICP %CP	NFC %	SS %	Starch %	ADF %	aND Fom%
1	13/04/23	20.4	79.6	12.2	12.9	35.9	0.7	5.5	3.2	24.6	31.2	7.5	15.0	23.2	42.8
2	19/12/22	19.8	80.2	11.8	19.6	34.4	1.0	5.4	4.7	24.0	32.5	10.9	11.5	15.9	38.3
3	16/05/23	22.7	77.3	11.0	16.8	38.9	1.0	5.9	4.0	23.9	28.6	2.2	17.4	21.0	44.4
4	06/06/23	22.8	77.2	10.3	19.8	40.1					37.2	11.8	14.1	22.1	37.2
5	15/06/23	32.6	67.4	10.1	14.7	45.0	0.7	4.9	3.1	20.7	26.0	2.3	8.8	26.9	45.7
6	17/01/23	48.0	52.0	10.9	15.5	44.0	0.6	3.8	1.5	9.6	39.3	4.8	22.0	21.3	31.0
7	08/12/22	17.8	82.2	11.3	17.8	33.1	0.9	5.1	4.3	23.9	26.7	10.2	3.3	19.4	44.5
8	24/01/23	18.7	81.3	12.1	21.7	41.2	1.5	7.0	4.8	21.9	25.2	5.0	13.7	14.9	37.0
9	08/03/23	20.8	79.2	10.5	17.4	56.8	1.1	6.4	4.6	26.3	18.3	2.2	13.2	25.0	51.3
10	17/11/22	36.6	63.4	11.2	18.2	32.8	1.3	7.2	4.5	24.9	31.7	4.9	19.0	20.4	38.8
11	07/03/23	22.8	77.2	10.6	19.5	55.5	1.2	6.2	4.6	23.6	17.7	3.9	11.7	25.4	48.3
12	N/A - dry cows only sampled, no diet information														
13	12/04/23	21.3	78.7	12.2	20.0	39.6	1.4	7.1	4.2	20.9	31.7	4.6	22.3	13.1	32.8
14	03/04/23	20.3	79.7	11.2	22.0	40.3	1.6	7.1	4.7	21.3	22.2	4.8	16.0	22.2	39.4
15	31/01/23	25.1	74.9	11.5	19.5	35.5	1.2	6.0	4.9	25.2	31.4	4.2	19.0	18.4	38.7
16	19/05/23	50.9	49.1	10.7	17.2	49.0	0.6	3.4	1.4	7.9	37.4	3.6	23.6	23.3	30.7
17	20/01/23	46.0	54.0	10.8	16.6	46.0	0.8	4.7	1.6	9.5	35.1	4.9	18.9	22.8	31.8
18	14/02/23	50.4	49.6	10.7	18.2	40.0	1.6	9.0	3.2	17.3	40.2	4.5	21.8	24.1	30.4
19	10/01/23	55.0	45.0	10.9	17.0	42.0	1.1	6.4	1.7	9.8	39.0	8.7	22.0	22.0	35.0
20	09/01/23	49.7	50.3	10.4	13.5	40.0	0.6	4.4	1.8	13.1	41.1	2.3	30.7	24.3	32.3
21	28/02/23	51.2	48.8	10.9	18.3	38.0	1.4	7.5	2.7	14.9	39.4	2.8	22.5	22.9	28.6
22	16/02/23	43.6	56.4	10.1	14.8	46.0	0.9	6.1	1.5	9.9	34.0	5.4	16.9	27.1	38.2
23	30/11/22	45.7	54.3	10.6	17.5	35.0	1.0	6.0	2.5	14.1	35.6	2.2	21.2	24.3	33.6
23	30/11/22	49.3	50.7	10.8	17.0	34.0	1.7	10.2	3.3	19.4	36.1	2.8	20.5	22.3	32.8
24	19/01/23	48.4	51.6	11.4	18.3	34.0	1.1	6.3	2.8	15.2	42.2	3.7	30.3	18.4	28.3
25	01/12/22	36.7	63.3	10.4	17.5	39.0	1.0	5.6	3.0	17.1	31.4	13.8	6.7	26.2	37.9
26	22/03/23	50.0	50.0	10.7	13.8	33.0	0.9	6.5	1.8	13.0	21.7	38.1	5.4	21.6	24.4
27	01/03/23	45.3	54.7	10.9	18.7	43.0	1.5	8.1	2.7	14.2	36.9	3.1	23.1	22.7	30.6
28	15/02/23	53.6	46.4	9.8	14.5	42.0	1.2	8.3	1.8	12.4	31.5	3.9	16.1	29.9	40.7
29	18/01/23	43.4	56.6	11.1	16.0	46.0	0.8	5.2	1.5	9.3	45.0	1.9	29.7	19.6	25.8
30	10/01/23	62.5	37.5	11.4	18.0	30.0	1.0	5.3	2.6	14.4	38.1	3.5	22.8	18.7	32.3

ADF = acid detergent fibre; ADICP = acid detergent insoluble crude protein; aND Fom = amylase NDF organic matter; CP = crude protein; DM = dry matter; kg = kilograms; ME = metabolizable energy; MJ = megajoules; NDICP = neutral detergent insoluble crude protein; NFC = non-fibre carbohydrates; SP = soluble protein; SS = simple sugars

Table A8 (2): Peak milk cohort wet chemistry dietary data

Farm ID	Lignin %	Crude Fat %	Ash %	Ca %	P %	Mg %	K %	S %	Na %	Cl %	Fe PPM	Zn PPM	Cu PPM	Supplemental feed in dairy parlour bail
1		3.2	9.9	0.49	0.49	0.20	2.80							5kg barley ,3kg pellet
2	4.5	4.1	9.7	0.50	0.46	0.24	2.80		0.75	0.78	302	52	14	2.6kg wheat, 2.6kg DDG, 2.6kg commercial pellet
3	4.1	3.6	9.5	0.53	0.40	0.26	2.70		0.83	0.83	353	54	16	4kg wheat, 2kg corn silage, 0.4kg soybean, bicarb, monensin, lime
4	3.4		9.4	0.67	0.56	0.27	1.83	0.28	0.50	0.68	375	49	8	5kg pellet, 2.5kg barley, 3kg DDG
5	2.6	4.1	9.4	0.72	0.41	0.37	1.94	0.24	0.68	1.40	473	70	14	3.2kg wheat, 2.6kg DDG on feed pad
6	2.8	4.9	9.4	0.45	0.50	0.23	2.34	0.23	0.57	0.73	337	34	7	4.2kg wheat, 2kg corn silage on feed pad
7	4.8	4.4	10.6	0.55	0.45	0.25	3.10		0.86	0.89	328	54	15	1kg speciality pellets, 3kg DDG
8	3.1	4.4	11.7	0.52	0.46	0.46	3.53		0.21	1.96	324	72	0	4kg barley, 0.35kg speciality pellet
9	2.0	3.3	9.3	0.44	0.43	0.41	3.07		0.39	1.14	216	59	16	5.2kg barley, mix (dicalcium phosphate, lime, gypsum, dolomite)
10		3.9	8.6	0.63	0.40	0.20	1.42							9kg barley, 1.5kg canola, 0.25kg limestone, 0.03kg MgOx
11	2.8	4.0	10.3	0.47	0.49	0.42	2.86		0.38	0.98	208	66	15	Variable rate 3:1 chickpea:triticale mix, salt, lime, acid buffer, MgOx, mineral, vitamins, mycotoxin buffer. Average 4.6kg/cow
12	N/A - dry cows only sampled, no diet information													
13	2.9	4.7	10.4	0.39	0.47	0.40	3.08		0.39	1.59	275	57	15	6kg corn silage, 2kg commercial pellet
14	3.2	4.3	11.4	0.44	0.49	0.38	3.25		0.62	1.66	302	51	15	4kg wheat, 2kg barley, 1.6kg canola, bicarb/minerals
15	4.2	3.6	9.3	0.50	0.47	0.27	2.37		1.02	0.70	301	54	14	7.5kg barley, 1.4kg canola, bicarb, 0.4kg mix (16% dicalcium phosphate, 6% minerals, 37% oil, 40% Ca pellet, MgOx)
16	6.0	5.7	9.1	0.68	0.52	0.34	1.41	0.23	0.25	0.64	937	81	20	0.3kg wheat
17	4.0	6.6	9.9	0.89	0.44	0.35	1.24	0.27	0.76	1.30	768	78	24	2kg canola, 1.6kg wheat, 0.4kg lupin
18	6.3	5.1	6.2	0.57	0.47	0.31	1.53	0.25	0.11	0.28	298	32	9	0.2kg barley, 0.05kg canola
19	3.6	4.4	6.3	0.70	0.24	0.30	1.20	0.20	0.56	0.45	249	36	10	No feed in the bail
20	9.5	3.0		0.80	0.40	0.33	1.90	0.21	0.33	1.12	496	45	13	2.5kg barley
21	5.1	5.9	7.7	1.21	0.55	0.31	1.00	0.30	0.51	0.54	446	79	16	No feed in the bail
22	5.1	6.2	6.9	0.41	0.45	0.27	1.40	0.22	0.27	0.64	625	60	16	No feed in the bail
23	5.8	5.9	7.4	0.94	0.53	0.28	1.16	0.24	0.62	0.49	518	92	20	No feed in the bail
23	4.8	6.5	7.7	1.13	0.48	0.28	1.19	0.22	0.73	0.50	509	96	19	No feed in the bail
24	5.4	4.4	6.9	0.88	0.54	0.29	0.97	0.26	0.45	0.41	393	81	16	No feed in the bail
25	5.0	4.6	8.6	0.70	0.61	0.28	1.86	0.30	0.25	0.68	488	38	8	No feed in the bail
26	9.1	5.5		0.70	0.50	0.36	1.30	0.21	0.39	0.76	552	46	11	No feed in the bail
27	5.3	6.0	7.7	1.08	0.51	0.27	1.22	0.25	0.51	0.51	352	74	15	No feed in the bail
28	9.5	4.9	8.4	0.42	0.45	0.25	1.62	0.22	0.05	0.40	905	30	7	No feed in the bail
29	4.6	4.1	9.2	0.98	0.46	0.31	1.44	0.25	0.45	0.44	756	75	16	No feed in the bail
30	7.9	6.4	8.0	1.10	0.46	0.24	0.93	0.23	0.48	0.51	288	48	12	No feed in the bail

