Final report

Q fever: A new approach to combatting an old Australian livestock problem

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Abstract

Coxiella burnetii, the bacterium causing Q fever, presents a significant public health challenge for the Australian livestock industry, with domestic ruminants being the main reservoirs. While typically subclinical in animals, *C. burnetii* can cause reproductive failure, however this is not well understood. This project report describes a longitudinal study in a dairy herd which showed that seropositive cattle at calving were less likely to become pregnant, and calves from cows with positive PCR vaginal swabs had higher mortality rates shortly after birth. These findings were then applied to a study in a beef herd which found that seropositive cows were 2.2 times more likely to leave the herd within 270 days, possibly due to poorer reproductive performance. The research has advanced understanding of coxiellosis in Australian cattle, suggesting significant production losses may occur. This project report provides evidence-based recommendations for future research to assess the pathogen's impact on the livestock industry which includes a focus on understanding the pathogenesis of *C. burnetii* and its role in reproductive failure and calf mortality, the risk of exposure in abattoirs, and the feasibility of developing an Australian vaccine. Raising awareness among farmers and veterinarians and improving diagnostic tools are also recommended. The project also developed a beef herd simulation model to estimate the effects of reproductive issues and calf losses, which will be valuable for future assessments.

Executive summary

Background

*Coxiella burnetii i*s the bacterium responsible for the zoonotic disease, Q fever. This disease poses an ongoing public health challenge for the Australian livestock industry, with data indicating that domestic ruminants are the main reservoirs of human infection. While infection with *C. burnetii* is typically subclinical in animals, clinical disease primarily manifests as reproductive failure, a phenomenon that is poorly understood and whose economic impact has not been measured.

In cattle, *C. burnetii* has been documented as a cause of sporadic abortion, premature delivery, stillbirth, and weak offspring (APSW) complex. However, its role in retained foetal membranes, metritis/endometritis, and infertility/subfertility in cattle herds remains unclear. Studies are needed to understand the pathogenesis of the disease and to identify associations between *C. burnetii* infection and reproductive performance. In sheep, *C. burnetii* has been documented to cause sporadic and epidemic abortion, with neonatal weakness and mortality also reported. However, studies focusing on the reproductive impacts in Australian sheep are rare, particularly in regions heavily impacted by *C. burnetii*, such as northern New South Wales and Queensland.

A highly effective, vaccine is available in Australia for the prevention of Q fever in humans. However, the vaccination process is costly and complicated, and many people in the meat and livestock industry remaining unvaccinated and therefore should be considered as only one component of the industry's approach to managing the impact of this disease. An inactivated *C. burnetii* vaccine is available for use in cattle, sheep, and goats in Europe, which is effective in limiting the clinical manifestations of the disease and reducing bacterial shedding, thus limiting infection in both animals and humans.

This project aims to address some of the *C. burnetii* knowledge gaps and concerns by investigating cattle and sheep health and reproductive issues associated with infection in the Australian context. The development and optimization of practical diagnostic tools will facilitate research into the pathogenesis of coxiellosis and the evaluation of disease control and prevention methods. A thorough understanding of the immunology associated with *C. burnetii* infection is crucial for developing and evaluating vaccines intended for use in cattle and sheep.

Objectives

- Refinement and validation of existing and new assays for use in investigating the pathogenesis and impact of coxiellosis in cattle.
- Evaluate livestock health and reproductive consequences of *C. burnetii.*
- Define *C. burnetii* shedding and infection risks associated with processing.
- Quantify the productive or reproductive losses associated with coxiellosis and use this data to develop a model that will begin to evaluate the economic impact of coxiellosis on animal production at the herd level and inform the potential value of livestock vaccination as a risk management strategy.
- Isolate and characterise *C. burnetii* strains.
- Develop evidence-based guidelines for management of coxiellosis in herds.

Methodology

- Various methodologies were utilised to investigate infection in cattle herds and sheep flocks in this study including molecular techniques (a three gene multiplex PCR), serology (an ELISA and cytokine release assays.
- Survival analysis was used to investigate associations between measures of *C. burnetii* exposure and outcomes of interest.
- *Coxiella burnetii* isolation was undertaken in Vero cell cultures and MLVA was used to genotype *C. burnetii* strains in positive tissue samples.

Results/key findings

Detailed reproductive data was collected through a longitudinal study in an intensive dairy herd during pregnancy, at calving and on multiple occasions up to 200 days into lactation showing that cattle that were seropositive at calving were less likely to become pregnant than those that were seronegative at calving. *Coxiella burnetii* exposure was not strongly associated with foetal loss or abortion however calves born from cows with a positive PCR vaginal swab were more likely to die within a few days of birth than calves from cows that tested negative.

A longitudinal beef cow study using an analytic approach using the outcome 'departure from the herd' determined that animals that were seropositive prior to breeding were 2.2 times more likely to depart the herd during the following 270 days than seronegative animals and we hypothesise that this may be due to worse reproductive performance in the seropositive group.

Infection with *C. burnetii* was common in beef cattle presenting at abattoir, with evidence of infection in herds from a wide geographic area and very high seroprevalences in some mobs. Seroprevalence was higher in cows of parity 6 or above than in younger cows, steers or heifers. By contrast, we could not identify a *C. burnetii* positive sheep flock despite trialling multiple methodologies across ~ 25 different flocks.

A final key project outcome was the isolation of *C. burnetii* strains from cattle enrolled in the study (held in archival storage) that have also been isolated from Australian Q fever patients across multiple clonal complexes, confirming the Q fever public health risk that cattle infected with *C. burnetii* present. Furthermore *C. burnetii* strains identified in cattle are not specific to cattle but rather are also found in other animal species, including wildlife. Within a cattle herd, multiple types of *C. burnetii* can co-circulate.

Benefits to industry

This discovery research project has advanced the knowledge of the pathogenesis of coxiellosis in Australian cattle herds with the findings suggesting that *C. burnetii* may be causing important production losses. The project has provided evidence-based recommendations for areas where future research should be directed to understand the true impact of this pathogen on the Australian livestock industry. The considerable knowledge around best practice for sampling and diagnosis of this pathogen both in the animal and in farm environments is available to inform future research and diagnostic efforts. *Coxiella burnetii* isolates are in archival storage providing a unique resource for future projects or vaccine development.

A beef herd simulation model was developed to provide robust estimates of the effects of low reproductive performance, increased calf losses and/or low calf growth rates in temperate zone

seasonal calving cow and calf beef herds. This model will be a valuable ongoing tool for assessing effects of both infectious and non-infectious causes as robust effect estimates become available.

Future research and recommendations

Future research in beef cattle and herds should be directed towards:

- further understanding the pathogenesis of infection with *C. burnetii* and investigating the role *C. burnetii* plays in reproductive failure and calf mortality across a larger number of Australian beef herds
- understanding how the high seroprevalence at slaughter translates into risk of exposure of people in abattoirs to the pathogen along the slaughter line,
- further assessment of the geographic distribution and prevalence of *C. burnetii* in Australian cattle herds and potentially in sheep flocks, and
- detailed review of the economic benefits and costs, feasibility and risks of a program to develop an Australian vaccine for animals 4.3.1 against *C. burnetii* to control infection in animals and humans.

Considering the widespread occurrence of *C. burnetii* across stock classes, management systems and rainfall zones, ongoing effort to raise or maintain awareness among famers and veterinarians is needed. This could include developing and establishing provision of best practice diagnostic tools in government and commercial veterinary laboratories for testing of samples of animal and environmental (dust) origin, and communication of recommended investigatory procedures for field advisers.

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1. Background

Coxiella burnetii is the bacterium responsible for the zoonotic disease, Q fever - the most common, non-food-borne, notifiable zoonosis in Australia with annual notifications in humans ranging from 457 to 605 cases annually over the last 10 years (https://nindss.health.gov.au/pbi-dashboard). This disease is an ongoing challenge for the Australian livestock industry from a public health perspective with data obtained from human Q fever notifications indicating that domestic ruminants are the main reservoirs of human infection (Graves and Islam 2016).

For the most part, infection with *C. burnetii* is subclinical in animals, however clinical disease (known as coxiellosis) is reported to occur largely in the form of reproductive failure (Agerholm 2013), and this may potentially add to the on-going challenge faced by the red meat industry in dealing with this disease. However, the impact of coxiellosis on reproductive performance in the Australian red meat industry is poorly understood and the subsequent economic losses incurred have not been measured.

In studies conducted in cattle in other countries, *C. burnetii* has been documented, and is generally accepted, as a cause of sporadic abortion, premature delivery, stillbirth and weak offspring (APSW) complex, however there is much confusion and conjecture in the literature as to its role in retained foetal membranes, metritis/endometritis, and infertility/subfertility in cattle herds (Agerholm 2013). The impacts of *C. burnetii* infection upon calf growth and development are largely unknown. In the only study conducted in the Australian context, Wood (2020) analysed serological results from beef cattle managed on commercial properties located in Queensland and the Northern Territory against a large dataset investigating causes of reduced reproductive performance in beef cattle across northern Australia (The Northern Beef Fertility Project: CashCow; Meat and Livestock Australia; B.NBP.0382) with results suggesting that high levels of *C. burnetii* exposure at the property level was associated with reduced pregnancy rates in those herds. However, the author concluded that the study design did not allow for causality to be inferred and that further research was needed to confirm this hypothesis. Ideally, studies need to be conducted that allow for understanding of the pathogenesis occurring at the individual cow level to account for any identified associations between *C. burnetii* infection and reproductive performance.

In sheep, *C. burnetii* has similarly been documented overseas to be a cause of sporadic and epidemic abortion with neonatal weakness and mortality also reported (Agerholm 2013) however studies focussing on reproductive impacts in Australian sheep are rare. The only published study of *C. burnetii* in Australian sheep conducted in the last decade (funded by Meat and Livestock Australia; B. AHE.0318), reported very low *C. burnetii* seroprevalences of 0.08% in primiparous ewes and 0.36% in mature ewes and *C. burnetii* was not detected in aborted or stillborn lambs by quantitative PCR (Clune et al, 2022). However, this study was conducted in flocks located in southern Australia (Victoria, South Australia and Western Australia), an area with low Q fever notification rates in humans and therefore also likely lower rates of infection in animals. Thus, research is warranted in sheep flocks in regions more heavily impacted by *C. burnetii* including northern New South Wales and Queensland.

As with animals, the usual route of infection for humans is via inhalation of aerosols containing the organism. Those working in livestock-associated industries including abattoir workers, shearers, farm workers, rural contractors and veterinarians are particularly at risk (Eastwood 2018). A highly

effective, locally produced vaccine is available in Australia for prevention of Q fever in humans (Q-VAX®; Seqirus, Victoria, Australia). However, the process of vaccination is costly and complicated resulting in many people in the meat and livestock industry remaining unvaccinated despite a strong awareness of the disease and the importance of vaccination. Members of the public not identifying as directly associated with livestock industries may still be at risk of infection and may not seek vaccination. For example, those residing close to abattoirs or along stock transport routes may be at risk due to aerosolisation of bacteria from these livestock associated activities. Thus, while the currently available human vaccination is very effective at preventing disease in people, the strong support and advocation of its use should be considered as only one component of the industry's approach to managing the impact of this disease from a public health perspective. In Europe, an inactivated *C. burnetii* vaccination is available for use in cattle, sheep and goats (Coxevac; Ceva Santé Animale, Libourne, France) and studies have shown that it is effective in both limiting the clinical manifestations of the disease (a reduction in abortions and improvement in reproductive performance in comparison with absence of vaccination) and reducing the shedding of the bacteria both in intensity and duration. Thus, the animal vaccine provides benefits in limiting the impact of infection for both animals and humans (Gisbert *et al*, 2024).

This project seeks to address some of these knowledge gaps and concerns by investigating cattle and sheep health and reproductive issues associated with *C. burnetii* infection in the Australian context. The development and optimisation of logistically useful and effective diagnostic tools will allow for research not only into the pathogenesis of coxiellosis, but also for detection of infected herds moving forward, and evaluation of methods of disease control and prevention. A thorough understanding of the immunology associated with *C. burnetii* infection would be crucial in the development and evaluation of vaccines intended for use in cattle and sheep.

If reliable estimates of the effects of *C. burnetii* on reproductive performance, calf survival and/or growth rates can be obtained, a model may be developed which allows for exploration of the economic consequences of these effects under various scenarios. This model could also be used to explore the economic impacts of numerous other agents and factors, provided estimates of their effects on reproductive performance, calf survival and/or growth rates are available. Thus, this model could have much wider application for the beef cattle industry than just exploring the effects of *C. burnetii* and informing the potential value of livestock vaccination as a risk management strategy.

2. Objectives

The project objectives as outlined in the research agreement and the success in meeting those objectives are as follows:

1. **Refinement and validation of existing and new assays** for use in investigating the pathogenesis and impact of coxiellosis in cattle. These are assays aimed at detecting, isolating, and typing the organism (PCR and/or genotyping or whole genome sequencing) and quantifying the host immune response to infection (serological [ELISA or IFA] and cell mediated [e.g., interferon gamma or other cytokine assays]). The usefulness of cell culture methodologies in measurements of bacterial strain virulence will be investigated.

   With over 8,000 samples processed as part of the project, extensive work has been done on **Objective 1** in refining and validating new and existing assays (see Section 3. Methodology). This project utilised a three-gene multiplex PCR to detect shedding in placental tissue, swabs, milk, faeces, and dust and, for each of these sample types, extensive work was done

in optimising the extraction process to ensure amplifiable DNA of high quality was obtained with appropriate controls in place to ensure detection of contamination and minimise false positives. In addition, a notoriously logistically difficult cytokine release assay was optimised to detect interferon gamma and Interleukin-10 with great success. Following a collaboration with German colleague, Dr Ben Bauer, a dust sampling methodology was established and optimised within our laboratory and successfully utilised to detect *C. burnetii* in dust samples on the intensive dairy farm. While cell culture models utilising avirulent strains of *C. burnetii* have been established in our laboratory their use has not yet been extended to the investigation of strain virulence due to difficulty accessing PC3 facilities that was encountered throughout the project.

2. **Evaluate livestock health and reproductive consequences of *C. burnetii.*** *Coxiella burnetii* is a difficult organism to work with due to its propensity to cause disease in people. Historically, this has limited the conduct of basic field research to quantify the cost of the disease in livestock. The opportunity to conduct this work is made possible by the new tools that have been developed and plan to be refined in Objective 1. With these tools it is possible to detect the organism without having to culture all samples (culture requires high level containment). Coxiella will be studied in sheep, dairy beef and beef cattle enterprises. This component of the project will involve strategic sampling of stock through the production cycle with a particular focus on the reproductive cycle to determine when stock are most likely to be infected and to determine the health and reproductive consequences of these infections. It is also anticipated that this work will determine the optimal sampling and testing strategies to identify infected stock and infection risk. This work will assist in identifying classes of stock that should be vaccinated should a livestock vaccine be developed.

   Coxiella burnetii infection has been studied in both dairy and beef enterprises with sampling conducted throughout the reproductive cycle to meet **Objective 2**. The optimal sampling and testing strategy identified in the intensive dairy herd enabled more focussed sampling in a *C. burnetii* endemic beef herd as outlined in Section 4.1 and 4.2. Unfortunately, despite several attempts using multiple appropriately controlled methodologies, we were unable to identify an endemic sheep flock (see Section 4.5) in which to conduct a similar longitudinal study however a strategy for moving forward in investigating coxiellosis in Australian sheep flocks has been proposed for future research in Section 5.