Ca = calcium; Cl = chloride ion; Cu = copper; DDG = dried distiller's grain; Fe = iron; K = potassium; kg = kilogram Mg = magnesium; MgOx = magnesium oxide; Na = sodium; P = phosphorous; PPM = parts per million; S = sulphur; Zn = zinc

Table A9: Dry cohort diet and housing qualitative information.

Farm ID	System	Sample date	Far off	Close up
1	Pasture	25/01/23	Heifers and cows separate on multiple extensive paddocks: sparse native pastures with kikuyu (*Cenchrus clandestinus*, previously *Pennisetum clandestinum*) base	Heifers and cows together: cereal hay, very limited kikuyu pasture and 3 kg of formulated, grain-based transition diet
2	Pasture	23/01/23	Heifers join dry cows based on visual appraisal. Extensive pasture: dense, mature kikuyu, native grasses, 3kg every 2d of milker concentrate (38% wheat, 38% dried distillers grain (DDG), 24% mineral mix)	Heifers and cows together: Cereal hay, minimal pasture, 4kg commercial transition feed pellet.
3	Pasture	30/01/23	Heifers and cows separate on extensive pastures: Mature kikuyu, kikuyu silage, couch (*Elymus repens*), *Paspalum* spp.	Heifers and cows together: Transition lead feed, oaten hay, minimal kikuyu pasture
4	Pasture	06/04/23	Heifers and cows separate: Ryegrass paddocks. No silage	Heifers and cows together: 3wks cereal hay 3kg, springer pellets 4kg, bare paddocks
5	Pasture(N/A, no dry cows samples)			
6	Pasture	17/01/23	Heifers and cows separate on bare paddocks: heifer – grazed alfalfa, mature ryegrass; cows – corn silage 27%, ryegrass silage 63%, wheat straw 6% canola meal 4%	Heifers and cows together on dry lot: Corn silage 26%, canola meal 4.5% wheat grain 7.4%, oat hay 28%, barley 7.4% ryegrass silage 20%, anionic protein meal and wheat middlings 6.5%
7	Pasture	08/12/22	Heifers and cows separate: heifers in two groups – extensive pastures with very mature kikuyu, woody weeds, clover; cows – extensive pastures kikuyu and mature ryegrass	Heifers and cows separate: kikuyu as main forage. No transition pellet
8	Pasture	24/01/23	Heifers and cows separate on extension pasture: Kikuyu, sparse mature ryegrass, clover, *Poa* spp.	Heifers and cows together: Low quality kikuyu silage, transition feed 2kg, sparse kikuyu pasture, sparse mature ryegrass

Table A9: Dry cohort diet and housing qualitative information.

Farm ID	System	Sample date	Far off	Close up
9	Pasture	08/03/23	Heifers and cows separate on extensive pasture: heifers – kikuyu, rhodes grass (*Chloris* spp.), minimal annual ryegrass; cows – *Setaria* spp., rhodes grass, kikuyu	Heifers and cows together: 3kg grain (50% barley, 50% wheat), 2kg springer ration, anionic salt, minimal kikuyu, rhodes grass silage (*Chloris* spp.)
10	Pasture	17/11/22	Heifers and cows separately on extensive pastures: mature kikuyu, woody weeds, *Poa* spp., couch (*Elymus repens*), *Paspalum* spp., clovers	Heifers and cows together: 15kg corn silage, 3.5kg barley, 6.5kg millet silage, 250g anionic salts
11	Pasture	07/03/23	Heifers and cows separate: Heifers - run through milking robot, 2kg milker ration, kikuyu dominant pasture; cows - extensive pastures – Kikuyu, couch (*Elymus repens*), woody weeds, *Rumex* spp., *Paspalum* spp.	Heifers and cows separate: heifers - run through robot, 2kg milker ration, kikuyu dominant, no zeolite; cows - oaten hay, 14d zeolite
12	Pasture	10/03/23	Heifers and cows separately on extensive paddocks: Kikuyu dominant, couch (*Elymus repens*), *Paspalum* spp., clover, rushes (*Jancus* spp.)	Heifers and cows together: 3wks oaten hay and 3kg springer ration.
13	Pasture	12/04/23	Heifers and cows separate on multiple extensive paddocks: Mixed pasture, mature kikuyu, mature ryegrass, clover, woody weeds.	Heifers and cows together: bare paddock, commercial lead feed pellet, cereal hay
14	Pasture	27/01/23	Heifers and cows separate, across multiple (> 4) properties with extensive pastures: native pastures (*Poa* spp), couch (*Elymus repens*), *Paspalum* spp., kikuyu, clover, woody weeds	Heifers and cows together: Cereal hay, kikuyu, cereal based transition feed
15	Pasture	31/01/23	Heifers and cows separate on extensive pastures: Kikuyu and ryegrass base	Heifers and cows together: Limited kikuyu and ryegrass, some oats (30%), oaten hay (50%), mix(barley, $MgCl_2$, oil, minerals) (20%).
16	Confinement	20/03/23	Heifers and cows on separate pastures, multiple locations: heifers - straw and cereal silage; cows - 35kg/head TMR mix of straw 13%, cereal silage 87%, minerals	Heifers and cows on separate paddock: heifers - anionic minerals added to far-off diet; cows - 1kg of canola meal added to added to far-off diet and anionic minerals

Table A9: Dry cohort diet and housing qualitative information.

Farm ID	System	Sample date	Far off	Close up
17	Confinement	13/02/23	Heifers and cows separate on bare paddocks: Corn silage, straw, cereal hay, mineral mix	Heifers and cows together on dry lot: Corn silage, negative dietary cation difference, grain, canola, cereal hay
18	Confinement	14/02/23	Heifers on pasture and cows (dry lot): heifers - kikuyu, ryegrass; cows - lactating ration TMR refusals, straw.	Heifers and cows together on dry-lot: corn silage, almond hulls, canola meal, oat hay, straw.
19	Confinement	10/01/23	Heifers and cows separate on bare paddocks: Corn silage, cereal hay, mineral mix	Heifers and cows together: bare paddock, 4-5kg of a barley/wheat based, BioChlor monensin, flavomycin, trace elements lead feed including lupins and canola, ad lib cereal hay with a small amount of brewers grain and citrus pulp
20	Confinement	09/01/23	Heifers and cows separate on extensive pastures: lucerne dominant	Heifers and cows separate: 4-5kg of a barley based BioChlor, monensin, flavomycin, trace elements lead-feed including lupins and canola, *ad lib* cereal hay with a small amount of corn silage
21	Confinement	28/02/23	Heifers and cows separate in free-stalls: Cereal silage, cotton seed, minerals	Heifers and cows together in free-stalls: Cereal silage, straw, zeolite
22	Confinement	16/02/23	Heifers and cows separate on extensive pasture: heifers - adlib hay; cows - on basic hay for 1-2 weeks, move to different dry paddock with ad lib hay and straw.	Heifers and cows together on pasture: 3 weeks prior to calving, hay and lead feed pellets on minimum pasture that is kikuyu dominant with *Paspalum* spp.
23	Confinement	30/11/22	Heifers and cows separate on pasture: Kikuyu and fescue (*Festuca arundinacea*) pasture, TMR of lactating ration refusals - corn silage forage base	Heifers and cows together on pasture: at 250d carried calf, TMR with zeolite, corn silage forage based
24	Confinement	19/01/23	Heifers and cows separate in compost bedded pack barns: lactating cow refusals (23%), straw (15%), low protein cereal (53%), canola (6.5%), mineral mix (3%)	Heifers and cows separate in compost bedded pack barns: corn silage (36%), straw (16%), canola (13%), cereal (7.5%), concentrate mix (16%), zeolite

Table A9: Dry cohort diet and housing qualitative information.