3. **Define *C. burnetii* shedding and infection risks from farm through processing** – Shedding of most infectious organisms is influenced by environmental stressors such as transport, lot feeding and lairage. Sampling (e.g., blood, viscera, faeces, swabs) will occur throughout the process from paddock to processing plant to determine where changes in bacterial shedding and immunological responses occur. These results will aid in understanding the points of greatest public risk as well as inform usefulness of vaccination and effective vaccination protocols.

   An extensive study was conducted in a large processing plant located in southern Queensland to meet **Objective 3**. The study outlined in Section 4.4, utilised seroprevalence as a measure of previous *C. burnetii* exposure to demonstrate that cows are the class of with the highest seroprevalence. A seroprevalence study was chosen after results from MLA funded research conducted by Wood (2020) became available whereby the researcher was unable to detect *C. burnetii* DNA in a large number of samples collected at abattoir. Unfortunately, due to privacy concerns we were unable to conduct trace back studies to properties of origin to determine if transportation induced bacterial shedding as originally

planned. However, the results of the longitudinal study conducted on a beef property confirms that cows had the highest seroprevalence on farm with serologically positive animals being 2.2 times more likely to be culled than seronegative cows (see section 4.2).

4. **Economic modelling** - There are significant WH&S safety costs incurred by abattoirs associated with Q fever. The current livestock-related costs associated with coxiellosis are unknown and cannot be calculated without basic preliminary research to quantify the productive or reproductive losses incurred. Objective 2 will provide the data required to begin to evaluate the economic impact of coxiellosis on animal production at the herd level. This modelling will also inform the potential value of livestock vaccination as a risk management strategy providing an indication of the financial constraints of this approach.

   Significant progress has been made on the development of an economic model (**Objective 4**) however this component of the project has been considerably delayed as Richard Shephard, a central collaborator in this component of the project, was quite unwell for much of the first half of 2024. As a result, the anticipated date for completion of this component of the project is 31st October 2024.

5. **Isolation and molecular characterisation of *C. burnetii* strains** obtained from cattle and sheep collected during sampling. Characterisation of the strains will utilise genotyping methodologies which studies conducted by collaborators, Australian Rickettsial Reference Laboratory (ARRL), have demonstrated to be appropriate for Australian isolates and will also utilise cutting edge methodologies such as next generation or whole genome sequencing. The focus will be on correlation of strains of animal origin with those previously obtained from humans (held in a collection by the ARRL) to infer which livestock species or strain may need to be prioritised with vaccination. In addition, the isolates will be analysed for genes associated with, or predictive of, virulence.

   Considerable success has been achieved in meeting **Objective 5** (see Section 4.6) with nine *C. burnetii* strains isolated in Vero cells from samples collected in the study in meeting Objective 2. These isolates are currently in archival storage with the Australian Rickettsial Reference Laboratory's *C. burnetii* collection. Attempts were made to grow these isolates in the axenic media which has successfully grown *C. burnetii* isolates obtained overseas however, to date, no Australian isolate (both cattle and those from humans) has been able to be grown in this media. Future research could be directed towards understanding the additional media requirements of Australian *C. burnetii* strains. MLVA genotyping has identified strains three *C. burnetii* strains (CbAU05, CbAU07 and CbAU09) that have also been isolated from Australian Q fever patients. Whole genome sequencing (WGS) was attempted on PCR positive tissue samples obtained in this study using two methodologies (Nanopore and Illumina) however this was unsuccessful due to the inability of both methodologies to elicit long reading frames. The ability to culture *C. burnetii* strains in axenic media will likely enable WGS to be conducted more successfully in the future.

6. **Evidence based management** - Based on the outcomes and findings of the study, evidence-based guidance and recommendations regarding management of coxiellosis and Q fever will be developed in consultation with industry representatives and communicated with the livestock industry and public health agencies.

   The results of this study have been presented to a variety of industry representatives including livestock veterinarians, other *C. burnetii* researchers overseas and representatives from Australian Meat Processors (AMPC). In addition, the research team has sought

feedback on the study outcomes from Meat & Livestock Australia. Discussions regarding the relevance of the study outcomes for red meat industry resulted in the research team developing key messaging and recommendations and these are outlined in Sections 5 and 6 of this report, thus meeting **Objective 6.**

3. Methodology

3.1 Animals and sampling

3.1.1 Animal Ethics

Animal ethics approval was sought and gained from the University of Sydney Animal Ethics Committee to identify *C. burnetii*-endemic cattle and sheep farms and then to investigate whether *C. burnetii* has an impact on reproductive performance in the cattle and sheep residing on those farms (Project numbers: 2021/2014; 2022/2191; and 2022/2241).

3.1.2 Animal sampling

Due to the variety of experiments conducted as part of this project, details of animal sampling are provided in the results section for each component of the project.

3.2 Molecular methodology

3.2.1 *Coxiella burnetii* PCR

3.2.1.1 DNA extraction

DNA was extracted using the Biosprint® 96 One-For-All Vet Kit (Qiagen, Australia) according to the manufacturer's protocol for purification of viral nuclei acids and bacterial DNA from animal tissue homogenates, serum, plasma, other body fluids, swabs, and washes. For liquid samples such as sera and whole milk, 160 µl was added to 40 µl of proteinase K and incubated at 56 ˚C overnight (15-20 hours) in a dry block heater (Ratek, Victoria, Australia). For vaginal swabs, one of the swab's tips was removed using heat-sterilised scissors and homogenised in 500 µl of sterile phosphate buffered saline (PBS) solution by vortexing for 10 times for 3 seconds, after which 160 µl of the homogenate was added to 40 µl of proteinase K and incubated overnight in a dry block heater. For placental samples, an approximately 25 mg piece of tissue was homogenised in 1 ml of Buffer RTL supplied in the kit using a high throughput bead mill with 5 mm stainless steel beads (TissueLyser; Qiagen, Australia) at 25 Hz. Following this, 160 µl of the tissue homogenate was added to 40 µl of proteinase K and incubated in a dry block heater overnight. Following the incubation of these samples, 140 µl of each homogenate was loaded directly into the corresponding lysate S-block according to the Biosprint® 96 One-For-All Vet Kit (Qiagen) manufacturer's instructions. Negative extraction controls which contained only RTL buffer and Proteinase K were included for every 8 – 12 samples.

3.2.1.2 Quantitative PCR detecting *Coxiella burnetii* DNA

Detection and quantification of *C. burnetii* DNA in extracted samples was performed using an optimised multiplex qPCR assay targeting the two single copy genes: *groEL* (heat shock operon; *htpAB*) and *com1* (the outer membrane protein-coding gene) and the multicopy insertion sequence gene: IS*1111.* Details of primers and PCR conditions are shown in Table 1. Each reaction

contained 5µL 1X SensiFAST Probe No-ROX Kit (BioLine, Australia), primers and probe, 2µL DNA and nuclease free water in a total volume of 10µL. Amplification and fluorescence detection was performed in a Bio-Rad-CFX Real-Time PCR Thermocycler (Bio-Rad laboratories Pty Ltd, Gladesville, NSW, Australia) according to the following cycling parameters: initial denaturation at 95^{o}C for 3 minutes followed by 40 cycles of denaturation at 95^{o}C for 10 seconds and annealing and extension at 60^{o}C for 40 seconds. Each qPCR run included no template controls (NTC) and positive controls containing 1,100, 110 and 11 copies of the *C. burnetii* genome per reaction (Amplirun® Vircell, Granada, Spain). The lower limit of detection for these qPCR assays was determined to be 11 copies of the *C. burnetii* genome per reaction which corresponded to a cycling or quantification threshold (Cq) of ~34, ~36 and ~35 for IS*1111*, *com1* and *htpAB* respectively. Samples were initially screened as a single qPCR reaction, and any sample producing amplification for any gene target was subsequently repeated in duplicate.

The overall classification of samples for the presence or absence of *C. burnetii* DNA was based on the number of gene targets amplified, the 11-copy cut-off Cqs for each target gene and the reproducibility of the triplicate reactions.

3.2.1.3 Quantitative PCR detecting host species DNA – bovine endogenous control

Primers targeting the mitochondrial DNA (mtDNA) gene, bovine cytochrome b (BCB), were designed as an endogenous control to confirm the presence of DNA and to confirm DNA integrity. The primer sequences were designed based on an alignment of the BCB gene (Table 1). For the BCB assay, each reaction contained 5µL 1X SensiFAST Probe Lo-ROX Kit (BioLine, Australia), primers and probe, 2µL DNA and nuclease free water in a total volume of 10µL. Two NTCs containing nuclease free water rather than DNA were used per run to ensure reagents were contamination free. The positive control used was DNA extracted from a bovine sample confirmed as positive in a previous study. The reactions were performed using a Bio-Rad CFX96 Touch Real-Time PCR detection system (Bio-Rad Laboratories) for 40 cycles involving an initial 3 minutes of denaturation at 95 ˚C and 40 seconds of annealing and extension at 60 ˚C, followed by 39 cycles of 10 seconds of denaturation at 95 ˚C, and 40 seconds of annealing and extension at 60 ˚C. The results were viewed and exported from Bio-Rad CFX Manager (Bio-Rad Laboratories).

3.2.1.4 Dust sample endogenous control

To confirm the presence and integrity of extracted DNA from the dust samples, a commercially available product, RT-qPCR Extraction Control Red (redEC; Meridian Bioscience, Ohio, United States), was used according to the manufacturer's instructions. The product utilises *Escherichia coli* cells in a known concentration to determine the success of the extraction process without impacting sample DNA detection. The redEC primer in conjunction with a Quasar labelled probe is included in the kit. Each reaction contained 5µL 1X SensiFAST Probe No-ROX Kit (BioLine, Australia), primers and probe, 2µL DNA and nuclease free water in a total volume of 10µL. Amplification and fluorescence detection was performed in a Bio-Rad-CFX Real-Time PCR Thermocycler (Bio-Rad Laboratories) according to the following cycling parameters: initial denaturation at 95^{o}C for 3 minutes followed by 40 cycles of denaturation at 95^{o}C for 10 seconds and annealing and extension at 55^{o}C for 30 seconds. Each qPCR run included NTCs.

Table 1. Sequence, product lengths, and concentrations of *Coxiella burnetii* and endogenous control (Bovine cytochrome B) gene primers used in the multiplex PCR.

Primer	Primer sequences (5'-3')	Product length (bp)	Reference
IS*1111*[A] Forward primer Reverse primer Probe	 CGCAGCACGTCAAACCG TATCTTTAACAGCGCTTGAACGTC FAM[E]- ATGTCAAAAGTAACAAGAATGATCGTAAC- BHQ1[F]	146	Adapted from deBruin *et al*., 2011
***htpAB*[B]** Forward primer Reverse primer Probe	 GTGGCTTCGCGTACATCAGA CATGGGGTTCATTCCAGCA FAM[a]-AGCCAGTACGGTCGCTGTTGTGGT- BHQ1[F]	114	Designed in house by Sullivan Nicolaides Pathology (Brisbane Queensland) using accession number: M20482 (Shapiro *et al*., 2020)
***com1*[C]** Forward primer Reverse primer Probe	 AAAACCTCCGCGTTGTCTTCA GCTAATGATACTTTGGCAGCGTATTG Quasar670[G]- AGAACTGCCCATTTTTGGCGGCCA-BHQ2[H]	76	Adapted from Lockhardt *et al*., 2011
***BCB*[D]** Forward primer Reverse primer Probe	 GAGGCGGATTCTCAGTAGACAAAG GAGCCTGTTTCGTGGAGGAATA Quasar 670[G] - CCCTTACCCGATTCTTCGCTTTCCA-BHQ2[H]	121	*Bos taurus* Mitochondrial Cytochrome B gene (Genbank accession no. GQ358783.1)

[A] *C. burnetii* multi-copy insertion sequence *1111* (IS*1111*), [B] *C. burnetii* single copy heat shock operon (*htpAB*), [C]*C. burnetii* single copy outer membrane protein gene (*com1*), [D]Bovine cytochrome B (*BCB*), included as DNA extraction control, [E]6-Carboxyfluorescein, [F]Black Hole Quencher-1, [G]Quasar 670 Carboxylic Acid, [H]Black Hole Quencher-2

3.2.2 Multiple locus variable-number tandem repeat analysis (MLVA)

3.2.2.1 PCR primers and reactions

Coxiella burnetii DNA was amplified using conventional PCR reactions with primers (Table 2) directed against the three loci ms24, ms28, and ms33 (Vincent et al., 2016). Reactions were performed utilising a Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia). All PCR reagents were purchased from Promega (Promega, WI, USA) and PCR primers were synthesised by Integrated DNA Technologies (Baulkham Hills, NSW, Australia). Each reaction consisted of 1X GoTaq® reaction Buffer 1.5mM MgCl2, 0.2mM dNTPs, 400nM each forward and reverse primers 1UGoTaq® DNA Polymerase, 1.5µL of template DNA and sterile deionised water, in a total volume of 25µL. A NTC was used by substituting nuclease free water in place of DNA in each PCR reaction to ensure the absence of contamination in the reagents. The

cycling protocol was as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of amplification involving 30 seconds of denaturation at 95°C, then 30 seconds of annealing at 58°C, and 60 seconds of extension at 72°C, with a final extension step at 72°C for 7 minutes. Positive control DNA from attenuated Nine Mile Phase II Clone 4 (RSA439) was incorporated into the study. The PCR products were analysed on a 2.0% agarose gel stained with RedSafe™ Nucleic Acid Staining Solution (Scientifix, Clayton, Victoria, Australia). Five microliters of each of the reactions were loaded into each well and 5µL of 100bp HyperLadder™ (Bioline, Alexandria, Australia) was included in each row for amplicon sizing. The gel was run at 120V for 50 minutes in 1x TAE buffer before being visualised under ultraviolet light using Bio-Rad Gel Doc (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia).

Table 2. The published multiple locus variable-number tandem repeat analysis (MLVA) primer sequences for the three loci (ms24, ms28, and ms33) used to amplify the distinctive *C. burnetii* strains isolated in Australia.

Locus	Primer name	Primer sequence (5'-3')
ms24	ms24-F	FAM-ATGAAGAAAGGATGGAGGGACT
	NL_ms24-R	GCCACACAACTCTGTTTTCAG
ms28	ms28-F	TAGCAAAGAAATGTGAGGATCG
	ms28-R	ATTGAGCGAGAGAATCCGAATA
ms33	ms33-F	TAGGCAGAGGACAGAGGACAGT
	ms33-R	ATGGATTTAGCCAGCGATAAAA

3.2.2.2 Sizing of PCR products

The PCR products of the three described loci (ms24, ms28, ms33), which are presented as bands between 150-300 bp, were sent to the Australian Genome Research Facility (AGRF; Melbourne, Victoria, Australia) for fragment separation analysis to accurately determine the size of each amplicon, according to the published methodology by Vincent et al. (2016). The number of repeats for each locus was determined based on the size of the amplicon with the sample genotypes characterised by comparing with the pattern of the number of loci repeats defining the Australian MLVA genotypes CbAU01-14 and against the known genotype Nine Mile strain (RSA439) used as a positive control (https://microbesgenotyping.i2bc.paris-saclay.fr/databases/view/65).

3.3 Serology

3.3.1 Blood collection

Blood samples were collected directly into 10mL serum collection tubes containing silica to activate clotting and a gel for separation during centrifugation (Becton, Dickinson and Company, UK). Samples were gently mixed by inversion before storage and transportation at 4 °C. On arrival at the laboratory, samples were centrifuged (Centrifuge 5810R; Eppendorf, Australia) at 4,000 x g for 15 minutes, after which the serum was removed and stored at –20 to -45 °C before processing.

3.3.2 Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

Antibody detection was performed on serum samples using a commercially available ELISA kit (IDEXX Q Fever Antibody Test Kit; IDEXX Laboratories, NSW, Australia) following the manufacturer's instructions. All reagents and control sera for the ELISA were provided as a part of the kit. The microplate supplied with the kit is coated with combined phase I and phase II *C. burnetii* Nine Mile strain antigen. The serum samples were diluted 1:400 with wash buffer and 100 µl of serum was added to the wells of each plate in duplicate and incubated for one hour in a humid chamber at 37˚C. The plate was washed three times using an automated plate washer (Stat Fax 2600, Block Scientific, New York, US) before adding peroxidase labelled anti-ruminant IgG conjugate to the wells, followed by a further one-hour incubation at 37˚C. The plate was subsequently washed three times. The tetramethylbenzidine (TMB) substrate was then added, followed by incubation at room temperature in the dark to allow for colour development before the stop solution was applied. After completion of the ELISA protocol, the optical densities in each well were read using a spectrophotometer at a wavelength of 450nm (Polar Star Optima, BMG LabTech, Victoria, Australia) and analysed using Mars Data Analysis Software (BMG LabTech). A to positive ratio (S/P%) cut off ≥ 40% was used for classifying positives, samples with S/P% < 30% were classified as negative, and samples with S/P% ≥ 30% but < 40% were classified as suspect. All suspect samples were retested, and unless otherwise stated, repeat samples falling in the suspect category were classified as negative.

3.4 Measures of cell mediated immunity.

3.4.1 Cytokine stimulation assay for Interferon gamma and Interleukin 10

3.4.1.1 Blood Collection

Blood (10 mls) was collected from the coccygeal vein (located in the tail) of cattle into commercially available vacuum sealed polyethylene terephthalate (PET) blood collection tubes containing 158 USP units of spray dried lithium heparin (BD Vacutainer, Becton Dickinson). Following collection, blood tubes were gently inverted to incorporate the anticoagulant and transported to the laboratory at ambient temperature or on ice until processing.

3.4.1.2 Blood Stimulation

Ex vivo stimulation of each cow's whole blood (collected in lithium heparinised tubes) using a series of six treatments containing mitogens, antigens and negative (media only) control was performed within 18 hours following sample collection, to measure the ability of the cow's immune cells to produce cytokines IFN-γ and IL-10 in response to stimulation with *C. burnetii*.

All experiments were conducted in tissue culture treated 48 well polystyrene plates (Corning® Costar®, Corning). The culture medium used for all experiments consisted of RPMI medium 1640 (Sigma-Aldrich®), containing 10% fetal bovine serum (FBS; Invitrogen), 2mM L-glutamine (Sigma), and 100 μg/ml penicillin-streptomycin (Invitogen). All plates were pre-loaded with either media only, or media to which mitogens or antigens were added, and frozen at -45 °C until use. Formaldehyde inactivated whole cell Nine Mile strain *C. burnetii* phase 1 antigens (Dolfinin, Bratislava, Slovakia) at a concentration of 1/100 dilution of the stock were used in all experiments in this study. Pokeweed mitogen (PWM; Sigma) was used as non-specific T and B cell positive controls at 5 μg/ml to ensure that cells were viable and capable of responding to antigenic stimulation. A rough form lipopolysaccharide (LPS) (which lacks the O-antigen region) derived from *Escherichia coli* K12 (LPS-EK; Invitrogen) at 0.01 μg/ml was also included as an innate system positive control.

Blood collected into Lithium heparin tubes were transported to the laboratory within 16-18 hours following collection and placed in a low-speed rotator to homogenise the sample. Culture plates containing antigens and mitogens were thawed in the incubator and allowed to reach 37 ˚C before adding 300 μl of blood sample to each of the six treatment types. The one-part blood to one-part culture media mixture was placed into the incubator (5% CO_2) for 48 hours during which time the erythrocytes settled to the bottom of the well leaving a supernatant containing secreted cytokines. At the end of the 48-hour cell culture, 300 μl of plasma supernatant was transferred from each well of the 24 well plate to a tube of the 96 well racked storage system and fitted with a lid and then placed at -20 ˚C storage until analysed in an ELISA.

3.4.1.2 Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

Cytokine detection was performed on supernatant samples aspirated from the whole blood stimulation plates using an in-house IFN-γ and IL-10 sandwich ELISA. The 96 well microplate (Nunc MaxiSorb) was coated with a mouse anti-bovine IFN-γ (produced in house) or mouse anti-bovine IL-10 (Bio-Rad) at a concentration of 1.5 μg/ml or 2.5 μg/ml diluted in PBS or wash buffer (0.5% tween20 in RO water) respectively and incubated at 4 ˚C overnight. The next day, plates were washed manually with 300 μl of wash buffer three times. Next, 50 μl of samples and standards (Bovine IFN-γ Reagent, bovine IL-10 Yeast-derived Recombinant Protein, Kingfisher Biotech) were either diluted 1:2 in PBS for the IFN-γ ELISA or added undiluted for the IL-10 ELISA in duplicate and incubated for one hour at RT. The plate was washed three times before adding 50 μl per well of a biotin conjugated secondary antibody (mouse anti bovine IFN-gamma-Biotin, Serotec; mouse anti bovine IL-10-Biotin, Bio-Rad) at a concentration of 0.5ug/ml in PBS for the IFN-γ ELISA or wash solution for the IL-10 ELISA and incubated for 1h at RT. At the end of the incubation, the plates were washed three times as above and then 50 μl per well of HRP-Streptavidin (KRL Laboratories) was added to each well at 0.167 μg/ml or at 0.333 μg/ml and incubated at RT for 20 mins or 30 mins for the IFN-γ and IL-10 ELISA respectively. The plate was washed five times. Then tetramethylbenzidine (TMB) substrate set (BD OptEIA™) was added at 100 μl per well and incubated in the dark. The development of the plates was monitored using the 620 nM wavelength on an absorbance plate reader until the top standard reached 0.45 to 0.5 or the negative control exceeded 0.05. When this point was reached, the reaction was stopped by adding 100ul per well of 2M sulfuric acid. The optical densities were read using a spectrophotometer at 450nM and were converted to ng/ml based on the standard curve. The cytokine response is reported with the unstimulated control (media background) subtracted, unless otherwise stated. Sample to positive ratios (S/P ratio [%]) were also calculated according

to the following formula: ([OD *C. burnetii* stimulation – OD media background control]/ [OD stimulation positive control (PWM) – OD media background]) x 100.

3.5 Statistical analysis

Various statistical methodologies were utilised in the studies conducted across this overall project. Details of the methodologies used are provided with the results of each sub-project in Section 4.

4. Results

4.1 Investigation of impact of *Coxiella burnetii* on production parameters and reproductive performance in a data-intensive dairy cattle system.

While the ultimate aim of this project was to investigate the impact of *Coxiella burnetii* on production parameters and reproductive performance in extensive beef production systems, the project investigators identified early in the project development that the logistics of frequent sampling, especially around the time of calving, would be difficult in extensive beef enterprises. Therefore, it was decided to conduct initial studies in a data-intensive dairy system which would afford greater opportunities for sampling as part of the routine management practices conducted by those enterprises and provide greater meta-data on productive and reproductive performance. Subsequently, optimised and streamlined sampling strategies and key learnings could then be transferred to the extensive beef production systems.

4.1.1 Longitudinal study of *Coxiella burnetii* in an endemic herd

A longitudinal study was conducted to gain insight into patterns of *C. burnetii* infection, particularly bacterial shedding and host immune responses around calving and into the subsequent lactation, ultimately to allow for analysis of the association between *C. burnetii* status and (re)productive parameters. Sampling for the experiments in this study occurred in an intensively managed dairy cow herd located in New South Wales, Australia.

The longitudinal sampling study commenced in mid-May 2022 with Longitudinal sampling timepoint 1 (L1; pre-calving) and concluded with the final sampling (L5; mid-lactation) which was conducted in mid-December 2022. Sampling at calving (L2) and in early lactation (L3 and L4) occurred in between these two timepoints. A summary of the sampling timepoints, the number of animals sampled, sample types and numbers collected for each sample type at each timepoint is presented in Fig. 1 and Table 3.

A total of 8,456 samples were collected over the five timepoints with 144 animals remaining in the study at 190 days post calving. The initial total animal number at L1 was based on a generous sample size calculation to allow for expected losses of animals from the longitudinal study due to management and health issues associated with a working commercial cattle enterprise.

The samples collected included:

1. blood (serum for serology and PCR; whole blood for the cytokine release assays; whole blood for harvest of plasma and buffy coat for PCR)
2. placenta (for PCR; L2 only)

3. colostrum (for PCR; L2 only)
4. milk (for PCR; L3-5 only)
5. faeces (for PCR)
6. vaginal swabs (for PCR)

4.1.1.1 Presence of *Coxiella burnetii* DNA detected by multiplex PCR

The percentage of each sample type testing positive for *C. burnetii* on multiplex PCR is presented in Table 3.

In summary, of the 205 placentas collected at parturition (L2 timepoint), 23.4% (48/205) were strongly positive on all three genes via multiplex *C. burnetii* PCR (48/205). A further 43.4% (89/205) were classified as 'suspect' positive. Suspect positive samples were either those not positive on all three genes or with higher threshold or quantification cycle (Ct or Cq) values meaning lower amounts of target nucleic acid in the sample.

Figure 1. Summary of the sampling strategy and number of cows sampled for a longitudinal study of a *C. burnetii* endemically infected Australian intensive dairy herd.

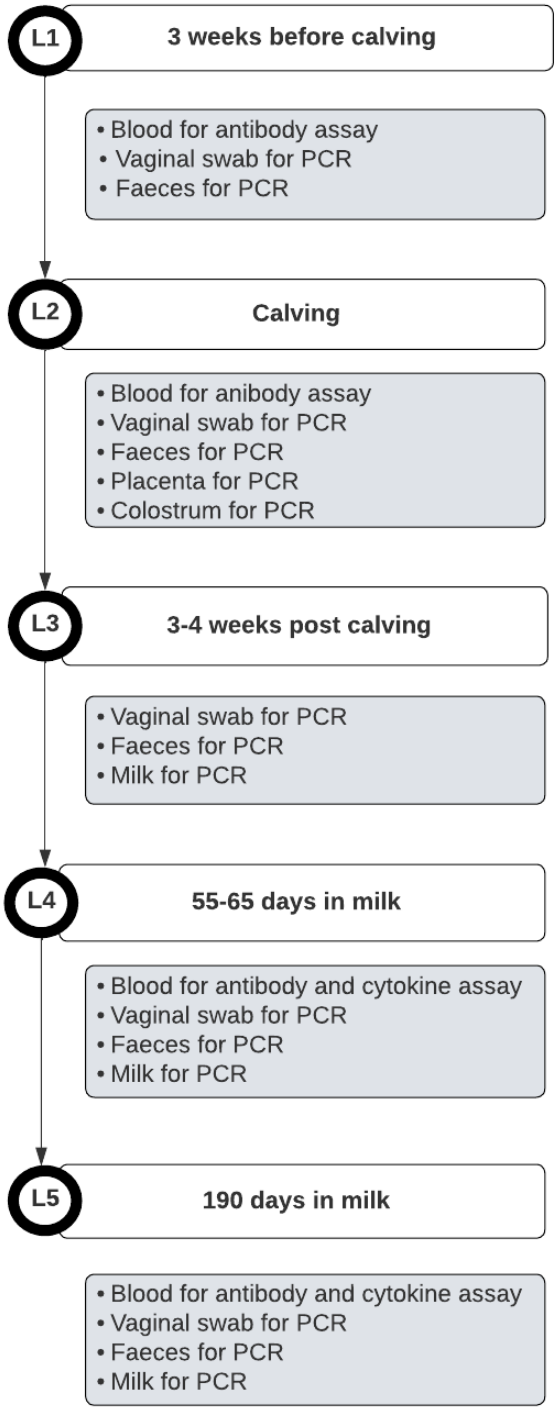


Table 3. Summary of animal sampling and preliminary PCR results for the longitudinal study of *Coxiella burnetii* in data-intensive cattle operation.

***** Suspect positive placentas – not positive on all three genes or very late cycling threshold values.

Longitudinal Sampling Timepoint	Stage in Cycle	Animals Sampled	Samples Collected at Each Timepoint	Total Number of Samples	PCR Results (% samples positive)			
					Placenta	Vaginal swabs	Colostrum or Milk	Faeces
L1 (mid-May 2022)	21 days prior to calving	396	Blood for serology, cytokine assays and PCR; vaginal swabs and faeces for PCR	1,680	N/A	3.0	N/A	1.2
L2	At calving	205	Blood for serology, cytokine assays and PCR; vaginal swabs, placenta, colostrum and faeces for PCR	1, 435	23.4 strongly positive; 43.4 suspect positive*	3.9	27.0	29.5
L3	21-28 days post calving	201	Blood for serology, cytokine assays and PCR; vaginal swabs, milk and faeces for PCR	2,010	N/A	2.0	5.0	10.5
L4	55-65 days post calving	185	Blood for serology, cytokine assays and PCR; vaginal swabs, milk and faeces for PCR	2,035	N/A	0	3.8	4.3
L5 (mid-December 2022)	190 days post calving	144	Blood for serology, cytokine assays and PCR; vaginal swabs, milk and faeces for PCR	1,296	N/A	4.9	9.2	2.1
Total Number of samples				8,456				

4.1.1.2 Serology – *Coxiella burnetii* ELISA

The results of the analysis of serum samples collected at L1, L2, L4 and L5 are presented in **Table 4**.

At the commencement of the longitudinal study (i.e., at L1 – or 21 days prior to calving) the seroprevalence of previous exposure to *C. burnetii* as measured by antibody in an ELISA was 32.5%. The seroprevalence dropped at calving (an immune phenomenon associated with calving) and then rose again by L4 and L5 to be 30.8% and 27.3% respectively.

Table 4. *Coxiella burnetii* seroprevalence (as determined by ELISA) in a longitudinal study conducted in an intensive dairy herd.

	Longitudinal 1 21 days prior to calving		**Longitudinal 2 At calving**		**Longitudinal 4 55-65 days post calving**		**Longitudinal 5 190-200 days post calving**	
ELISA Result	**Number**	**%**	**Number**	**%**	**Number**	**%**	**Number**	**%**
Positive	100	32.5	41	21.7	52	30.8	36	27.3
Negative	196	63.6	147	77.8	113	66.9	96	72.7
Suspect	12	3.8	1.0	0.5	4	2.4	0	0
Total	**308**	**100**	**189**	**100**	**169**	**100**	**132**	**100**

4.1.1.3 Cell mediated response – *Coxiella burnetii* cytokine stimulation/release assays.

Interferon-gamma is a pro-inflammatory cytokine associated with cell mediated immunity (CMI) or T Helper 1 (Th1) cell responses. It is produced by T cells and natural killer cells and stimulates macrophage microbicidal activity which has been demonstrated to be essential in control of infections associated with other intracellular bacteria such as those in the *Mycobacteria* genus. Interleukin-10 is an anti-inflammatory cytokine associated with T regulator cells (Treg) which functions to regulate the inflammatory response. It functions to inhibit cytokine production by Th1 cells while promoting B cell proliferation and differentiation. Assessment of these cytokines was chosen in this study because humans with chronic Q fever have demonstrated overproduction of IL-10 and high IFN-γ responses, and it was hypothesized that cows that mount ineffective immune responses to *C. burnetii* may follow similar pathways to humans with chronic Q fever.