Farm ID	System	Sample date	Far off	Close up
25	Confinement	01/12/22	Heifers and cows on separate pasture: heifers - TMR with corn silage, grass silage, grain, DDG; cows - TMR with corn silage grass silage, grain fines, DDG	Heifers and cows together on pasture: 28d prior to calving, corn silage wheat hay, springer pellet
26	Confinement	22/03/23	Heifers and cows separate on pasture: Grass silage, sorghum, cereal hay, wheat and canola	Heifers and cows together on pasture: Grass silages, sorghum, cereal hay, wheat, canola and zeolite. Cows 14d, heifers 10d.
27	Confinement	01/03/23	Heifers and cows in separate free-stalls: Cereal silage (58%), lactation cow refusals (20%), concentrate mix (12%), straw (8%), lucerne silage (3%)	Heifers and cows separate in free-stalls: Cows - Corn silage (35%), straw (16%), cereal silage (14%), concentrate/meal (23%), almond hulls (4%), lucerne silage (3%). Heifers - corn silage (24%), cereal silage (21%), straw (14%), concentrate/meal (22%), almond hull (6%)
28	Confinement	15/02/23	Heifers and cow separate in dry lots: Wheat silage (35%), corn silage (24%), straw (12%), almond hulls (12%), vetch (*Vicia* spp.) silage (11%), canola (5%), limestone (1.3%)	Heifers and cow separate in dry lots: Corn silage (34%), straw (18%), wheat grain (16%), wheat hay (16%), canola (13%), commercial lead feed (3.5%)
29	Confinement	18/01/23	Heifers and cows on separate dry-lots: wheat vetch (55%), wheat straw (18%), almond hulls (27%)	Heifers and cows together in compost bedded pack barn: Corn silage (22%), wheat and vetch (*Vicia* spp.) silage (42%), mixed pellet (36%)
30	Confinement	10/01/23	Heifers and cows separate on pasture: 2.5kg wheat, 0.5kg canola, mature ryegrass	Heifers and cows together on bare pasture: wheat grain (20%), dried wheat distillers (20%), anionic protein meal, minerals and wheat middlings (7%) ryegrass silage (17%), oat hay (36%)

Table A10: Marginal predictions for the 100 d in-calf rate (following logistic regression: 100DICR) and hazards of pregnancy (following Weibull parametric survival analysis: HPREG) with independent variables of production system, parity, and the season a cow became eligible for breeding, and interactions.

	100DICR (95%CI)		Relative HPREG (95%CI)	
System	Pasture	Confinement	Pasture	Confinement
Parity				
1st	38.16 (30.08, 46.24)^{efgh}	40.39 (36.68, 44.11)^{fh}	1.14 (0.93, 1.34)^{ehij}	1.33 (0.86, 1.79)^{il}
2nd	39.61 (32.92, 46.31)^{gh}	38.15 (33.49, 42.81)^{dfh}	1.18 (0.90, 1.47)^{jkl}	1.19 (0.77, 1.61)^{gik}
3rd	34.38 (28.16, 40.60)^{cdef}	34.41 (29.50, 39.31)^{deg}	1.02 (0.76, 1.28)^{ehi}	1.05 (0.68, 1.41)^{cfhj}
4th	32.00 (26.36, 37.65)^{cd}	28.40 (23.36, 33.44)^{bc}	0.92 (0.68, 1.15)^{dfg}	0.84 (0.53, 1.16)^{bde}
> 4th	25.68 (20.68, 30.68)^{ab}	23.33 (19.83, 26.84)^{a}	0.75 (0.53, 0.97)^{abc}	0.65 (0.39, 0.90)^{a}
Season				
Summer	28.47 (20.55, 36.39)^{ab}	30.55 (27.15, 33.95)^{a}	0.91 (0.81, 1.02)^{ab}	1.08 (0.70, 1.45)^{ab}
Autumn	32.33 (25.41, 39.26)^{ab}	34.35 (29.13, 39.56)^{bc}	1.15 (0.81, 1.49)^{ab}	1.18 (0.76, 1.60)^{ac}
Winter	41.22 (34.61, 47.83)^{c}	33.85 (29.08, 38.62)^{bc}	1.19 (0.88, 1.49)^{bc}	1.05 (0.67, 1.44)^{ab}
Spring	33.28 (26.57, 39.98)^{ab}	32.46 (26.93, 37.99)^{ab}	0.90 (0.71, 1.08)^{a}	1.00 (0.63, 1.36)^{ab}
Global	33.95 (27.97, 39.94)^{a}	32.93 (28.94, 36.92)^{a}	1.04 (0.82, 1.25)^{a}	1.08 (0.70, 1.45)^{a}

Cells that share a superscript letter are not different at the 5% confidence level, within each model subsection. As marginal hazard ratios are calculated holding other variables at their mean, there are no referent groups to report.

Table A11: Marginal predictions for the DIM of first mastitis event (following linear regression) and hazards of mastitis (following Weibull parametric survival analysis: HMAST) by parity and production system.

	DIM first mastitis event (95%CI)		Relative HMAST (95%CI)	
System	Pasture	Confinement	Pasture	Confinement
Parity				
1st	98.5 (67.2, 129.8)^{abc}	133.1 (112.8, 153.5)^{c}	1.00 (referent)^{ab}	1.29 (0.44, 2.13)$^{a\ c}$
2nd	98.3 (80.8, 115.8)^{ab}	128.8 (117.1, 140.6)^{c}	1.85 (1.44, 2.26)^{cdef}	2.56 (0.86, 4.27)^{bdgi}
3rd	103.4 (73.6, 133.3)^{abc}	115.4 (105.4, 125.3)^{bc}	2.25 (1.74, 2.77)^{gh}	3.48 (1.28, 5.67)^{ehj}
4th	92.7 (78.1, 107.4)^{a}	114.9 (104.2, 125.6)^{bc}	2.66 (1.80, 3.52)^{gh}	3.88 (1.46, 6.30)^{fhk}
> 4th	103.6 (83.1, 124.1)^{abc}	99.8 (91.4, 108.2)^{ab}	3.80 (2.35, 5.25)^{ijk}	4.31 (1.77, 6.85)^{hk}
Global	100.0 (91.1, 108.8)^{a}	117.0 (107.6, 126.3)^{b}	2.13 (1.62, 2.64)^{a}	2.85 (1.10, 4.61)^{a}

Cells that share a superscript letter are not different at the 5% confidence level, within each model subsection. As marginal hazard ratios are calculated with other variables held at mean, there are no referent groups in the global category.

Table A12: Marginal predictions for the DIM of first lameness event (following linear regression) and hazards of lameness (following Weibull parametric survival analysis: HLAME) by parity and production system.

	DIM first lameness event (95%CI)		Relative HLAME (95%CI)	
System	Pasture	Confinement	Pasture	Confinement
Parity				
1st	112.6 (81.1, 144.1)[ab]	120.2 (84.6, 155.7)[ac]	1.00 (referent)[ab]	0.82 (0.04, 1.61)[ab]
2nd	166.5 (125.5, 207.6)[abcde]	167.0 (147.5, 186.6)[e]	0.87 (0.57, 1.16)[ab]	0.76 (0.22, 1.31)[a]
3rd	161.1 (123.3, 198.8)[cde]	156.6 (137.8, 175.5)[cde]	0.96 (0.74, 1.17)[ab]	1.08 (0.23, 1.93)[abc]
4th	170.5 (139.6, 201.3)[de]	149.3 (124.5, 174.0)[bde]	1.32 (1.04, 1.61)[acd]	1.39 (0.24, 2.54)[abcd]
> 4th	170.3 (130.2, 210.3)[abcde]	136.7 (113.6, 159.9)[abcd]	2.20 (1.12, 3.27)[cd]	1.58 (0.36, 2.81)[bd]
Global	152.4 (135.0, 169.7)[a]	143.1 (123.5, 162.6)[a]	1.19 (0.92, 1.44)[a]	1.04 (0.24, 1.84)[a]

Cells that share a superscript letter are not different at the 5% confidence level, within each model subsection. As marginal hazard ratios are calculated with other variables held at mean, there are no referent groups in the global category.

Table A13: Detailed information on Mouse Splash® Lipidomix standards (Avanti Lipids, Birmingham, AL) that were used as internal standards to correct for run-order and batch effects and to quantify the analysed lipids.