The distribution of the results of the cytokine stimulation assays for interferon gamma (IFN-γ) and interleukin 10 (IL-10) in whole blood for 162 individual cows at the L4 (55-65 days post calving) sampling time point are presented in Figs. 2 and 3**.**

Figure 2. Individual cow interferon gamma response at 55-65 days post calving (L4) in a whole blood cytokine *Coxiella burnetii* antigen stimulation assay.

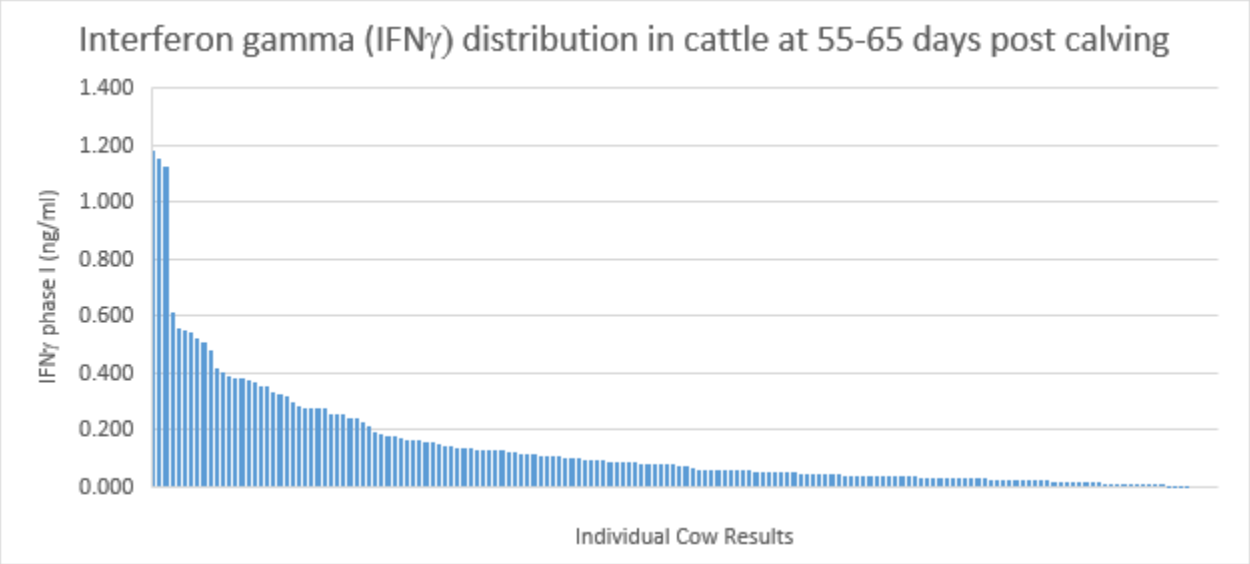
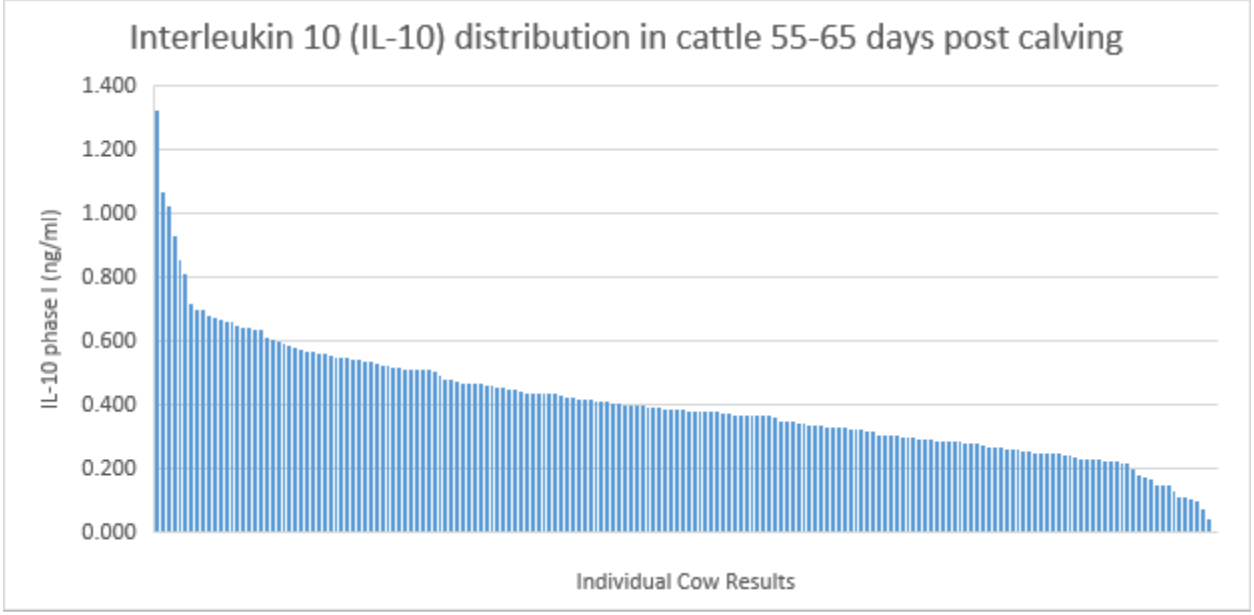


Figure 3. Individual cow interleukin 10 response at 55-65 days post calving (L4) in a whole blood cytokine *Coxiella burnetii* antigen stimulation assay.



4.1.1.4 Analysis for measurable impact of *Coxiella burnetii* on production and reproduction

Study Objectives

The primary objective of this investigation was to identify if cows with exposure to *C. burnetii* had impaired health, survival, and fertility, and to investigate if their offspring had impaired survival and health.

Fieldwork and Laboratory Testing

Section 4.1.1 outlines cow enrolment, sampling and laboratory testing. The enrolment dataset included measures of *C. burnetii* exposure, which were based on the testing of samples collected at stages L1 to L5. See Table 3 for an overview of test results used as measures of *C. burnetii* exposure in analysis.

Statistical Methods

Measures of health, survival, and fertility were extracted as raw data from the farm management software (DairyComp 305, Valley Ag Software, Tulare, USA) and imported in R programming environment (R Core Team, 2018). Data were inspected for duplicates, missing values, and outliers and then merged with the enrolment dataset for analysis. Survival analysis was used to investigate associations between measures of *C. burnetii* exposure and outcomes of interest show in Table 5.

Kaplan Meier failure curves were used to plot the percentage of individuals that had experienced the outcome of interest over time following enrolment. Competing risk survival analysis was used alongside conventional survival analysis for outcomes where subjects (cows or calves) could potentially experience events that would prevent them from experiencing the outcome of interest. For example, a cow is no longer at risk of abortion after calving (i.e., calving is a 'competing event'). This analytical approach allows estimation of the cumulative incidence following enrolment. Cox proportion hazards regression (a 'conventional' model approach to survival analysis) and a competing risk regression modelling approach (Fine and Gray, 1999), were used to yield hazard ratios (HR) and sub-distribution hazard ratios (SHR), respectively. In the context of this study the HR and SHR close to 1 implied a comparable risk of the outcome (e.g., pregnancy) between subjects exposed and unexposed to *C. burnetii*. If the hazard ratio exceeded 1, it suggested an increased risk of the outcome event among exposed subjects, while a hazard ratio less than 1 indicated reduced risk in the exposed group. Biologically plausible interactions between pairs of exposures were also investigated for their association with outcomes of interest. Continuous exposure measures were evaluated for linear and non-linear relationships with the outcome of interest. The proportional hazards assumption was evaluated using Schoenfeld residuals. No adjustments were made for multiple comparisons.

Table 5. Outcomes evaluated for association with measures of *Coxiella burnetii* exposure using survival analysis.

Outcome of interest	Time at risk
Cows enrolled at L1	
Pregnancy	From first calving after enrollment until pregnancy, censoring, or a competing event
Departure from the herd	From calving after enrollment until death, sale, or planned sale from the farm.
Abortion	From first pregnancy after enrolment until abortion, censoring, or a competing event (e.g., calving, departure)
Calves born from study cows	
Death	From birth until death or censoring
Scours	From birth until first case of diarrhea (identified by farm workers), or a competing event (e.g., death)
Respiratory disease	From birth until first case of respiratory disease (identified by farm workers), or a competing event (e.g., death).

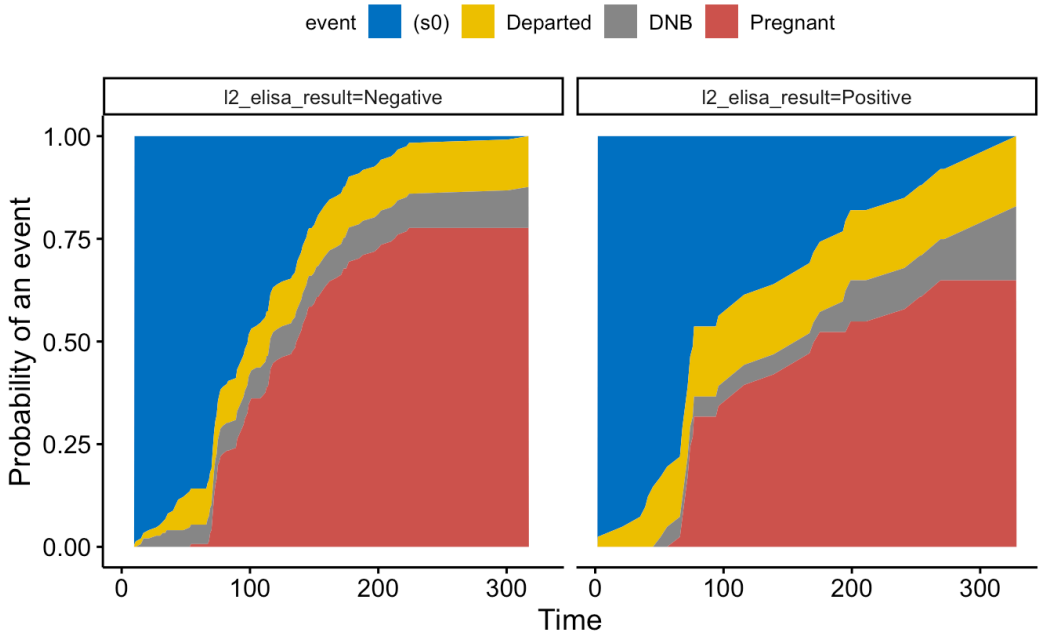
Results and Discussion

Analysis was conducted with 23 measures of exposure and six outcome measures of interest, equating to 96 models. We found that seropositive cows at L2 (calving) had HR and SHR of 0.6 (95% CI: 0.3 to 0.9) and 0.7 (95% CI: 0.5 to 1.1), respectively, for pregnancy indicating worse reproductive performance in *C. burnetii* exposed cows (Fig 4). We also found that cows that were seropositive for *C. burnetii* at the L5 sampling time point were 2.3 times more likely to depart from the herd or be removed from the breeding program, when compared to seronegative cows (HR = 2.3, 95% CI: 1.1 – 5.1). Furthermore, we found that the calves born from cows with a positive vaginal swab for *C. burnetii* at calving were 3.4 times (95% CI: 1.0 – 11.2) more likely to die than calves with negative mothers. Our analysis failed to identify clear associations between placenta PCR status at birth and outcomes of interest. Furthermore, no measures of exposure were strongly associated with foetal loss, which is consistent with the findings from our case control study (see 4.1.2).

Given the observed associations between *C. burnetii* exposure and measures of health and fertility in our dataset, it is plausible that *C. burnetii* exposure could impact beef herds in a similar way. However, it should be noted that this study was conducted in a single data-intensive cattle herd, and that many exposure-outcome combinations were evaluated, which increases the risk of identifying non-causal associations. Therefore, these findings should be replicated in a beef herd.

The finding that seropositive cows at calving had poorer reproductive performance (and further that this was not associated with placenta PCR results) suggests that the type of host immune response (i.e., humoral or cell mediated) following *C. burnetii* infection may have an impact on the subsequent reproductive outcomes.

Figure 4: The cumulative incidence of pregnancy (outcome of interest, red) and competing events (departure and DNB, yellow and green respectively) from calving (Time = 0) until 300 days post-calving. DNB = Do not breed.



4.1.2 Early Pregnancy Loss Case Control Study

Objectives

The objective of this case control study was to investigate associations between measures of *C. burnetii* exposure (blood antibody, vaginal swab PCR, and faecal PCR) and foetal loss in the first 100 days of gestation.

Fieldwork and Laboratory Testing

Blood (for serology via IDEXX ELISA), vaginal swabs (polyester - FLOQ and rayon; Copan Diagnostics) and faeces (for PCR) were collected from cows that had experienced foetal loss during the first 100 days of gestation (cases) and cows that had conceived at the same time and remained pregnant (controls). Control animals were matched by pen, lactation, conception date and days in milk. Pregnancies were the result of artificial insemination (AI) and embryo transfer (ET) and were diagnosed at day 32-39 after conception.

Sampling for this study commenced on 04/07/2022. A sampling end point was established on 30th August 2023 at which analysis was conducted on 81 cases and 135 controls, with stopping rules being established prior to analysis. Findings from interim analysis were not sufficiently conclusive to meet the *a priori* stopping rules. Consequently, an additional 45 cases and 90 controls were enrolled in late 2023 to increase the precision of effect estimates.

Statistical Methods

Exposure odds ratios (OR) were calculated to estimate the associations between measures of *C. burnetii* exposure and foetal loss. The OR was calculated using the tabular method (does not account for matching) and conditional logistic regression modelling (accounts for matching). Univariable

conditional logistic regression with no covariates was performed and multivariable modelling with conception method (AI or ET) as a covariate was performed.

Stratified models were used to compare the associations between *C. burnetii* exposure and foetal loss in AI and ET pregnancies. All statistical analyses were conducted in the R programming environment (R Core Team, 2018), using the package 'survival' (Therneau, 2023). The 95% confidence interval (CI) for the OR and Wald's test p-value were computed. A statistically significant result was observed if the 95% CI excluded the null value of OR = 1.0 (where OR of 1 = no effect; OR < 1 indicates a protective effect; OR > 1 indicates a risk effect), and Wald's test p-value was less than 0.05.

Results and Discussion

From the 115 cases and 187 controls included in the final analysis, 32.8% (99 / 302) of animals were positive for *C. burnetii*-antibody. *Coxiella burnetii* DNA was detected in 0.0% of flocked vaginal swabs (0/149), 0.0% of rayon vaginal swabs (0/216) and 0.5% faecal samples (1/216). Shedding was detected in 0.0% (0/81) of cases and 0.7% (1/135) of control animals. The finding that *C. burnetii* was rarely detected in vaginal swabs and faeces in pregnant and non-pregnant cows indicates that *C. burnetii* is unlikely to be shed via these routes, and such diagnostic pathways are unlikely to be a useful for investigating foetal losses in cattle. All subsequent statistical analysis utilised serological results only and swabs and faecal samples were not collected from the cows enrolled in late 2023.

The distribution of antibody ELISA units was similar between cases and controls, as shown in Figure 5. Cases and controls are compared for variables measured in the study in Table 6. The prevalence of *C. burnetii* seropositivity was 33.0% (38/115) in cases and 32.6% (61/187) in control animals. Odds ratios for the association between Coxiella seropositivity and foetal loss are presented in Table 7. Multivariable conditional logistic regression modelling with conception method (AI vs ET) as a covariate observed an OR = 0.89 (95% CI: 0.54 - 1.46, P = 0.63) for foetal loss in seropositive vs seronegative cattle. A similar association was observed in AI pregnancies (OR = 0.84, 95% CI: 0.40 – 1.79; P = 0.66) and ET pregnancies (OR = 1.05, 95% CI: 0.49 – 2.27; P = 0.90). This finding indicates that under the conditions in this study, where seropositivity is relatively common (~35%), it appears unlikely to be a major contributor to foetal loss in the first 100 days of gestation, as evidenced by OR estimates being close to 1. This conclusion is consistent with the concurrent longitudinal study, where seropositivity was not observed to be a risk factor for abortion.