Compound Name	Molecular Weight	Exact Mass	Chemical Formula	Conc. (µg/mL)[1]	Conc. µM[1]
15:0-18:1(d7) PC	753.11	752.61	$C_{41}H_{73}D_7NO_8P$	75.3	100
15:0-18:1(d7) PE	711.03	710.56	$C_{38}H_{67}D_7NO_8P$	5.0	7
15:0-18:1(d7) PS (Na Salt)	777.02	776.53	$C_{39}H_{66}D_7NNaO_{10}P$	15.5	20
15:0-18:1(d7) PG (Na Salt)	764.02	763.54	$C_{39}H_{67}D_7NaO_{10}P$	3.8	5
15:0-18:1(d7) PI (NH4 Salt)	847.13	846.60	$C_{42}H_{75}D_7NO_{13}P$	16.9	20
15:0-18:1(d7) PA (Na Salt)	689.94	689.50	$C_{36}H_{61}D_7NaO_8P$	6.9	10
18:1(d7) Lyso PC	528.72	528.39	$C_{26}H_{45}D_7NO_7P$	23.8	45
18:1(d7) Lyso PE	486.64	486.35	$C_{23}H_{39}D_7NO_7P$	1.0	2
18:1(d7) Chol Ester	658.16	657.64	$C_{45}H_{71}D_7O_2$	164.5	250
C18(Plasm)-18:1(d9) PC	781.19	780.67	$C_{44}H_{77}D_9NO_8P$	15.6	20
15:0-18:1(d7) DAG	587.98	587.55	$C_{38}H_{61}D_7O_5$	8.8	15
15:0-18:1(d7)-15:0 TAG	812.37	811.77	$C_{51}H_{89}D_7O_6$	28.4	35
d18:1-18:1(d9) SM	738.12	737.64	$C_{41}H_{72}D_9N_2O_6P$	14.8	20
C18(Plasm)-18:1(d9) PE	739.11	738.62	$C_{41}H_{71}D_9NO_8P$	3.7	5

1: Concentrations are based on isotropic purity of each individual compound

Table A14: Parameterization for the genetic algorithm variable selection for regression analysis in PLS_Toolbox (Ver 9.3.1, Eigenvector Research Inc, Manson, WA) procedure for both the dry and peak-milk cohorts. We refer to the PLS_Toolbox instruction manual for further details.

Parameter	**Value**
Population size	100
Window width	1
% Initial terms	35
Penalty slope	0
Maximum Generations	100
% at Convergence	50
Mutation Rate	0.005
Crossover type	Single
Regression choice	Multivariate linear regression
Cross-validation parameters	Random
Cross-validation splits	10
Cross-validation iterations	5
Replicate runs	5
X-block pre-processing	External parameter orthogonalization (class: breed/farm, 1 partial components), autoscale
Y-block pre-processing	Log10, mean centre

Eigenvector Research. 2011. Genetic Algorithms for Variable Selection. Accessed Jun 27 2024. https://www.wiki.eigenvector.com/index.php?title=Genetic_Algorithms_for_Variable_Selection.

Table A15: Fatty acid profile of kikuyu grass (*Cenchrus clandestinus*, previously *Pennisetum clandestinum*). Reported as g/kg ± se from six samples per group. (Lean, I.J., unpublished)

	Without Nitrogen	With Nitrogen
C16:0	5.30 ± 0.29	4.85 ± 0.73
C16:1	0.43 ± 0.04	0.37 ± 0.07
C18:0	0.53 ± 0.05	0.40 ± 0.06
C18:1	1.34 ± 0.13	1.09 ± 0.12
C18:2	5.43 ± 0 0.46	4.52 ± 0.53
C18:3	10.73 ± 1.06	9.39 ± 1.64
Polyunsaturated	16.36 ±1.50	14.11± 2.14

Table A16: Cow exit schema provided to farm managers for all culling cases. Cows could have up to three recorded exit reasons.

Termination Type	Category	Description
Sold	Low production or transferred to beef production.	Low production or transferred to beef production.
Sold	Old age	Old age
Sold	Udder	Chronic clinical mastitis (milk quality visibly affected) or based on pathogen identification (e.g. mycoplasma, S.aureus, Pseudomonas)
		Chronically elevated individual somatic cell count (milk quality not visibly affected)
		3 Teat cow
		Physical udder health problem (ruptured suspensory ligament, teat injuries)
		Ease of milking
		Other (*add comment*)
Sold	Reproduction	Abortion
		Failure to conceive (failure to conceive after three or more services and no reproductive tract lesion identified by veterinarian)
		Other (e.g. reproductive tract pathology including infections, cystic ovarian disease, and other identifiable lesions, *add comment*)
Sold	Type Defect	Type Defect
Sold	Temperament	Temperament
Sold	Sold for Dairying	Sold for Dairying
Sold	Lameness	Leg
		Foot
		Lameness, other (*add comment*)
Sold	Injury	Injury - **(Right censored at date of exit)**
Sold	Miscellaneous disease	Miscellaneous disease (bloat, gastrointestinal disease, and others)
Sold	Eye cancer	Eye cancer
Sold	Other	Other (*add comment*)
Other	Lost or missing	Unknown date and reason for cow exit – **(lost to follow up, excluded)**

Table A17: Cow exit schema provided to farm managers for all mortality cases. Cows could have up to three recorded exit reasons.

Termination Type	Category	Description
Died	Metabolic Disease	Milk fever
		Grass tetany
		Ketosis
		Other metabolic disease (add comment)
Died	Gastrointestinal	Bloat
		Gastric accidents (e.g. Displaced abomasum, caecal torsions)
		Diarrhoea
		Johnes Disease
		Other (e.g. Acidosis, haemorrhagic bowel, Johnes, add comment)
Died	Other	Respiratory disease
		Neurological (excl. paralysis)
		Botulism
		Plant toxicity
		Other (add comment)
		Reason unknown
Died	EBL	Enzootic bovine leukosis
Died	Udder	Acute mastitis
		Udder Injury
		Other udder (add comment)
Died	Calving Difficulties	Dystocia
		Uterine prolapse
		Acute metritis
		Other calving (add comment)
Died	Paralysis	Paralysis
	Lameness	Lameness not paralysis or accident
Died	Accident	Broken limb - **(Right censored at date of exit)**
		Other accident (add comment) - **(Right censored at date of exit)**

Table A18: The complete list of plasma lipid classes and species targeted and quantified in the liquid chromatography mass spectrometry analysis.

Lipid class	Abbreviation	Species targeted
Phosphatidylcholine	PC	PC(31:1), PC(31:0), PC(30:1), PC(30:0), PC(29:0), PC(28:0), PC(33:1), PC(33:0), PC(32:2), PC(32:1), PC(32:0), PCN1, PC(34:3), PC(34:2), PC(34:1), PC(35:3), PC(35:2), PC(35:1), PC(37:3), PC(37:2), PC(36:5), PC(36:4), PC(36:3), PC(36:2), PC(36:1), PC(40:7), PC(40:6), PC(40:5), PC(40:4), PC(38:6), PC(38:5), PC(38:4), PC(38:3), PC(38:2)
Lyso-phosphatidylcholine	LPC	LPC(14:0), LPC(16:1), LPC(15:0), LPC(18:3), LPC(18:2), LPC(17:1), LPC(17:0), LPC(16:0), LPC(20:5), LPC(20:4), LPC(20:3), LPC(18:1), LPC(18:0), LPC(22:6), LPC(22:5), LPC(20:0)
Ether-linked phosphatidylcholine	PC-O	PC(O-34:0), PC(O-33:2), PC(O-32:2), PC(O-32:1), PC(O-30:0), PC(O-34:3), PC(O-34:2), PC(O-34:1), PC(O-38:5), PC(O-38:4), PC(O-37:5), PC(O-36:4), PC(O-36:3), PC(O-36:2), PC(O-36:1)
Phosphatidylethanolamine	PE	PE(35:2), PE(35:1), PE(34:3), PE(34:2), PE(34:1), PE(33:1), PE(36:5), PE(36:4), PE(36:3), PE(36:2), PE(36:1), PE(40:6), PE(40:5), PE(38:6), PE(38:5), PE(38:4), PE(38:3), PE(38:1)
Lyso-phosphatidylethanolamine	LPE	LPE(18:3), LPE(18:2), LPE(18:1)
Ether-linked phosphatidylethanolamine	PE-O	PE(O-34:3), PE(O-34:2), PE(O-34:1), PE(O-33:2), PE(O-33:1), PE(O-32:2), PE(O-32:1), PE(O-40:6), PE(O-40:5), PE(O-38:5), PE(O-38:4), PE(O-36:5), PE(O-36:4), PE(O-36:3), PE(O-36:2), PE(O-36:1)
Phosphatidylinositol	PI	PI(33:1), PI(33:0), PI(32:1), PI(32:0), PI(31:0), PI(40:6), PI(40:5), PI(38:6), PI(38:5), PI(38:4), PI(38:3), PI(38:2), PI(37:4), PI(37:3), PI(37:2), PI(36:5), PI(36:4), PI(36:3), PI(36:2), PI(36:1), PI(35:3), PI(35:2), PI(35:1), PI(34:3), PI(34:2), PI(34:1), PI(34:0)
Sphingomyelin	SM	SM(31:1), SM(30:1), SM(28:1), SM(34:4), SM(32:2), SM(32:1), SM(34:2), SM(34:1), SM(33:2), SM(33:1), SM(36:4), SM(36:2), SM(36:1), SM(35:2), SM(35:1), SM(38:2), SM(38:1), SM(37:1), SM(41:3), SM(41:2), SM(41:1), SM(40:3), SM(40:2), SM(40:1), SM(39:2), SM(39:1), SM(44:5), SM(44:4), SM(44:2), SM(43:4), SM(43:3), SM(43:2), SM(43:1), SM(42:3), SM(42:2), SM(42:1)
Triacylglycerol	TG	TG(54:1), TG(54:2), TG(54:3), TG(52:1), TG(52:2), TG(52:3), TG(51:1), TG(51:2), TG(50:1), TG(50:2), TG(50:3), TG(49:0), TG(49:1), TG(49:2), TG(48:0), TG(48:1), TG(48:2), TG(47:0), TG(47:1), TG(46:0), TG(45:0)