Figure 5: Density plot comparing the distribution of *Coxiella burnetii* antibody ELISA values for cases and control cows.

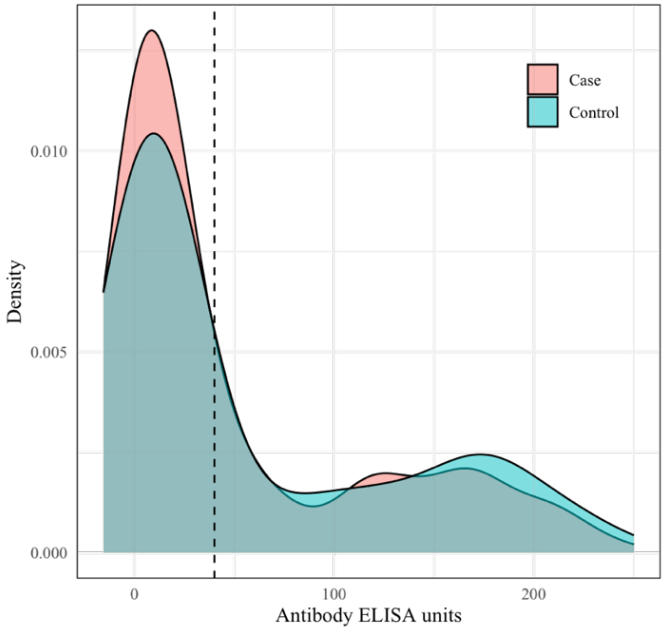


Table 6: Comparison of cases and controls for serostatus for *Coxiella burnetii* and other reproductive risk factors for foetal loss.

	Cases (N=115)	**Controls (N=187)**
Coxiella seropositive	38 (33.0%)	61 (32.6%)
Date at enrolment[1]		
2022-07-04	9 (7.8%)	16 (8.6%)
2022-07-19	16 (13.9%)	28 (15.0%)
2022-08-02	14 (12.2%)	24 (12.8%)
2022-10-13	18 (15.7%)	29 (15.5%)
2023-08-22	20 (17.4%)	32 (17.1%)
2023-08-23	6 (5.2%)	9 (4.8%)
2023-11-20	32 (27.8%)	49 (26.2%)
Conception method		
AI	44 (38.3%)	101 (54.0%)
ET	71 (61.7%)	86 (46.0%)
Parity[1]		
1	20 (17.4%)	35 (18.7%)
2	55 (47.8%)	89 (47.6%)
3	18 (15.7%)	27 (14.4%)
4	10 (8.7%)	18 (9.6%)
5	8 (7.0%)	11 (5.9%)
6	3 (2.6%)	6 (3.2%)
7	1 (0.9%)	0 (0%)
Conception to enrolment (d)[1]		
Mean (SD)	61.3 (15.2)	60.2 (15.1)
Median [Min, Max]	59.0 [38.0, 109]	58.0 [36.0, 111]
Calving to enrolment (d)		
Mean (SD)	154 (36.3)	157 (41.6)
Median [Min, Max]	148 [111, 302]	145 [106, 315]
Conception to first positive pregnancy diagnoses after conception (d)		
Mean (SD)	36.5 (10.9)	43.2 (17.1)
Median [Min, Max]	33.0 [30.0, 82.0]	34.0 [30.0, 80.0]
[1]Cases and controls were matched according to X		

Table 7: Associations between *Coxiella burnetii* seropositivity (measured by an ELISA-antibody test on serum) and abortion during the first 100 days of gestation.

	No. seropositive/no.	No. seropositive/no.	OR (95% CI)

	cases (%)	controls (%)	
Tabular methods			
Whole dataset	40 / 126 (31.7)	80 / 251 (31.9)	0.99 (0.63 to 1.57)
Final dataset	38 / 115 (33.0)	61 / 187 (32.6)	1.02 (0.62 to 1.67)
Logistic regression[1]			
Unconditional model	38 / 115 (33.0)	61 / 187 (32.6)	1.03 (0.64 to 1.66)
Conditional model			
AI pregnancies only	14 / 44 (31.8)	31 / 101 (30.7)	0.84 (0.40 to 1.79)
ET pregnancies only	24 / 71 (33.8)	30 / 86 (34.9)	1.05 (0.49 to 2.27)
Adjusted for conception method	-	-	0.89 (0.54 to 1.46)

4.2 *Coxiella burnetii* in extensive beef cattle production systems

The intensive dairy system study determined that serology was the most useful sample available to use to strategically sample beef cattle herds particularly given that the opportunities for sampling whereby cattle are yarded for normal management procedures occur relatively less frequently.

The strategy for investigations into extensive beef herds two stages:

Stage 1 – Use of serological assays to identify beef cattle enterprises where *C. burnetii* is endemic and which could be enrolled in Stage 2.

Stage 2 – Collection of blood from animals residing within endemic herds in a longitudinal study throughout the production cycle through one year and analysis of those samples via *C. burnetii* ELISA to identify exposed animals. These exposure variables would then be analysed to identify associations with outcomes derived from herd records.

4.2.1 Results of Stage 1: serological Screening in the Queensland Beef Cattle Production System

Three beef cattle enterprises (one in Queensland and two in northern NSW) were identified and screened as described for Stage 1 above as follows.

Property 1: located in Queensland

The results for a Queensland property are presented in Table 8 and 9. The overall seroprevalence for previous exposure to *C. burnetii* for this property was 5% with the highest seroprevalence in pregnant cows at 15%. While *C. burnetii* was identified as endemic on this farm, no further sampling was conducted at this property as it was determined that it would be logistically difficult to carry out the stage 2 sample collections in this location.

Table 8. *Coxiella burnetii* seroprevalence (as determined by ELISA) in an extensive beef farm located in Queensland.

ELISA Result	Number of animals	%
Positive	4	5.0%
Suspect	2	2.5%
Negative	74	92.5%
Total	**80**	**100.0%**

Table 9. *Coxiella burnetii* seroprevalence (as determined by ELISA) in an extensive beef farm located in Queensland by animal status.

	Pregnant				Empty			
	Heifers		Cows		Heifers		Cows	
	n	%	n	%	n	%	n	%
Positive	1	5.0%	3	15.0%	0	0.0%	0	0.0%
Suspect	1	5.0%	1	5.0%	0	0.0%	0	0.0%
Negative	18	90.0%	16	80.0%	14	100.0%	26	100.0%
Total	**20**	**100.0%**	**20**	**100.0%**	**14**	**100.0%**	**26**	**100.0%**

Property 2: located in Northern New South Wales

A total of 60 animals were sampled the first northern NSW farm, however no animals returned a positive serological result from this farm.

Property 3: located in Northern New South Wales

A total of 65 samples were obtained from two separate mobs on this farm. Mob 1 (cows) showed a seroprevalence of 26 %, whilst the second mob (heifers) returned a 3 % seroprevalence.

4.2.2 Results of Stage 2: Serological Screening in a Northern NSW Beef Cattle Production System

The second northern NSW farm agreed to participate in Stage 2, which was intended to be a longitudinal observational study that measured seroprevalence for *C. burnetii* at three time points (pre-breeding, at pregnancy diagnosis, and after subsequent calving). The objectives of the proposed longitudinal study were to: 1) Describe differences in seroprevalence by management group (grouped by age) and time, and 2) identify if seropositive animals had worse reproductive performance than seronegative animals.

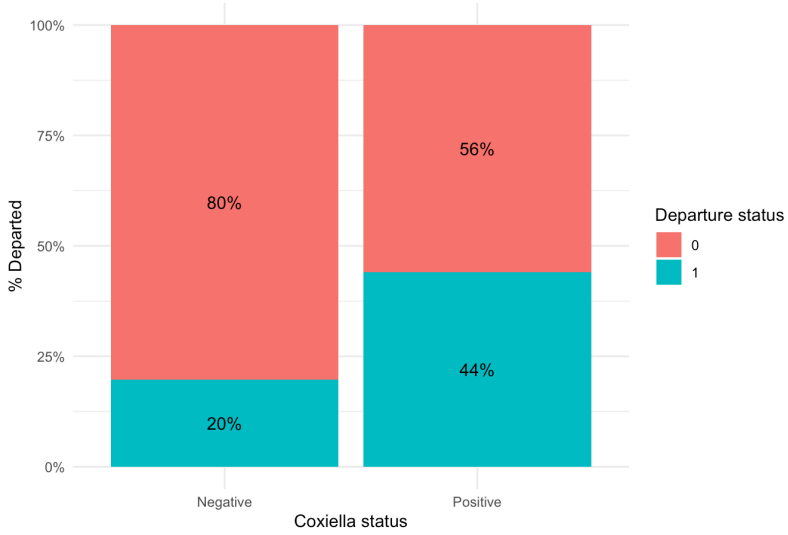
A total of 270 subjects were enrolled between 25/08/2023 and 22/09/2023, which was prior to the start of the mating period for the farm. The seroprevalences of *C. burnetii* at this time-point are shown Table 10**.** The seroprevalence in heifers and 3-year-olds were 0% and 3.6%, respectively, which is lower than what was observed in older groups (ranges 10.3 to 30.7%).

Table 10 *Coxiella burnetii* seroprevalence (as determined by ELISA) by age group at pre-breeding in an extensive beef farm located in northern NSW. Animals of different age groups remain as discrete mobs and are managed separately without mixing. (n=number)

	Heifers		3-year-old		6-year-old		7-year-old		8-year-old	
	n	%	n	%	n	%	n	%	n	%
Positive	0	0	1	1.8	3	10.3	14	26.9	6	11.6
Suspect	0	0	1	1.8	0	17.2	2	15.4	1	11.6
Negative	72	100	54	96.4	26	72.4	36	57.7	54	76.7
Total	72	100	56	100	29	100	52	100	61	100

Due to unexpected events at the study site, all heifer animals were lost to follow-up, and we were not able to measure pregnancy status in enrolled subjects during the follow-up period. Consequently, the analytical approach was adjusted to only include non-heifer animals, using the outcome variable 'departure', which was measured by comparing animal inventory lists collected at enrolment and in June 2024. Study subjects that were absent at the second inventory were assumed to have departed from the herd during the follow-up period. The farmer estimated that almost all departures were due to culling (i.e., not died on farm) during March following pregnancy testing. He estimated that the main reasons for culling were for empty-status as pregnancy diagnosis (50%) and for bad foot conformation (50%). Fig. 6 compares the percentage of animals that departed the study during the follow-up period. Seropositive animals (11 / 25, 44%) were 2.2 times more likely to depart the herd than seronegative animals (23 / 162, 20%).

Figure 6. Comparison of *Coxiella burnetii* serostatus (as determined by ELISA) and departure from the herd in cows in a northern New South Wales beef herd.



Multivariable models (generalised linear models and generalised linear mixed models) were used to adjust risk ratio estimates for clustering/confounding by mob and to explore for effect heterogeneity (Table 11). Adjusted risk ratio estimates (1.7 and 2.1) were similar to the unadjusted estimate (2.2). Findings from the interaction model did not find evidence of heterogeneity of effects (similar stratum specific risk ratio estimates, P > 0.05 for interaction term).

Table 11. Summary of effect estimates (risk ratio) from statistical models evaluating the relationship between *Coxiella burnetii* exposure status at enrolment (pre-breeding) and risk of departing the herd in the following 9 months.

Model	Risk ratio (95% CI)	Description
GLM Unadjusted	2.2 (95% CI: 1.2 to 3.7	Generalised linear model (binomial family, log link) without adjusting for group
GLM Fixed effect	1.7 (95% CI: 0.9 to 3.0	Generalised linear model (binomial family, log link) with a fixed effect added for management group (mob).
GLMM	2.1 (95% CI: 1.1 to 3.7	Generalised linear mixed model (binomial family, log link) with a random effect (intercept) added for management group (mob).
Interaction model	Estimates were all > 1 (ranging from 1.2 to 3.8).	Generalised linear model (binomial family, log link) with an interaction term for Coxiella status x management group. The P-value for the interaction term was > 0.05.

These findings indicate that seropositive animals in this study were more likely to depart the herd than seronegative animals. We hypothesise that this may be due to worse reproductive performance in the seropositive group. However, this requires further investigation.

In summary, the findings from the beef herd longitudinal study indicate that the association between *Coxiella burnetii* exposure and pregnancy rates observed in the data-intensive dairy herd, may also occur in a beef setting. However, further research in a larger number of beef cattle herds is needed to verify this.

4.3 A beef herd model to assess the physical and economic impacts of changes in reproductive performance, calf survival and/or growth rates.

4.3.1 Objectives

The aim of this component of the project is to develop a stochastic simulation model to provide robust estimates of impacts of changes in reproductive performance, calf survival and/or growth rates in pasture-based seasonal calving (i.e., restricted mating) beef breeder herds in temperate regions of Australia.

A secondary aim is to provide a preliminary estimate of an example impact of *Coxiella burnetii* in such herds based on estimates of both the prevalence of exposure and size of effect from lactating cows in a dairy herd.

4.3.2 Purposes of the model

The model provides estimates of the impacts on herd physical inputs and outputs of changes in reproductive performance, calf survival and/or growth rates in a pasture-based seasonal calving (i.e., restricted mating) beef breeder herd over a range of situations including:

- the suitability of the farm for cutting and feed silage and/or hay, and accessibility to reasonably priced grain
- various pasture growth rates (e.g., low, medium, high for each month or year)
- various stocking rates, and
- various levels of herd reproductive performance under the counterfactual.

The model has been designed to assist both those developing, and those assessing, research and development projects in understanding the likely magnitudes of, and variability in, impacts due to changes in reproductive performance, calf survival and/or growth rates. The model will help identify circumstances when impacts are largest and when these are least and will help define minimal reductions in reproductive performance etc that have important impacts.

The model will also assist those people along with veterinary practitioners, other advisers and herd managers in learning about system interactions that determine these impacts.

Finally, the model may be a useful support tool for herd managers when supported by an appropriate adviser in making decisions for their particular farm. The appropriateness of this use of the model will need to be assessed after the model has become available and been used for some time.

4.3.3 Model development methodology

Richard Shephard has developed the model by extensively modifying a dairy herd simulation model he had previously developed, including adding modules for calf growth, sales and purchases, and other aspects of the farm. Model logic and plausible parameter values will be heavily informed by inputs from Paul Cusack, Australian Livestock Production Services, with critical review by Sam Rowe

and John House from the University of Sydney and John Morton (Jemora Pty Ltd and collaborator for other components of the project).

4.3.4 Key features of the model

The simulated herd is a pasture-based seasonal calving (i.e., restricted mating) beef breeder herd. No sheep or other non-bovine species are run on the farm and no cropping is conducted on the farm. In addition to cull cows, sales of steers and surplus heifers have been incorporated into the model based on farm- and animal-level decision rules, and purchases of steers and/or heifers is allowed only where excess pasture is available, or where necessary following poor herd reproductive performance.

Both estimates of central tendencies and widths of distributions of key outcome variables due to both uncertainties about, and variability in, parameter values will be generated.

The model simulates events separately for each animal from conception or purchase to death or culling on a daily basis over time. This allows substantial flexibility in use of the model.

The default settings for the herd will reflect a well-managed herd. Herd management responses to reductions in reproductive performance, calf survival and/or growth rates will be inbuilt to reflect good management that minimises the consequences of adverse events.

The model will not be designed to assess effects of herd system changes on risk.