Table A19: The reported culling reasons for the dry-cohort cows (n = 717 cows). Up to three reported culling reasons per cow were permitted

Category	Sub-category	% of Cohort	% of Sold	Count
Low production	-	4.3	14.4	31
Old age	-	1.5	5.1	11
Udder	-	12.1	40.5	87
	Clinical mastitis	5.3	17.7	38
	Physical issue	3.8	12.6	27
	SCC	2.2	7.4	16
	3 Teats	1.3	4.2	9
	Ease of Milking	0.4	1.4	3
	Other Udder	0.1	0.5	1
Infertility	-	9.6	32.1	69
	Failure to conceive	7.9	26.5	57
	Abortion	1.5	5.1	11
	Other repro	0.1	0.5	1
Type Defect	-	0.4	1.4	3
Temperament	-	0.8	2.8	6
Sold for Dairying	-	1.0	3.3	7
Lameness	-	2.8	9.3	20
	Foot	1.3	4.2	9
	Leg	1.3	4.2	9
	Other	0.4	1.4	3
Injury	-	0.4	1.4	3
Other	-	2.1	7.0	15
	Misc. disease	1.1	3.7	8

Table A20: The reported culling reasons for the peak-milk cohort cows (n = 794 cows). Up to three reported culling reasons per cow were permitted

Category	Sub-category	% of Cohort	% of Sold	Count
Low production	-	5.5	17.7	44
Old age	-	1.8	5.6	14
Udder	-	9.7	31.0	77
	Clinical mastitis	6.2	19.8	49
	SCC	1.4	4.4	11
	Physical issue	1.3	4.0	10
	3 Teats	0.8	2.4	6
	Ease of Milking	0.5	1.6	4
Infertility	-	11.3	36.3	90
	Failure to conceive	10.2	32.7	81
	Abortion	1.3	4.0	10
	Other reproduction	0.3	0.8	2
Type Defect	-	0.5	1.6	4
Temperament	-	0.8	2.4	6
Sold for Dairying	-	1.6	5.2	13
Lameness	-	1.8	5.6	14
	Leg	1.0	3.2	8
	Foot	0.8	2.4	6
Injury	-	1.0	3.2	8
Other	-	2.9	9.3	23
	Misc. disease	2.0	6.5	16
	Eye cancer	0.3	0.8	2

Table A21: The reported mortality reasons for the dry-cohort cows (n = 717 cows). Up to three reported culling reasons per cow were permitted

Category	Sub-category	% of Cohort	% of Died	Count
Metabolic	-	0.4	4.1	3
	Milk fever	0.4	4.1	3
Gastrointestinal	-	1.4	13.5	10
	Diarrhoea	0.6	5.4	4
	Other GI	0.4	4.1	3
	Gastric accidents	0.3	2.7	2
	Bloat	0.1	1.4	1
Other	-	2.1	20.3	15
	Reason unknown	1.0	9.5	7
	Other	0.8	8.1	6
	Respiratory disease	0.3	2.7	2
Udder	-	2.5	24.3	18
	Acute mastitis	2.5	24.3	18
Calving	-	1.4	13.5	10
	Dystocia	1.3	12.2	9
	Other calving	0.1	1.4	1
Lameness	-	0.7	6.8	5
	Other lameness	0.7	6.8	5
	Paralysis	0.6	5.4	4
Accident	-	1.7	16.2	12
	Other accident	1.0	9.5	7
	Broken limb	0.7	6.8	5

Table A22: The reported mortality reasons for the dry-cohort cows (n = 794 cows). Up to three reported culling reasons per cow were permitted

Category	Sub-category	% of Cohort	% of Died	Count
Metabolic	-	0.6	7.6	5
	Milk fever	0.6	7.6	5
Gastrointestinal	-	0.6	7.6	5
	Gastric accidents	0.3	3.0	2
	Diarrhoea	0.3	3.0	2
	Other GI	0.1	1.5	1
Other	-	2.8	33.3	22
	Other	1.4	16.7	11
	Reason unknown	1.3	15.2	10
	Respiratory disease	0.1	1.5	1
Udder	-	1.9	22.7	15
	Acute mastitis	1.8	21.2	14
	Other udder	0.1	1.5	1
Calving	-	0.3	3.0	2
	Dystocia	0.3	3.0	2
Lameness	-	0.8	9.1	6
	Other lameness	0.8	9.1	6
Accident	-	1.5	18.2	12
	Other accident	1.1	13.6	9
	Broken limb	0.4	4.5	3

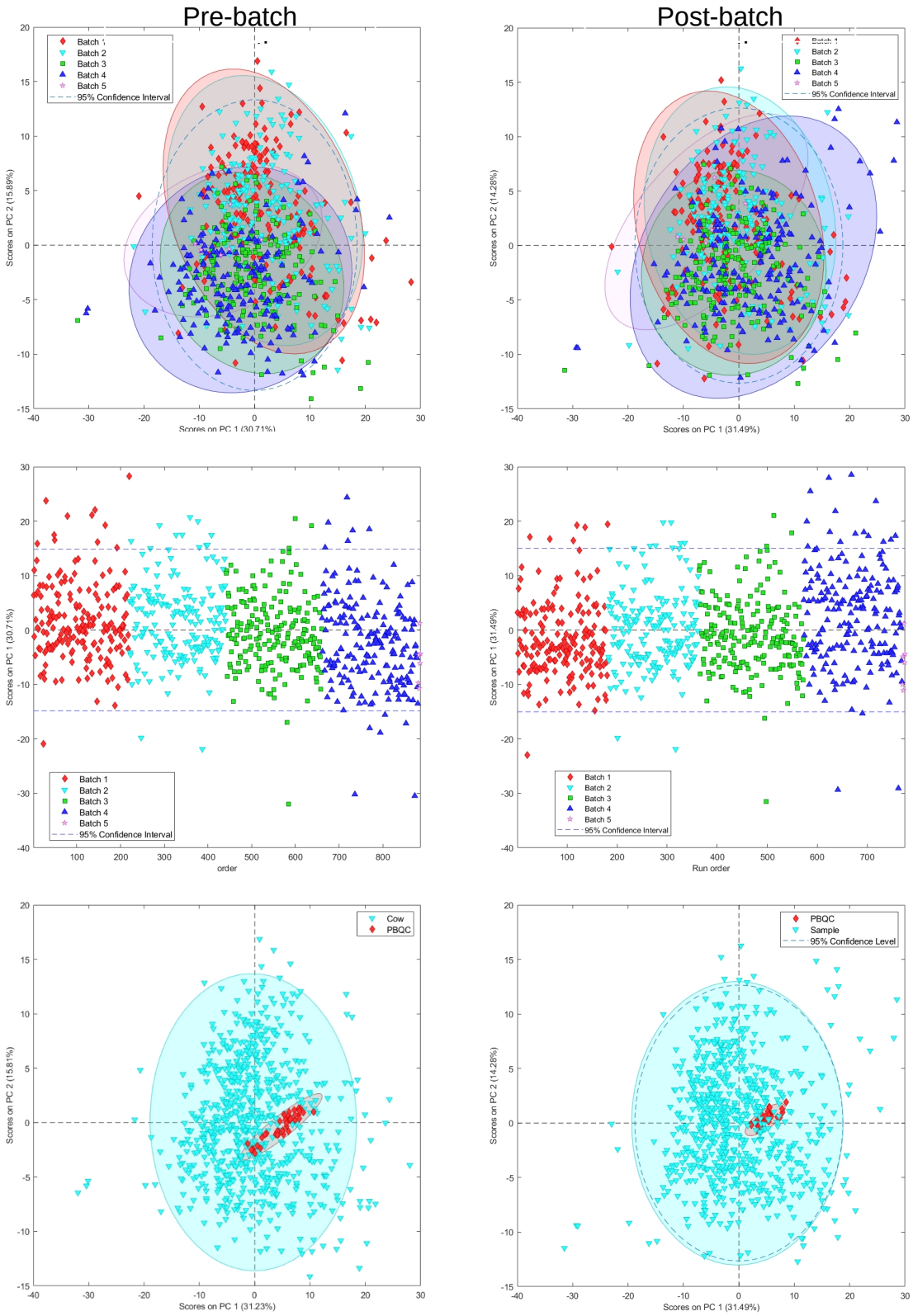

Figure A1: Principal component analysis of batch and run-order effects of the dry cohort. Lipid peaks were auto-scaled. Though evidence of strong batch effects were absent, correction visually tightened the pooled quality control samples indicating improved data quality. Analysis performed in and figures produced in PLS_Toolbox (Ver 9.3.1, Eigenvector Research Inc, Manson, WA).

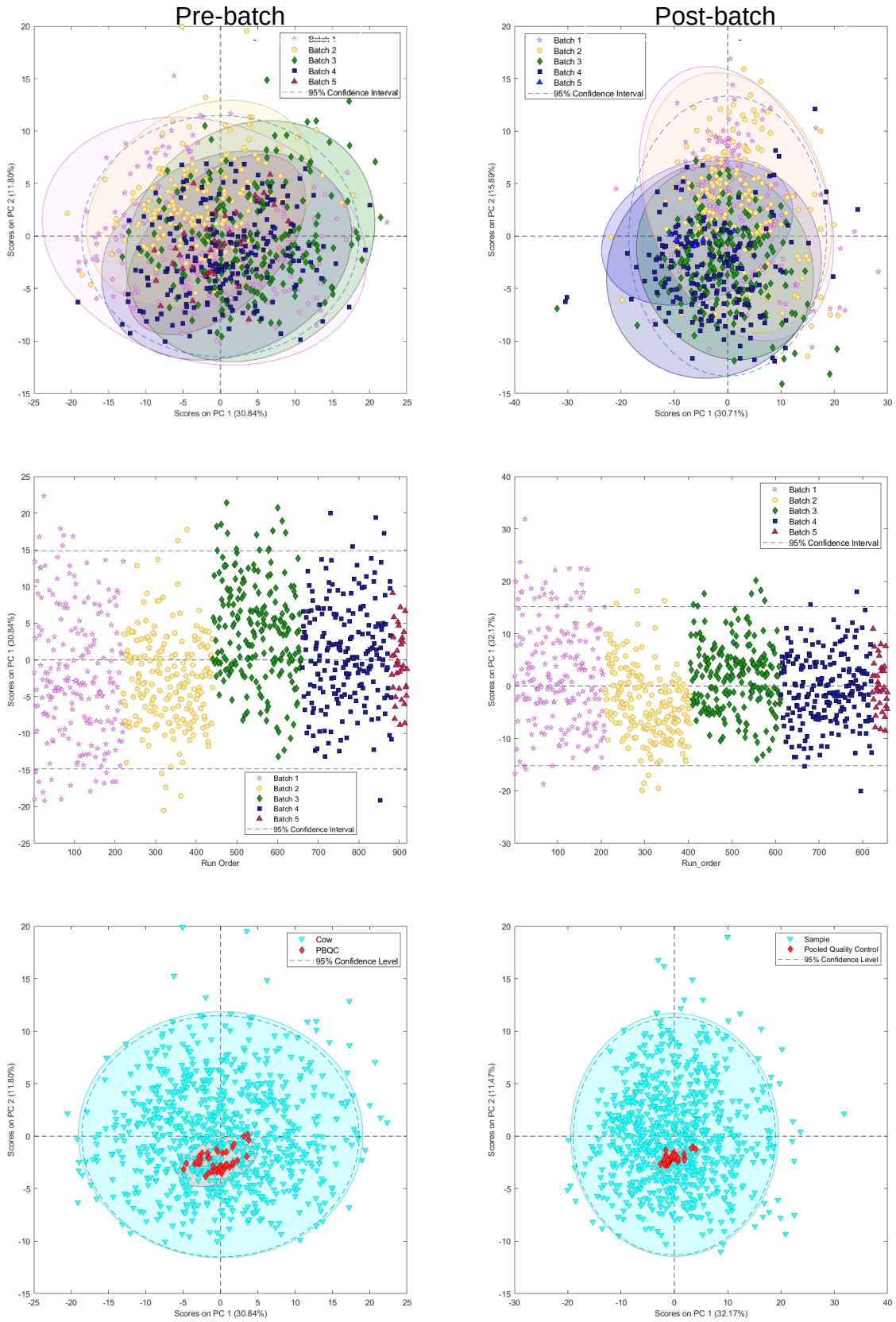

Figure A2: Principal component analysis of batch and run-order effects of the peak-milk cohort. Lipid peaks were auto-scaled. The is evidence of moderate run-order effect in batch 3. The pooled quality control samples indicating improved data quality post-correction. Analysis performed in and figures produced in PLS_Toolbox (Ver 9.3.1, Eigenvector Research Inc, Manson, WA).

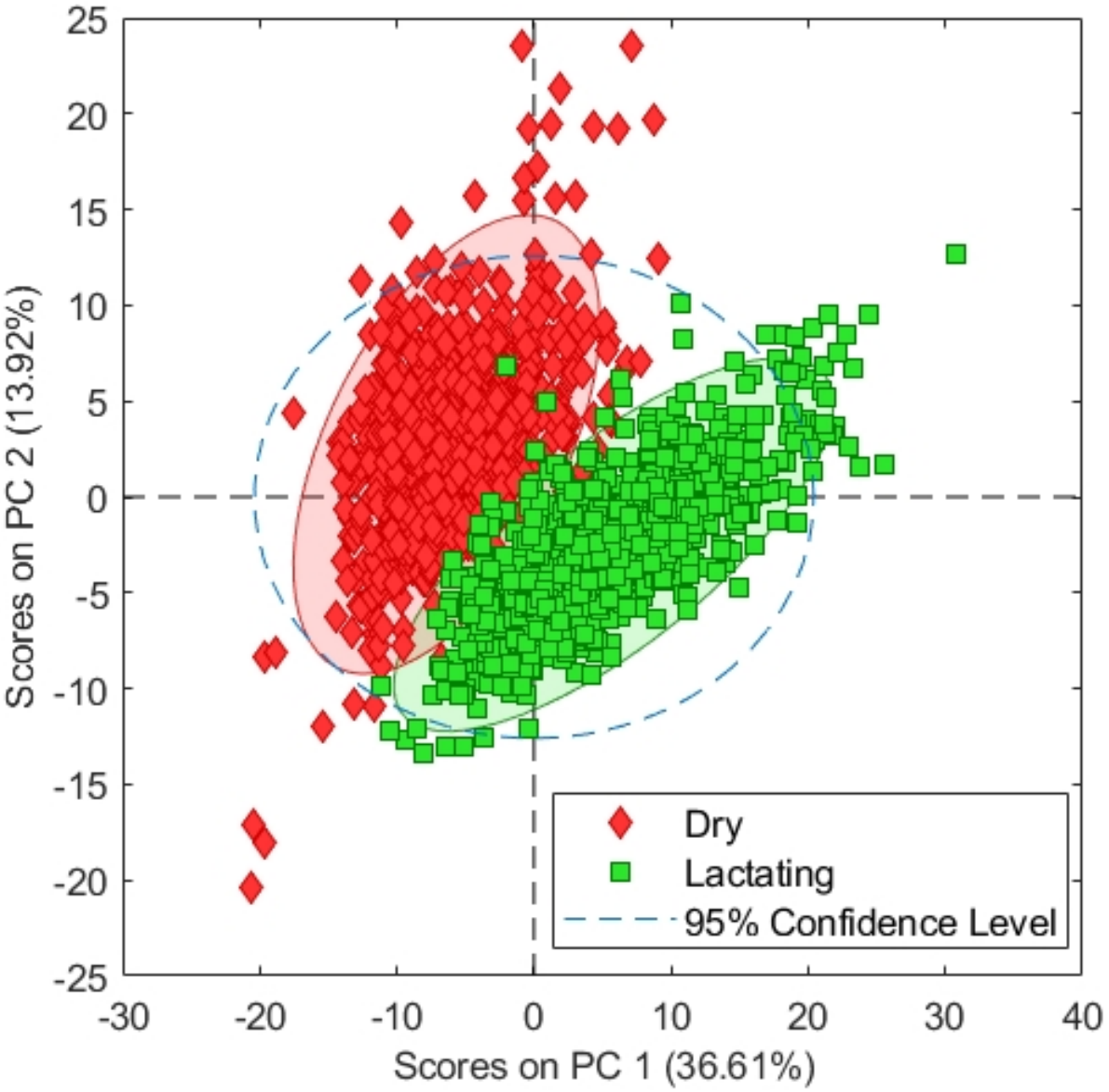

Figure A3: Principal component analysis that included both dry and lactating cohorts, autoscaling pre-processing of lipid data. A decision was made to perform analysis separately for the two cohorts. Analysis performed in and figures produced in PLS_Toolbox (Ver 9.3.1, Eigenvector Research Inc, Manson, WA).