The model is flexible in that it can be used for assessing impacts of numerous causes of reduced reproductive performance, calf survival and/or growth rates. The model does not directly assess the impact of exposures such as *C. burnetii* nor of interventions such as a *C. burnetii* cattle vaccine. Rather, estimates of various infectious agents (including but not limited to *C. burnetii*) and other factors (e.g., synchronisation and AI programs) on reproductive performance, calf survival and/or growth rates under specified conditions are obtained and inputted by the user. The user will be able to vary both the magnitude of these effects, the breeding group affected (i.e., yearlings or cows) and the number of females in the group exposed to that cause in any particular year. The model provides estimates of the impacts of those changes on herd physical inputs and outputs.

For example, assuming that exposure of cows to *C. burnetii* before calving decreases reproductive performance in the lactation commencing with that calving (as appears to have occurred in the study dairy herd), the estimated proportion of cows exposed and size of that effect in exposed cows will be entered as inputs and the consequences of the decreased reproductive performance on herd physical inputs and outputs assessed by comparing those under that to the counter factual of no such cows exposed.

Other features of the model are as follows:

- The user will be able to specify different hazards of conception for each day of the breeding period.
- A 300 or 400-cow breeder herd will be modelled. Nothing in the model will be affected by economies of scale so there is no point in modelling larger herds.
- Herd size will be steady state i.e., the model will not incorporate herd rebuilding and herd reduction.
- The herd will have just four groups of cattle:
    - Cows (plus unweaned calves from calving to weaning)
    - Replacement heifers

    - Steers and excess heifers (heifers to be sold)
    - Bulls
- The user will specify hazards of conception for each day of the breeding period separately for each of the cow mob and the replacement heifer mob when mated as yearlings.
- The user will nominate:
    - whether silage and/or hay are cut and fed on the farm
    - whether grain can be fed on the farm
- Purchase of additional heifers and/or steers will be allowed under decision rules to be specified.
- Sale dates for steers and excess heifers will be based on decision rules including the following:
    - whether the herd has <30 days of standing feed
    - whether silage and/or hay and/or grain are fed on the farm
- Genetic effects will not be modelled.
- Use of artificial insemination will not be specifically included. However, as the user will specify hazards of conception for each day of the breeding period, if they wish, values can be chosen that would reflect a poor AI program, or alternatively, a successful AI program followed by various hazards of conception when bulls are with the mob.

4.3.5 Model delivery

The deliverables from this project component, on completion, will be:

- a non-technical description of how the simulated herd operates,
- some example comparisons of scenarios from the model, and
- availability of the model, critically reviewed for logic and robustness and usable by Richard Shephard, the major model developer and model coder.

The appropriateness of various applications of the model will need to be assessed after the model has become available and been used for some time. (This will be after the end of the current project.) For example, the desirability and appropriateness of making the model available for interactive use on-line will need to be assessed. If this application is desired and appropriate, it would be possible to do this using a Shiny app (Shiny is an R package that enables building interactive web applications that can execute R code on the backend). However, supporting documentation would need to be developed and modest ongoing funding would be required for ongoing web access fees.

4.3.6 Progress of this component of the project

As indicated in the project milestone report dated 30th June 2024, and as discussed in detail with Michael Laurence on 17th September, this component of the project has been considerably delayed as a central collaborator in this component of the project, was not available for much of the first half of 2024. As a result, delivery of this component of the project will be slightly delayed beyond 30th September 2024. The anticipated date for completion of this component of the project is 31st October 2024.

Most major coding has been completed. Once the model has been finalised, several scenarios will be run, including providing a preliminary estimate of an example impact of *C. burnetii* in pasture-based seasonal calving (i.e., restricted mating) beef breeder herds in temperate regions of Australia. The

latter will be done using estimates of both the prevalence of exposure and size of effect from lactating cows in a dairy herd from a separate component of the current project (see section 4.1-2). From that component, the prevalence of exposure (estimated as seroprevalence at calving using a *C. burnetii* ELISA described in Section 3.3.2) was 21.7% and the hazard ratio for conception for seropositive cows relative to seronegative cows was variously estimated at 0.6 (95% CI 0.3 to 0.9) using a Cox model and 0.7 (95% CI 0.5 to 1.1) using a Fine and Gray model that accounted for competing risks.

4.4 Seroprevalence of *Coxiella burnetii* for cattle processed in an Australian abattoir.

4.4.1 Aim of the study and study design

The aims of this study were:

a) to describe seroprevalences to *C. burnetii* in different classes of cattle slaughtered in an abattoir from a wide range of geographic sources;
b) to assess the extent of clustering of seropositivity to C. burnetii by lot; and
c) to assess some potential risk factors for seropositivity.

This study was conducted in an abattoir located in southern Queensland, Australia that processes cattle from a wide geographical region in eastern Australia including northern NSW, southern Queensland, and western Queensland. Samples were collected from cattle in December 2022, June 2023 and November 2023. Five classes of cattle representing major beef cattle classes produced in Australia were sampled including: pasture-based heifers, pasture-based steers, feedlot heifers, feedlot steers and cows. To identify a seroprevalence difference of 10 % between classes of cattle, for each class, 20 lots of cattle were selected containing 15 unique carcases. The aim was to test 300 carcases for each class and a total of 1,500 carcases (however only 288 carcases were available for pasture steers resulting in a total of 1,488 carcases from 72 properties being sampled). Additional data including pregnancy status and animal source was collected for each carcase. Lots were defined as source groups of cattle of the same class from the same property and processed sequentially.

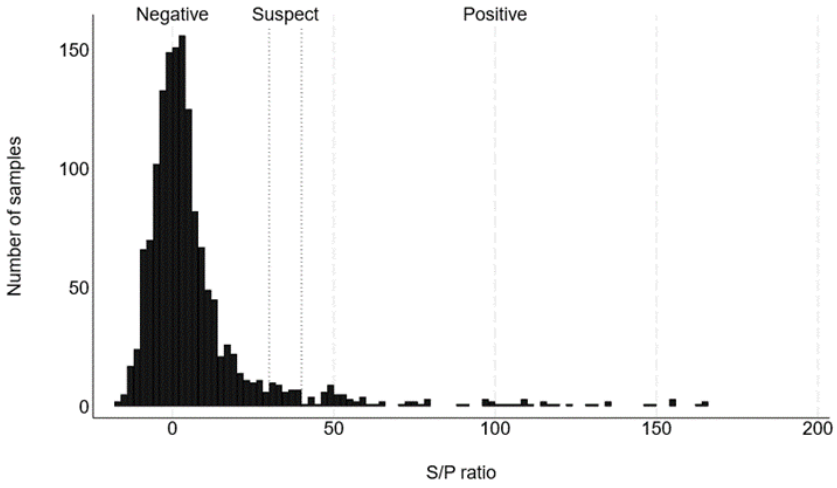
4.4.2 Sample collection and processing, and serology

Approximately 10 ml of free-flowing blood was collected from the chest cavity of each carcase post exsanguination and electrical stimulation into a blood collection tube containing silica to activate clotting and a gel for separation during centrifugation (Becton, Dickinson and Company, UK). Samples were gently mixed by inversion before transportation to the laboratory on ice. On arrival at the laboratory, samples were centrifuged (Centrifuge 5810R; Eppendorf, Australia) at 4,000 x g for 15 minutes, after which the serum was removed and stored at -45 °C until analysed in duplicate in the IDEXX ELISA kit to measure antibody against *C. burnetii* as outlined in Section 3.3.

4.4.3 Statistical Analysis

Statistical analyses were performed using Stata Statistical Software: Release 18 (StataCorp LLC, College Station, TX, USA). Seropositive animals were compared to non-seropositive animals, which included suspect and seronegative results (Figure 7)

Figure 7 Distribution of ELISA results (sample-to-positive ratio, S/P) for 1,488 carcases from a Queensland abattoir. Positive results (n = 124) are contrasted to negative and suspect results.



Clustering of seropositivity within lots was assessed for each class of cattle using intraclass correlation coefficients (ICC). If seroprevalences are the same in each lot within a class, there is no clustering of seropositivity by lot and ICC = 0. Alternatively, if all animals are seropositive in some lots and non-seropositive in the remaining lots, there is complete clustering of results by lot and ICC = 1. ICC was calculated after fitting constant-only two-level mixed-effects logistic regression models and using the latent variable threshold approach as:

random intercept variance/(π2/3 + random intercept variance).

Associated 95% confidence intervals for ICC were calculated using the logit transformation with standard errors calculated using the delta method. Stata's -melogit- and -estat icc- commands were used.

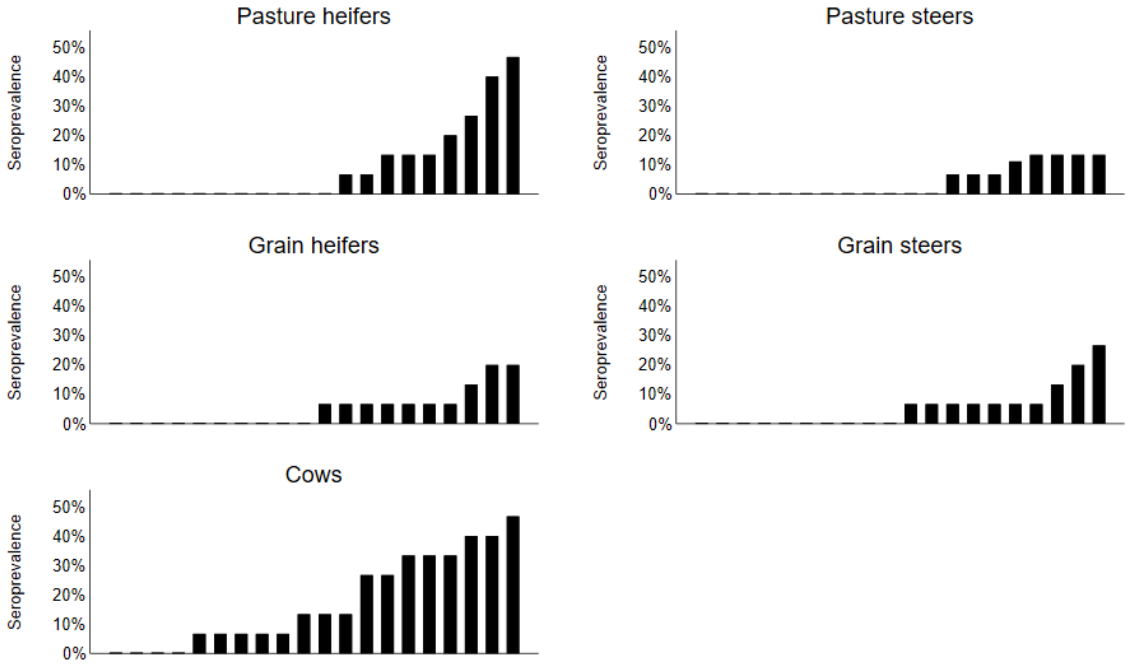
Seroprevalences were compared between population subsets (e.g., class, location) using multilevel mixed-effects logistic regression models as described above with fixed effects defining population subsets.

4.4.3 Results

A total of 1,488 carcases from 72 properties were sampled with 300 from each class except for pasture steers (n = 288; Table 13). All classes included 20 lots of cattle, with 15 animals per lot. The seroprevalence of antibodies to *C. burnetii* was 8.3% at animal level and 53% at lot level (Figure 8).

Figure 8. Distribution of within-lot seroprevalence of antibodies to *Coxiella burnetii* in cattle at slaughter. Each bar presents one lot of 15 animals.

Seroprevalence was highest in cows (17.7% at animal level, 80% at lot level), followed by pasture-fed heifers, and grain-fed animals, and lowest in pasture steers (4.2% at animal level, 40% at lot level). The difference in seroprevalence between cows and other classes was significant for each class



(Table 12). ICC was highest for pasture heifers (0.48) and lowest for pasture steers (0.05) (Figure 8; Table 12)

Table 12. Seroprevalence of antibodies to *Coxiella burnetii* for five classes of cattle sampled at slaughter in Queensland, Australia, 2022-2023. Intraclass correlation for seroprevalence within lot is shown based on 20 lots per class.

Class	Sampled	Positive (%)		Odds ratio for seropositivity (95% CI)	Intraclass correlation (95% CI)
		Animals	Lots		
Cows	300	53 (17.7)	16 (80)	reference	0.22 (0.08; 0.48)
Grain Heifers	300	15 (5.0)	10 (50)	0.22 (0.1; 0.6)	0.09 (0.04; 0.73)
Grain Steers	300	16 (5.3)	10 (50)	0.23 (0.1; 0.6)	0.17 (0.02; 0.61)
Pasture Heifers	300	28 (9.3)	9 (45)	0.39 (0.2; 0.9)	0.48 (0.19; 0.77)
Pasture Steers	288	12 (4.2)	8 (40)	0.18 (0.1; 0.5)	0.05 (0.0; 0.96)

Seropositive cattle, particularly cows, were detected in low and high rainfall zones (Figure 9). Seroprevalence was numerically lower in zone 2 (intermediate rainfall) than in zones 1 (high rainfall) or 3 (low rainfall) for cows and pasture-raised animals but the association between zone and seroprevalence was not significant when accounting for class of stock (Table 13). The number of observations for combinations of class, zone and sampling date was too small for meaningful analysis of temporal associations.

Figure 9. Origin of cows and pasture-raised animals sampled at slaughter (left) and seroprevalence of *Coxiella burnetii* antibodies in lots of cows (right) relative to rainfall (48-month rainfall map adapted from Australian Bureau of Meteorology).

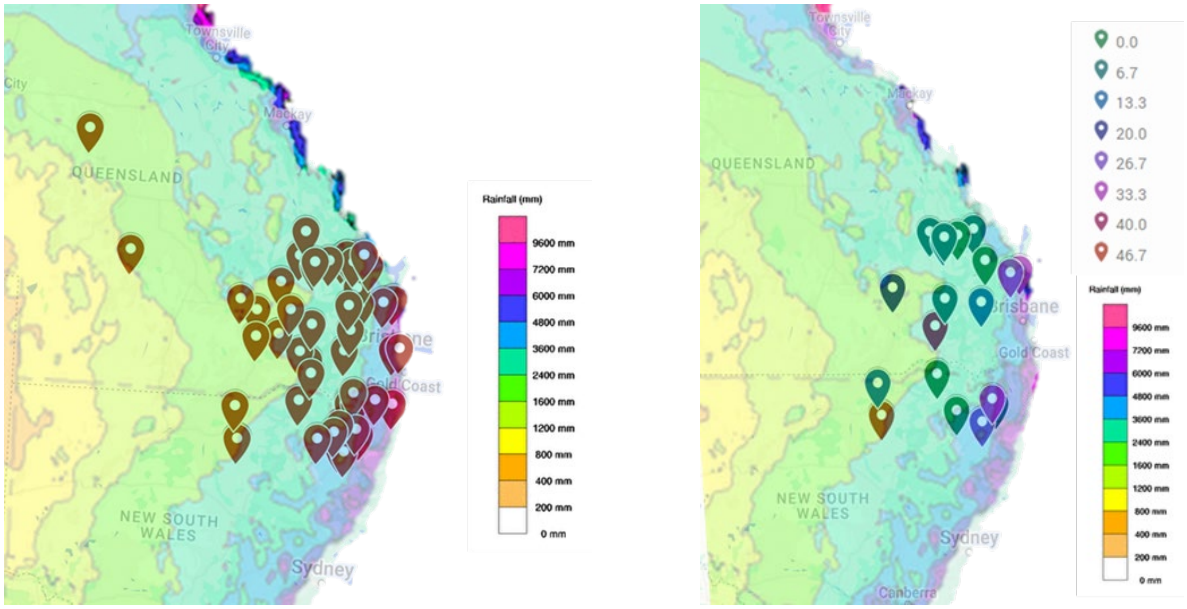


Table 13. Seroprevalence of antibodies to *Coxiella burnetii* at slaughter for three classes of cattle across three rainfall zones in New South Wales and Queensland (Zone 1, >3600 mm; Zone 2, 2400-3600 mm; Zone 3, < 2400 mm based on 48-month rainfall data from Australian Bureau of Meteorology).

	Class				
	Cow	**Pasture Heifers**	**Pasture Steers**	**Total**	**Odds ratio (95% CI)**
Zone 1					Reference level
Mean	22%	13%	1%	12%	
Range	0% - 33%	0% - 47%	0% - 7%	0% - 47%	
Total lots	6	9	6	21	
Zone 2					0.41 (0.17; 1.02) p = 0.055
Mean	9%	4%	4%	6%	
Range	0% - 40%	0% - 27%	0% - 13%	0% - 40%	
Total lots	10	7	8	25	
Zone 3					1.93 (0.75; 4.96) p = 0.172
Mean	32%	12%	8%	16%	
Range	13% - 47%	0% - 20%	0% - 13%	0% - 47%	
Total lots	4	4	6	14	
Total					
Mean	18%	9%	4%	10%	
Range	0% - 47%	0% - 47%	0% - 13%	0% - 47%	
Total lots	20	20	20	60	

4.4.4 Discussion

The seroprevalence of *C. burnetii* in beef cattle in NSW and Queensland in the current study (4.2% to 17.7% depending on class) was higher than that previously reported for Western Australia WA; (0.6% of 329 cattle; Banazis et al., 2010;, but the observed seroprevalences were similar to results from previous reports from Queensland (Qld) and Northern Territory (NT) cattle (seroprevalence 17% of 1344 cattle on-farm or at slaughter; Cooper et al., 2011; estimated prevalence of prior exposure 5% of 2012 breeding cattle; Wood et al., 2021). Studies differed in laboratory methodologies, which included IDEXX ELISA (this study and Banazis et al., 2010), in-house ELISA for phase I and phase II antibodies (Cooper et al., 2011) or indirect immunofluorescent assay (Wood et al., 2021).

Animal classes and age also differed by study. Most animals in the WA study (Banazis et al., 2010) were heifers or steers rather than cows. By contrast, the CashCow study (Wood et al., 2021) focussed on breeding mobs on farms with high quality cattle handling facilities and annual pregnancy testing. The earlier study from Qld and the NT (Cooper et al., 2011) included heifers,

cows, and beef cattle at slaughter (Townsville abattoir in Northern Qld) but did not provide a breakdown of seroprevalence by animal class. In our study, seroprevalence was higher in cows than in heifers or steers, which may have been due to a longer time period of exposure amongst cows (being older than heifers and steers) along with the fact that cows would have gone through at least one breeding and calving period noting that *C. burnetii* targets the reproductive system during pregnancy. The low seroprevalence in the WA study could be due, in part, to the high proportion of heifers and steers included in that study. By the same logic, however, the CashCow study, which focussed on breeding animals, could have been expected to yield a higher seroprevalence than that observed in our study but that was not the case. Likewise, lot-level seroprevalences (percentages of lots that had at least one seropositive animal) in our study (40 to 80%, depending on class) were higher than herd-level seroprevalences in the CashCow study (17 to 78%, depending on region). The difference was particularly noticeable for Southeast Queensland (17% herd level seroprevalence in Wood et al. (2021), a region that was included in our study.

In addition to the class of animal, environmental and management conditions may affect the prevalence of pathogens and infectious diseases. Herds represented in the current study typically have higher rainfall, *Bos taurus* breeds, lower herd sizes, higher stocking rates and more intensive management than northern Australian production systems (Campbell et al., 2014; Greenwood et al., 2018). Northern production systems, as represented by the two studies from Queensland and Northern Territory, typically have larger herds and animals are generally *Bos indicus* cattle (Greenwood et al., 2018). Both systems supply cattle to feedlots which by purchasing cattle from grazing or breeding properties and feeding within a set area a designated supplement diet, usually including grain, for a set number of days to reach a target turn-off weight. Differences in seroprevalence or impacts of coxiellosis between subspecies or breeds of cattle have not been reported in any of the studies.

Seasonal trends in seroprevalence were observed in Qld beef cattle, with a significant increase in seroprevalence from April/March through May/June and July/August to Sept/Oct (Cooper et al., 2011). Our study design did not allow for robust analysis of seasonal effects, but we did observe spatial patterns. Seroprevalence was numerically (and in cows, statistically) higher in areas with high (>3200 mm; temperate region) or low (< 2400 mm, grasslands) rainfall compared to the area with intermediate (2400-3600 mm; subtropical) rainfall. Whether there is a biological association with rainfall itself or whether rainfall is a proxy for other environmental factors, e.g., cattle management systems or exposure to macropods which may carry *C. burnetii* (Tolpinrud et al., 2024), remains to be determined. Based on studies after abortion storms in goats, vegetation and soil moisture may limit the dispersion of C*. burnetii* via dust, whereas areas that are characterized by a combination of arable land, limited vegetation and deep groundwater pose a comparatively high risk of transmission (Van der Hoek et al., 2011). Airborne transmission of *C. burnetii* over a distance of a few kilometres has been described and modelled in the context of dairy production (cattle and goats) in Europe (Van der Hoek et al., 2011, Pandit et al., 2016). Those settings may bear some resemblance to the high rainfall areas in our study, which were usually along the coast with varying altitude, high pasture growth and intensive animal management as would typically align with southern Australian production systems (Campbell et al., 2014). They are quite distinct from the lower rainfall areas in our study (Zones 2 and 3), which are inland and reliant on rivers, dams and rainfall for water (Greenwood et al., 2018). The production system of Zone 3 is generally less intensive and operate as more typical northern production systems. Thus, mechanisms underpinning an association between environment and prevalence may be quite diverse. However, it is clear that no single environment or farm type is “safe” in terms of *C. burnetii* exposure.

Although it is tempting to interpret seroprevalence data in terms of transmission and human exposure risk, the correlation between antibody status and *C. burnetii* shedding is weak, as documented elsewhere in this report (see section 4.1) and in the scientific literature (Sadiki et al., 2023; Menadi et al., 2022). Thus, seroprevalence data inform the distribution of exposure to *C. burnetii* spatially and by class of cattle, so can be used as a guide as to which regions or cattle classes are of most interest in further studies on the shedding, transmission and impact of *C. burnetii* in cattle. The current study shows that *C. burnetii* is widespread, similar to that previously reported for the Northern beef industry. In addition, the low seroprevalence in heifers compared to cows suggests that separating the two age groups may help prevent transmission of the pathogen to young stock. In intensive systems heifers are often managed separately to cows for production reasons (Schatz & Partridge, 2012), and an additional benefit of this approach may be reduced exposure to *C. burnetii*. However, dispersal of *C. burnetii* via dust and aerosols (de Rooij et al., 2016) may still result in transmission between cows and heifers. Alternative prevention measures, such as vaccination, would require evidence of the reproductive impact of *C. burnetii* in beef cattle, and of the economics and logistic feasibility of vaccination development and use of vaccination as a control strategy.

4.4.5 Conclusions

Exposure of beef cattle to *C. burnetii* is not uncommon, with evidence of infection in herds from a wide geographic area and very high seroprevalences in some lots presenting at abattoir. Seroprevalences appear to be highest in cows. Exposure risk may vary with environmental conditions. Although seropositivity is not indicative of infectivity and human exposure risk, the high observed seroprevalences indicate that it is important to determine the production impact of *Coxiella* infection in beef production, as well as the risk of exposure of humans at slaughter, particularly when handling cows.

4.5 Impact of *Coxiella burnetii* on production parameters and reproduction in sheep enterprises

The aim of this component of the project was to identify sheep farms where *C. burnetii* is endemic, and then to conduct preliminary investigations to determine whether *C. burnetii* is having an impact on reproductive performance in the sheep residing on those selected farms.

4.5.1 Identification of *Coxiella burnetii*-endemic sheep flocks.

At the project outset, the intention was to identify infected farms via **cross-sectional abattoir sampling** however it was subsequently determined that this strategy was not possible due to privacy concerns from abattoirs.

In response, a new strategy was developed that involved collaboration with Dr Matthew Playford from Dawbuts Animal Health Pty Ltd (https://www.dawbuts.com/) whereby sheep producers who submit **flock faecal samples** to Dawbuts for diagnostic parasitology testing were invited to give their consent for these samples to be screened by *C. burnetii* multiplex PCR. This approach was based on published literature which stated that sheep were more likely to shed *C. burnetii* in faeces than other ruminant species such as goats and cattle (Rodolakis et al 2007). Following this approach, DNA was extracted from pooled faecal samples obtained from five farms and analysed via multiplex PCR against three *C. burnetii* genes as described in Section 3.2. The methodology for extracting DNA from ovine faecal samples was adapted to account for the presence of PCR inhibitors that are known to occur in faeces and based on knowledge gained through previous MLA-funded work on Johnes Disease performed in our laboratory. While we noted minimal inhibition in PCR of faecal samples in the cattle components of this project (See Section 4.1), there was considerable inhibition in ovine faecal samples necessitating the dilution of the DNA extracts (1:10) prior to use in the PCR reactions. No positive samples were identified from any of the farms tested however, it was unclear whether these were true negatives or whether the dilution of the DNA to minimise inhibition decreased the sensitivity of the assay so that it was unable detect low levels of *C. burnetii* shedding.

A third approach was adopted which involved direct recruiting of Dawbuts clientele from farms in regions associated with higher human Q fever notifications to **screen for *C. burnetii* endemicity via serology**. The focus was on farms which anecdotally reported having had staff members diagnosed with Q fever however subsequent sampling of these flocks did not identify serologically positive sheep.

In August 2023, the University of Sydney hosted international visiting scholar, Dr Ben Bauer from Hannover University in Germany. Dr Bauer has extensive experience in *C. burnetii* outbreak investigations in German flocks whereby **dust samples were utilised to screen for *C. burnetii* endemicity** (Bauer *et al,* 2020). It was decided to trial this methodology in this project under Australian conditions. Following, approval of a modification to the ethics protocol (2022/2241), a proof-of-concept and optimisation study was conducted using various swab types (polyester flocked, rayon and cotton swabs kept dry, stored in PBS or in a DNA preservation system at a known *C. burnetii* endemic property (the intensive dairy enterprise sampled for the cattle longitudinal study) as depicted in Figs. 10 A & B below to ensure that the sampling method would detect the presence of *C. burnetii* in dust.

Figure 10. Dust sampling to investigate for the presence of *Coxiella burnetii* on farm. A) Using a swab to collect dust from a ledge in a shearing shed; B) Dust evident on a flocked (polypropylene) swab stored in a conical tube containing PBS following collection.

Strongly positive *C. burnetii* multiplex PCR results were obtained from both flocked (polyester) and rayon swabs stored in conical tubes both as dry swabs and in PBS. The results from the flocked swabs soaked in PBS are presented in Table 14.

Table 14. Results from flocked (polyester) dust swabs soaked in PBS collected in the calving area of a dairy farm analysed with a three gene multiplex *Coxiella burnetii* PCR. Results are presented as cycling thresholds (Cq values).

Location in calving area	*Coxiella burnetii* gene (Cq)		
	IS*1111*	*Com1*	*htpAB*
Wall	18.9	23.8	23.4
Heifer Rail	28.1	31.9	31.6
Cow Rail	26.2	31.1	30.8

The dust sampling methodology was piloted in sheep flocks at the University of Sydney properties (Camden Campus and *Arthursleigh* in the Southern Highlands, NSW) in conjunction with serological testing to confirm previous exposure to *C. burnetii* in these flocks. No positive dust samples were detected on the University of Sydney properties however seropositive sheep were also not identified likely indicating that *C. burnetii* is not currently cycling through these flocks.

While no positive dust samples were identified from the University of Sydney sheep farms, the positive results from the intensive dairy dust sampling demonstrated that the optimised methodology was valid, so testing continued on sheep enterprises external to the University of Sydney. A call out via social media was made through collaborative partner, Sheep Connect NSW (https://www.sheepconnectnsw.com.au/), and via select Dawbuts Pty Ltd clientele to recruit participants to sample dust at strategic locations on their properties. A participant kit was mailed to

respondents containing specific sampling instructions, the swabs and return packaging. Participants were asked to collect dust swabs from three guided locations on their property and then post the swabs back to the University of Sydney via Express Post to be analysed in the laboratory. A total of 19 participants responded to the call out expressing an interest in being involved in the study and these were subsequently sent a sampling kit. Of these, 18 returned three swabs taken on their property from various locations (e.g., shearing sheds, on-farm abattoirs, stables, yard and pen rails, loading chutes and lambing sheds). Twelve properties were in NSW and there were 2 properties each in Queensland, Victoria and South Australia. One responder from Queensland did not return the swabs despite being sent two follow up emails. DNA was extracted from the returned swabs and analysed via *C. burnetii* multiplex PCR. All swabs returned a positive result in the endogenous control PCR indicating amplifiable DNA has been obtained, **however, no swabs returned a positive result on the multiplex *C. burnetii* PCR.**

4.5.2 Discussion of sheep study results of and recommended future studies

Despite using a variety of methods to identify an endemic sheep flock in which to investigate the impact of *C. burnetii* on production parameters and reproduction in Australian sheep flocks, we were unsuccessful in finding an endemic flock in which to conduct an investigation into the impact of *C. burnetii* on sheep reproduction within the time frame of this project. The only previously published study of *C. burnetii* in Australian sheep conducted in recent times (funded by Meat and Livestock Australia; B. AHE.0318), reported very low *C. burnetii* seroprevalences of 0.08% in primiparous ewes and 0.36% in mature ewes and *C. burnetii* was not detected in aborted or stillborn lambs by quantitative PCR (Clune et al, 2022). However, this study was conducted in flocks located in southern Australia (Victoria, South Australia and Western Australia), an area with low Q fever notification rates in humans and therefore also likely lower rates of infection in animals.

To accurately begin to determine the extent to which *C. burnetii* is impacting Australian flocks, it is important that studies are conducted in Queensland and New South Wales which are regions associated with high Q fever notification in humans and the recommended next steps for future research into *C. burnetii* in sheep would be to conduct a prevalence study to determine the extent to which sheep flocks are exposed to the pathogen in these regions. Possible ways in which this could be conducted could involve collaboration with government veterinary services (e.g. Local Lands Services in NSW) or government laboratories via the use of samples (blood or vaginal swabs) submitted for testing for routine diagnostics or other pathogens such as *Campylobacter* species. Alternatively, while we were unable to use abattoir sampling to identify an endemic property to recruit as a participant in this study due to privacy concerns, abattoirs may be more likely to agree to participate in a seroprevalence study via similar methodology to the one conducted in this project in beef cattle (see section 4.4) as stock suppliers could remain anonymous in such a study.

4.6 Isolation and characterisation of cattle *Coxiella burnetii* strains

4.6.1 Isolation of cattle *Coxiella burnetii* strains

Samples identified as strongly positive by *C. burnetii* multiplex PCR and archived in frozen storage were transported on dry ice to the Australian Rickettsial Reference Laboratory (ARRL) in Geelong, Victoria for attempted isolation by culture in Vero cells.