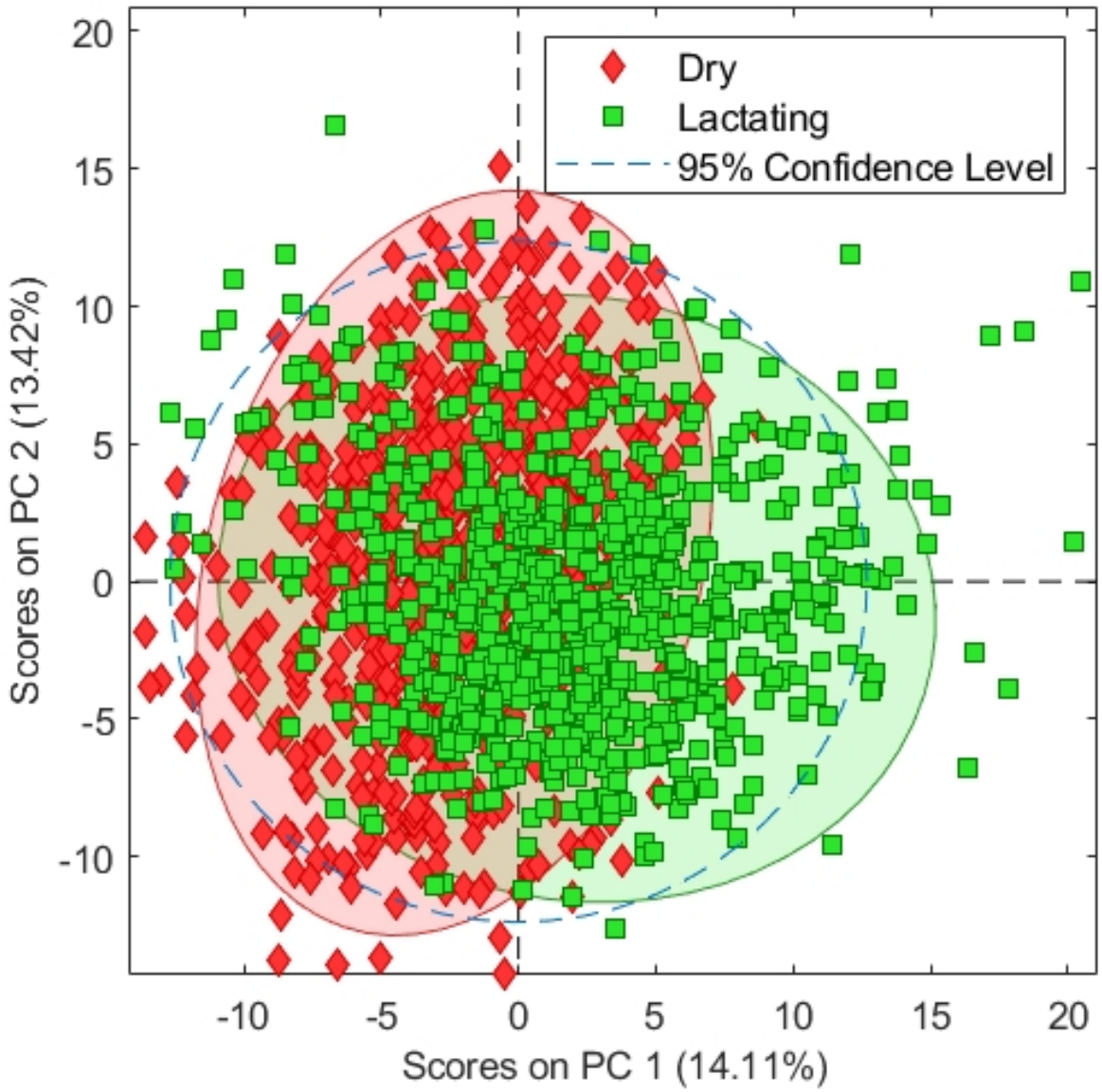

Figure A4: Principal component analysis with both dry and lactating cohort after external parameter orthogonalization (EPO) on the class variable of cohort. Direct comparison with Appendix Figure A3 displays how the variation association with a variable, in this case cohort, can be controlled. Analysis performed in and figures produced in PLS_Toolbox (Ver 9.3.1, Eigenvector Research Inc, Manson, WA).

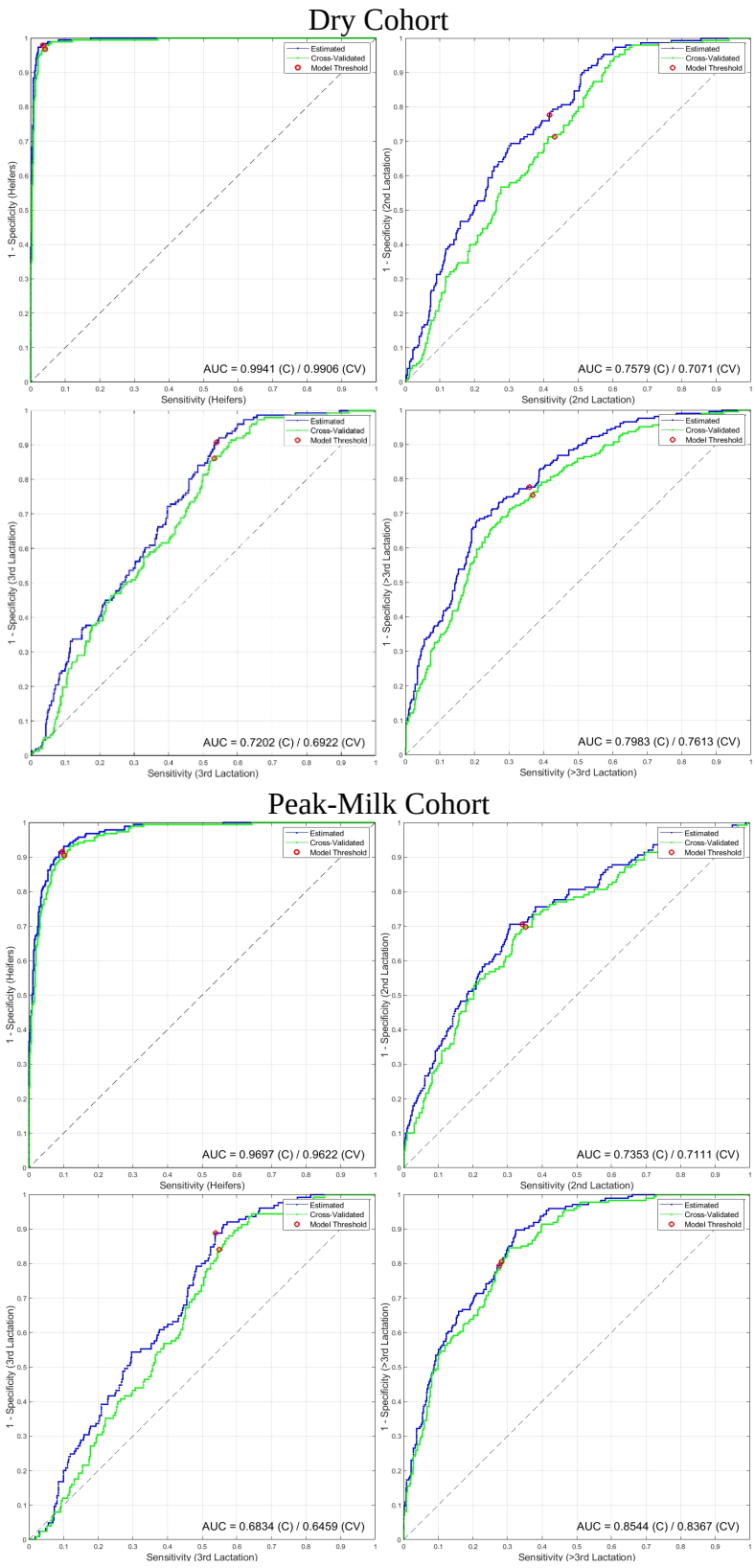

Figure A5: Receiver operator curves (ROC) for orthogonal partial least squares discriminate analysis between parity groups of heifers, 2nd, 3rd and > 3rd parity cows for the dry cohort (upper) and the peak-milk cohort (lower). Each ROC indicates the model's ability to discriminate a specific parity against all other parity groups combined (E.g. Heifers vs 2nd, 3rd and > 3rd cows). Discriminatory power between for the 2nd and 3rd parity animals was unsatisfactory. Analysis and figures produced in PLS_Toolbox (Ver 9.3.1, Eigenvector Research Inc, Manson, WA).

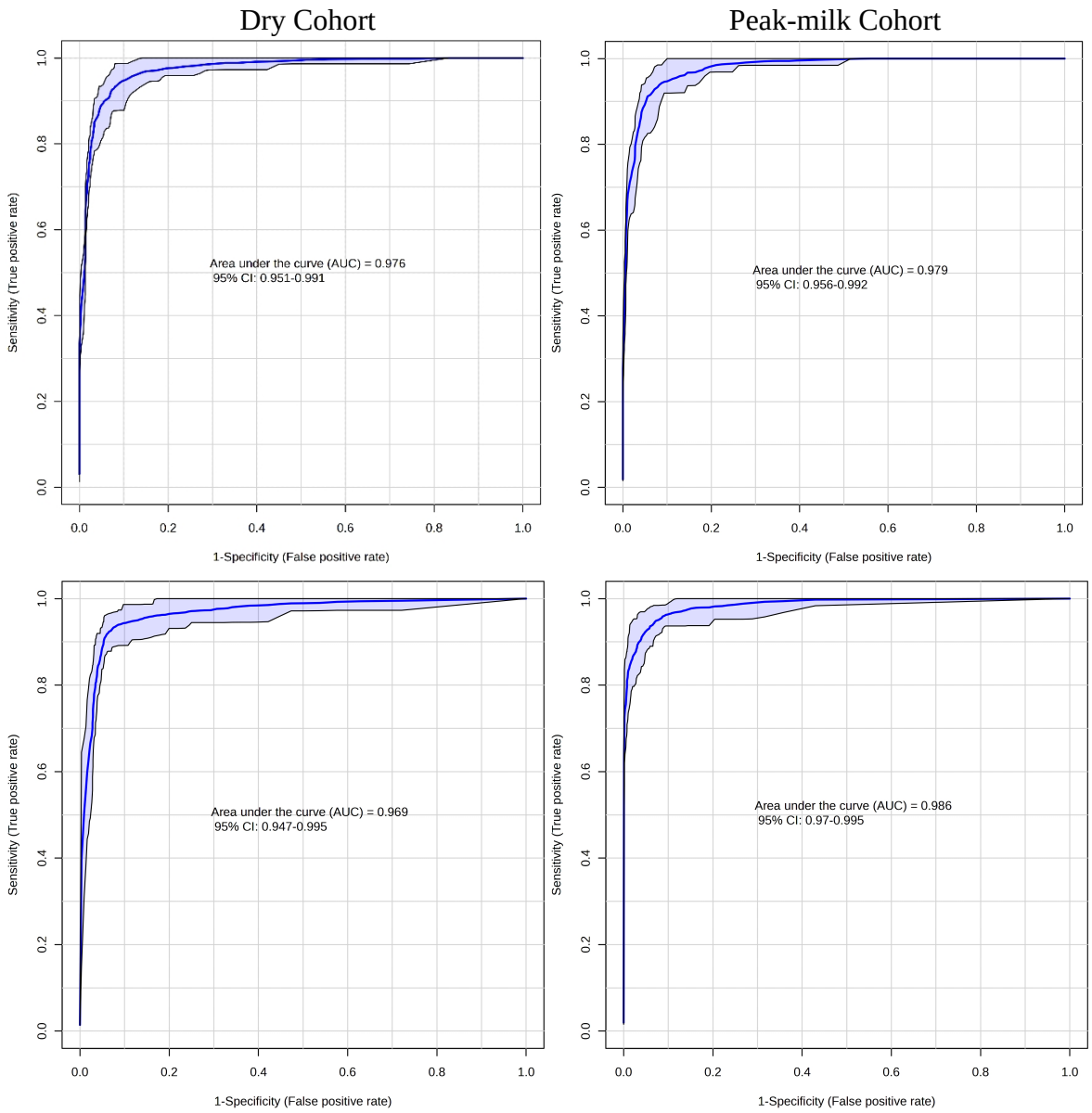

Figure A6: Receiver operator curves for discriminate analysis between heifers and > 3 lactation cows for the dry cohort (left) and the peak-milk cohort (right). The top row utilized support vector machine modelling, whereas the bottom row utilized random forest modelling. The best, according to AUC, support vector machine models utilized 15 and 100 lipids in the dry and peak-milk cohorts, respectively and the random forest models used 50 and 100 lipids, respectively. Analysis performed in and figures produced by MetaboAnalyst 6.0's online suite (https://www.metaboanalyst.ca).

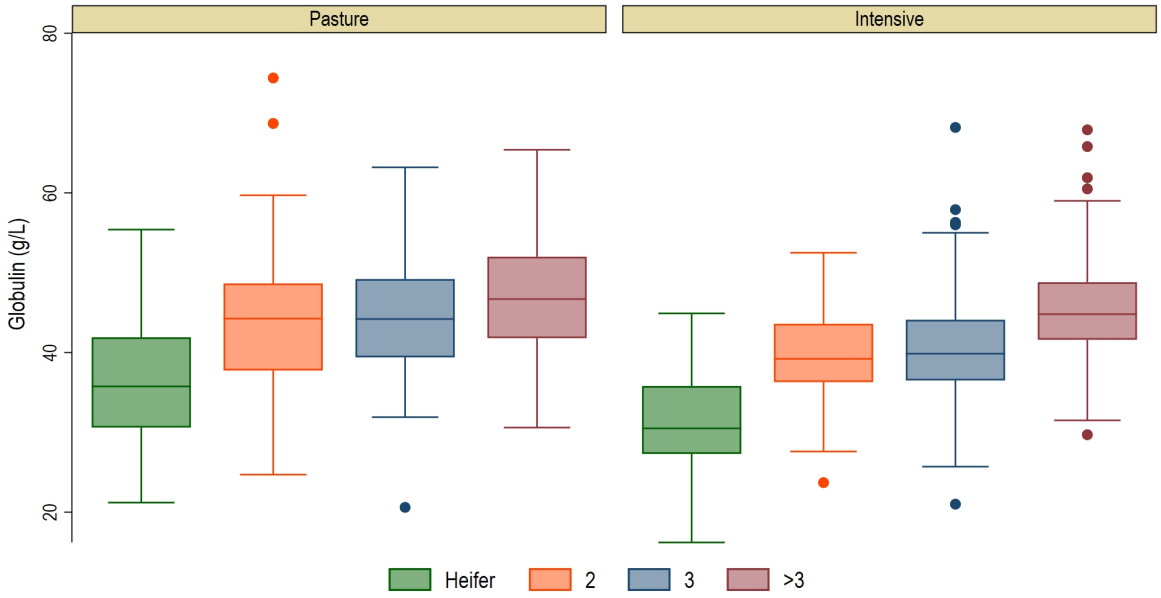

Figure A7: Box plots of the dry cow cohort distributions of globulin by parity and housing system. Box length is the interquartile range, with the median line drawn within the box. Whiskers span all data within 1.5 interquartile range, with outside values shown as dots.

Chapter 5: Final model specifications

Regression model pre-processing:

The external parameter optimization (**EPO**) method was superior to class-centre transformation to control for the variation in the data association with the effect of group (breed/farm). The optimum number of latent variables (**LV**) associated with the EPO adjustment differed for each model and were as follows: 5 and 4 LV in the orthogonal partial least square (**O-PLS**) regression models for the dry and peak-milk cohorts, respectively. And 2 and 1 LV in the multiple linear regression (**MLR**) model for the dry and peak-milk cohorts, respectively.

There was a moderate improvement by log-transforming age prior to mean centre transformation compared to mean centre transformation alone.

Regression model specifications:

The MLR model performed best, according to root mean squared error of prediction (**RMSEP**), using LASSO regression in the dry cohort (penalty value of $5.6x10^{-4}$) and ridge regression in the peak-milk cohort (penalty value of 45). After variable selection was first performed with the genetic algorithm (**GA**), LASSO regression (penalty 1.5x10-4 and 3.9x10-5 for dry and peak-milk, respectively) was chosen.

The O-PLS model had seven and five LV for the dry and peak-milk cohorts, respectively. The GA selected variable O-PLS model used 4 LV and 6 LV for the dry and peak-milk cohorts, respectively.

The GA variable selection method reduced the lipids to 63 in the dry and 55 in the peak-milk cohorts.

Discriminate analysis (DA):

Pre-processing was class-centred on breed/farm and auto-scaled.

The O-PLS DA model was specified with 5 and 7 LV in the dry and peak-milk cohorts, respectively. Random forest and support vector machine (**SVM**) utilised MetaboAnalyst 6.0's online suite of tools (https://www.metaboanalyst.ca) as described in the Chapter 5: *Methods and Materials*.