Coxiella burnetii is a fastidious bacterium and growth of *C. burnetii* in Vero cells is a slow process and can take up to 12 weeks before samples can be definitively declared as having 'growth' or 'no growth' via PCR. The ARRL inoculated Vero cell cultures with material from the intensive dairy study (see Section 4.1). Material from 58 placentas collected at the L2 (calving) sampling timepoint and 18 milk samples collected at L5 (190 days in milk) were inoculated onto Vero cell cultures resulting in **growth from nine samples (eight placenta samples and one milk sample)** as detected in PCR of culture supernatants.

Growth of these isolates was then attempted in specialised *C. burnetii* axenic media which has been successful in culture of *C. burnetii* isolates from Europe and USA (Sanchez et al, 2018), however these strains did not grow which is consistent with the outcomes of attempts to grow other strains isolated from Australian human Q fever patients (John Stenos, ARRL pers comm).

The reason for the lack of growth of Australian *C. burnetii* strains in axenic media is unknown but may be because these Australian cattle strains require additional growth factors not available in the current media. Future studies should be directed at identifying what is required for growth of these strains in axenic media as this will likely greatly assist future analysis of these strains in genomic studies.

The nine cattle *C. burnetii* isolates from this study will be kept in storage at -80 °C as part of the ARRL *C. burnetii* isolate archival collection for future studies.

4.6.2 Characterisation of cattle *Coxiella burnetii* strains by multiple locus variable-number tandem repeat analysis (MLVA)

Molecular genotyping is useful to differentiate between bacterial strains, provide insights into evolutionary changes, and correlate with virulence, zoonotic transmission, and clinical outcomes. Many molecular genotyping methods have been applied to *C. burnetii* strains including single nucleotide polymorphisms (SNP), multiple locus variable-number tandem repeat analysis (MLVA) and multi-spacer sequence typing (MST). When used to evaluate Australian isolates, SNP analysis was found to provide only a minimal degree of discrimination (Vincent *et al*., 2016), while MLVA and MST provided the greatest resolution and discrimination between different *C. burnetii* isolates obtained world-wide (Santos et al., 2012). MLVA was demonstrated to have the greatest discriminatory power for Australian isolates obtained from clinical human Q fever patients as published by project collaborators, the ARRL (Vincent *et al*. 2016). A proportion of the isolates identified by Vincent et al 2016 were categorised into two distinct clonal complexes (designated Clonal complex 1 and 2) in which all members of the complex shared identical alleles at 14 out of 15 loci with at least one other member. The remainder were designated singleton complexes which differed at two or more loci from any of the other genotypes and were represented by a single isolate.

As the Vincent *et al.* 2016 study demonstrated that the same degree of discrimination of the Australian isolates was achieved by analysing only three loci: ms24, ms28 and ms33, this

methodology utilising the loci ms24, ms28 and ms33 was employed to determine the genotype of the *C. burnetii* samples positive by PCR in this study.

The MLVA genotyping methodology was validated in our laboratory (see Section 3.2.2) by evaluating an initial subsample of three placenta samples that were strongly positive on *C. burnetii* multiplex PCR. The MLVA analysis was conducted simultaneously at both the University of Sydney laboratory (Camden NSW) and the ARRL in Geelong, Victoria to ensure that identical genotypes were obtained by both laboratories. There was 100% agreement between the two laboratories (Table 15) and two different genotypes were subsequently identified that had also been identified in Australian patients clinically diagnosed with Q fever (Vincent et al., 2016).

All strongly positive samples identified in 4.1.1 from the data-intensive cattle herd were subjected to MLVA genotyping for the three loci outlined above identifying three *C. burnetii* genotypes (CbAU05, CbAU07 and CbAU09) circulating within the herd (Table 16), all of which have been previously identified in Australian patients clinically diagnosed with Q fever (Vincent *et al*., 2016). Genotype CbAU07 falls into clonal complex 1 identified by Vincent *et al.*, whereas CbAU05 falls into clonal complex 2 and CbAU09 was a singleton genotype demonstrating the diversity of strains found in the cattle in this one herd.

In these animals, only one genotype was able to be identified within the PCR positive samples from each animal. The genotype that was detected most within the samples was CbAU07 which was identified in at least one animal across all sample types (placenta, vaginal swab, faeces and milk). One “super shedder” cow (number 11 in Table 16) was identified at sampling timepoint L2 (calving) which was strongly PCR positive on all sample types (placenta, colostrum, faeces and vaginal swab) and this cow was identified to be the only cow with genotype CbAU05. Additionally, only genotype CBAU07 was identified in the milk samples for which the three loci were amplifiable by PCR.

Table 15. Comparison of multilocus variable number of tandem repeats analysis (MLVA) results from two laboratories: University of Sydney, Camden, and Australian Rickettsial Reference Laboratory.

The samples are three placental samples from a single farm that were positive on a multiplex 3-gene *Coxiella burnetii* PCR. Genotypes CbAU05 and CbAU09 have both been previously identified in Australian patients clinically diagnosed with Q fever (Vincent et al., 2016).

Sample	Australian Rickettsial Reference Laboratory		University of Sydney, Camden Laboratory		Identified in Australian clinical Q fever patients?
	ms24, ms28, ms33	Genotype	ms24, ms28, ms33	Genotype	
1	21, 5, 4	CbAU09	21, 5, 4	CbAU09	Yes
2	21, 5, 4	CbAU09	21 ,5, 4	CbAU09	Yes
3	14, 5, 4	CbAU05	14, 5, 4	CbAU05	Yes
Nine Mile Clone 4 (Positive control)	27, 6, 9	Confirmed	27, 6, 9	Confirmed	No

Table 16. Multilocus variable number of tandem repeats analysis (MLVA) results from *Coxiella burnetii* PCR positive samples obtained from animals in a data-intensive cattle herd.

Not all samples were PCR positive from all animals and not all positive samples were able to be amplified in all three loci (ms24, ms28, ms33) examined.

Animal Number	Sample Type						
	Placenta	Vaginal Swab	Faeces 1	Faeces 2	Milk 1	Milk 2	Milk 3
1			CbAU07				
2					CbAU07	CbAU07	CbAU07
3	CbAU09						
4	CbAU07						
5					CbAU07	CbAU07	
6		CbAU07					
7	CbAU09						
8	CbAU07	CbAU07					
9					CbAU07	CbAU07	
10	CbAU09						
11	CbAU05	CbAU05	CbAU05	CbAU05			
12	CbAU07	CbAU07					
13	CbAU07						

4.6.3 Characterisation of *Coxiella burnetii* strains by whole genome sequencing

The focus for this project has been on the characterisation of *C. burnetii* strains via MLVA genotyping as outlined in Section 4.6.2 above however, opportunistically, a collaboration commenced with Dr Daniel Bogema, Dr Cheryl Jenkins and Dr Karren Plain at Elizabeth Macarthur Agricultural Institute (EMAI; Department of Regional NSW) to conduct more extensive characterisation of C. burnetii strains. This involved two different technologies: nanopore (Oxford Nanopore Technologies, UK) and a reversible dye terminator technique (Illumina, California, USA) to sequence tissues plus assembly, *in silico* purification of *C. burnetii* strains, followed by bioinformatic analysis including phylogenomics and comparison with previous whole genome sequencing, analysis for gene presence and/or association. Unfortunately, the *C. burnetii* in the tissue samples was too low and did not provide enough sequence data. Table 17 summarises the amount of sequence data produced for each sample in each run and how much of that aligned to a *C. burnetii* reference genome. It is estimated that approximately 30-40 times the amount of the *C. burnetii* data would be required to generate a quality genome via this method.

Table 17. Production of sequence data by reversible dye terminator technique (Illumina, California USA) and nanopore (Oxford Nanopore Technologies, UK) methodology from cattle placenta samples previously identified as positive by three gene multiplex *Coxiella burnetii* PCR.

Placenta sample identification	Total Illumina reads	Coxiella Illumina reads	Total Nanopore reads	Coxiella Nanopore reads
1	12,917,878	12,248	5,722,435	396
2	10,136,618	6,373	33,990,317	971
3	11,016,882	10,633	n/a	n/a
4	7,716,602	1,067	3,906,062	45
5	11,827,126	7,829	10,741,767	168

5. Conclusion

While the ultimate aim of this project was to investigate the impact of *C. burnetii* on production parameters and reproductive performance in beef production systems, the project investigators identified early in the project development that the logistics of frequent sampling, especially around the time of calving, would be difficult in extensive beef enterprises. Therefore, it was decided to conduct initial studies in a data-intensive dairy herd which would afford greater opportunities for sampling as part of the routine management practices conducted by those enterprises and provide detail on productive and reproductive performance. Subsequently, optimised and streamlined sampling strategies and key learnings could then be transferred to the extensive beef production systems. The findings of the studies in these two systems are detailed below.

Our detailed analysis showed that the consequences of *C. burnetii* exposure may depend on a multitude of factors, potentially including parity, gestation stage (reminiscent of the pathophysiology of BVDV), *C. burnetii* strain, and the host immune response illustrating the complexity of the pathophysiology of *C. burnetii* infection, and which contributes to the challenges of measuring its impact, and reconciling results from different herds, countries and production systems.

5.1 Key findings

Reproductive impact:
In the **data-intensive dairy system**, a multitude of sampling methods and sample types were evaluated, and detailed reproductive data was collected through a longitudinal study and a case-control study. In the longitudinal study, cattle were sampled repeatedly including during pregnancy, at calving and on multiple occasions up to 200 days into lactation. In the case-control study, cows that experienced early foetal loss after a pregnancy diagnosis were compared with control cows (cows that had maintained pregnancy). The sampling, testing and data-intensity of this production system enabled us to consider 6 outcome measures and 23 measures of exposure, including information on pathogen shedding, antibody status, and cell-based immune responses, which would not have been possible in a beef production system.
The longitudinal study showed that cattle that were seropositive at calving were less likely to become pregnant than those that were seronegative at calving. The case-control study showed that, once pregnant, *C. burnetii* exposure was not strongly associated with foetal loss, nor did we see an association between *C. burnetii* and abortion in this endemically infected herd. We did, however, detect an association with calf survival. Calves born from cows with a vaginal swab that tested positive for *C. burnetii* were more likely to die within a few days of birth than calves from cows that tested negative. Given the observed associations between *C. burnetii* exposure and measures of fertility and calf survival in our dataset, we hypothesised that it was plausible that *C. burnetii* exposure could impact beef herds in a similar way.

A total of 270 animals from a single Angus herd were enrolled into **a longitudinal beef cow study** in September 2023 (i.e., pre-breeding). At that time, the seroprevalence was 10%, with variation by mob. Seroprevalence in heifer and 3-year-old mobs were 0% and 3.6%, respectively, which is lower than what was observed in older mobs (ranges 10.3 to 30.7%). Unfortunately, due to unexpected events at the study site, the planned longitudinal study in this beef herd was curtailed and animals were lost to follow up. However, an adjusted analytic approach using the outcome ‘departure from

the herd' determined that animals that were seropositive prior to breeding were 2.2 times more likely to depart the herd during the following 270 days than seronegative animals and we hypothesise that this may be due to worse reproductive performance in the seropositive group. The findings from the beef herd longitudinal study indicate that the association between *C. burnetii* exposure and pregnancy rates observed in the data-intensive dairy herd may also occur in a beef setting. However, further research in a larger number of beef herds is needed to verify this.

Distribution in cattle and sheep:
Infection with *C. burnetii* was common in beef cattle, with evidence of infection in herds from a wide geographic area and very high seroprevalences in some mobs. Seroprevalence was higher in cows of parity 6 or above than in younger cows, steers or heifers. By contrast, we could not identify a *C. burnetii* positive sheep flock despite trialling multiple methodologies across ~ 25 different flocks.

Public health risk:
A final key project outcome was the isolation of *C. burnetii* strains from cattle enrolled in the study that have also been isolated from Australian Q fever patients across multiple clonal complexes, confirming the Q fever public health risk that cattle infected with *C. burnetii* present. Furthermore *C. burnetii* strains identified in cattle are not specific to cattle but rather are also found in other animal species, including wildlife. Within a cattle herd, multiple types of *C. burnetii* can co-circulate.

5.2 Benefits to industry

General:
This discovery research project has advanced the knowledge of the pathogenesis of coxiellosis in Australian cattle herds. Contrary to previous perceptions, the findings of this study suggest that the pathogen is not uncommon in the Australian cattle industry and may be causing important production losses. Consideration of this pathogen as a differential diagnosis for reproductive failure and early calf mortality in the field will aid in understanding how frequently this pathogen is impacting cattle production systems, and the magnitude of these impacts. The project has provided evidence-based recommendations for areas where future research should be directed to understand the true impact of this pathogen on the Australian livestock industry.

Diagnostic methods:
The research team has gained considerable knowledge around best practice for sampling and diagnosis of this pathogen both in the animal and in farm environments using a variety of sample types (faeces, blood, vaginal swabs, dust) and testing methods (detection of *C. burnetii* or the host immune response). While PCR of placental tissue was very sensitive at detecting *C. burnetii* infection, DNA was rarely detected by PCR in vaginal swabs and faeces in pregnant and non-pregnant cows. For on-farm studies in beef herds, serology remained the most logistically useful diagnostic tool to be used in studies in beef herds. For environmental sampling, dust was found to be very effective as a non-invasive method of detecting *C. burnetii* endemicity in the intensive dairy herd. In addition, sampling and shipping of dust from farms, yards or shearing sheds to a diagnostic laboratory is simple and convenient.

Economic tool:
A beef herd simulation model was developed to provide robust estimates of the effects of low reproductive performance, increased calf losses and/or low calf growth rates in temperate zone

seasonal calving cow and calf beef herds. This model will be a valuable ongoing tool for assessing effects of both infectious and non-infectious causes as robust effect estimates become available.

***Coxiella burnetii* strains:**

Nine *C. burnetii* isolates were grown in Vero cells and these are currently in archival storage in the ARRL *C. burnetii* collection. This is a unique resource for future projects or vaccine development.

6. Future research and recommendations

Project insights:

Prior to this study, *C. burnetii* was previously well accepted as a cause for serious public health concern (Q fever) but thought by many to have no impact on reproductive performance in Australian cattle. The findings of this study suggest that the pathogen is not uncommon in the Australian beef industry and may be causing important production losses. At the individual animal level, impacts can be diverse, making them difficult to measure but the cumulative impact on the industry could be significant. Consideration of this pathogen as a differential diagnosis for reproductive failure and early calf mortality in the field will aid in understanding how frequently this pathogen is impacting cattle production systems, and the magnitude of these impacts.

Future research:

Future research in beef cattle and herds should be directed towards:

- further understanding the pathogenesis of infection with *C. burnetii* and the role the immune response plays in varying reproductive outcomes following infection,
- investigating the role *C. burnetii* plays in reproductive failure and calf mortality across a larger number of Australian beef herds and different cattle production systems,
- understanding how the high seroprevalence at slaughter translates into risk of exposure of people in abattoirs to the pathogen along the slaughter line,
- a mechanistic understanding of the environmental risk factors for *C. burnetii*, e.g., vegetation, moisture, and wildlife exposure, so that causal factors can be identified and managed,
- further assessment of the geographic distribution and prevalence of *C. burnetii* in Australian cattle herds and potentially in sheep flocks, and
- a detailed review of the economic benefits and costs, feasibility and risks of a program to develop an Australian vaccine for animals against *C. burnetii* as a means of inducing optimal immune responses following challenge with the pathogen and reducing shedding and subsequent infection in animals and humans.

Development and adoption: Considering the widespread occurrence of *C. burnetii* across stock classes, management systems and rainfall zones, ongoing effort to raise or maintain awareness among famers and veterinarians is needed. This could include developing and establishing provision of best practice diagnostic tools in government and commercial veterinary laboratories for testing of samples of animal and environmental (dust) origin, and communication of recommended investigatory procedures for field advisers.

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