# Investigating causes of mortality in live export cattle

This thesis is presented for the degree of

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By

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# Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

.....
(Sarah Jo Moore)

“Sure, the big events attract a lot of attention,

but what really matters is what goes on every single day.”

Michael Tilson Thomas

# Abstract

This research project was initiated to provide industry with current, credible, scientific data on causes of death and risk factors for mortality in Australian live export cattle on long-haul voyages.

Animal data and necropsy samples were collected from animals that died on 20 research voyages during the study period March 2010 to September 2012. The average voyage mortality percentage was 0.37%. Respiratory disease was the most commonly diagnosed cause of death, accounting for 107/215 (49.8%) of deaths overall, and 107/181 (59.1%) of deaths for which a diagnosis could be made. In addition, pneumonia was identified in 33% of animals for which respiratory disease was not considered the primary cause of death. Other common causes of death included lameness (*n* = 22/181, 12.2%), ketosis (*n* = 12, 6.6%), septicemia (*n* = 11, 6.1%), and enteric disease (*n* = 10, 5.5%).

Quantitative polymerase chain reaction (qPCR) assays were developed to detect viruses and bacteria known to be associated with bovine respiratory disease (BRD) in necropsy and nasal swab samples: Bovine coronavirus (BCoV, *Betacoronavirus 1*), Bovine herpesvirus 1 (BoHV-1), Bovine viral diarrhoea virus (BVDV), Bovine respiratory syncytial virus (BRSV), Bovine parainfluenza virus 3 (BPIV-3), *Histophilus somni*, *Mycoplasma bovis*, *Mannheimia haemolytica* and *Pasteurella multocida*

Two-thirds (130/195) of animals from which lung samples were collected had histological changes and/or positive qPCR results suggestive of infectious lung disease: 93/130 (72%) had evidence of primary bacterial infection, 4 (3%) with primary viral infection, 29 (22%) with concurrent bacterial and viral infections, and for 4 (3%) the causative organism could not be indentified. *M. bovis*, *H. somni*, *P. multocida*, *M. haemolytica* and BCoV were significantly associated with respiratory disease during voyages.

Results from nasal swab and serological samples collected at entry to the pre-export assembly depot indicated that there were significant differences in nasal and seroprevalence between animals sourced from different properties. Combined nasal swab and serum results suggest that BCoV and BVDV are likely to be important infectious agents in the development of BRD in live export cattle while BPIV-3 is unlikely to play a major role. The contribution of BoHV-1, BRSV and bacteria of interest is difficult to determine.

Analysis of animal and voyage data collected by industry between January 1995 and December 2012 revealed that while there has been an overall reduction in voyage mortality rates since 2000, there remain significant differences in mortality rate between load and discharge regions. Examination of daily mortality data available for research voyages revealed that peak daily mortality risk occurs at 3-4 weeks post-departure. The development of methods for spatial analyses coupled with data available in the National Livestock Identification System database allowed the description of patterns of animal movement prior to export.

This study has improved our understanding of causes of death and risk factors for mortality in Australian live export cattle. We now have baseline data on the prevalence of BRD organisms in live export cattle that could be used to develop strategies for BRD prevention and control prior to loading and during voyages.

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# Communications arising from this thesis

## Scientific papers

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Mortality of live export cattle on long-haul voyages: pathological changes and pathogens. Journal of Veterinary Diagnostic Investigation, 2014 26(2):252-65 (Chapter 4).

**Moore, S.J.**, O'Dea, M.A., Perkins, N., O'Hara, A.J.
Estimation of the nasal and seroprevalence of organisms known to be associated with bovine respiratory disease in Australian live export cattle
Journal of Veterinary Diagnostic Investigation, *in press* (submitted 17th April 2014) (Chapter 5).

**Moore, S.J.**, Madin, B., Norman, G., Perkins, N.
Risk factors for voyage mortality in cattle exported live from Australia by sea
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**Moore, S.J.** Identifying causes of morbidity and mortality in cattle exported live to the Middle East. *Australian and New Zealand College of Veterinary Scientists, Science Week*, June 28th – 30th 2012, Surfers Paradise, Australia. Oral presentation.

**Moore, S.J.**, O'Dea, M.A., Creeper, J., Perkins, N., Barnes, A., O'Hara, A.J. Causes of mortality in live export cattle on long haul voyages. *Murdoch Post-Graduate Student Association Annual Conference*, October 3rd, 2013, Perth, Australia. Oral and poster presentation.

**Moore, S.J.**, O'Dea, M.A., Creeper, J., Perkins, N., Barnes, A., O'Hara, A.J. Causes of mortality in live export cattle on long haul voyages. *American College of Veterinary Pathologists Annual Meeting*, November 16th – 20th 2013, Montreal, Canada. Poster presentation.

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## Public media

Veterinary research highlights refocus for cattle export. Article by Geoff Vivian for Science Network Western Australia, 6th March 2014. Available at: http://sciencewa.net.au/topics/agriculture/item/2707-veterinary-research-highlights-refocus-for-cattle-export.

Respiratory disease causing export cattle deaths. Interview and article by Carmen Brown for ABC Radio, 11th March 2014. Available at: http://www.abc.net.au/news/2014-03-11/export-cattle-deaths-linked-to-respiratory-disease/5310412.

# Abbreviations

| | |
|---|---|
| ALES | Australian Live Export Standards (released 1997) |
| AMSA | Australian Maritime Safety Authority |
| AQIS | Australian Quarantine and Inspection Service (renamed DAFF Biosecurity in 2013) |
| ASEL | Australian Standards for the Export of Livestock (released 2004) |
| BCoV | Bovine coronavirus |
| BoHV-1 | Bovine herpesvirus 1 |
| BPIV-3 | Bovine parainfluenza virus 3 |
| BRSV | Bovine respiratory syncytial virus |
| BVDV | Bovine viral diarrhoea virus |
| DAFF | (Australian Government) Department of Agriculture, Fisheries and Forestry |
| DAFWA | Department of Agriculture and Food, Western Australia |
| ELISA | Enzyme-linked immunosorbent assay |
| ESCAS | Export Supply Chain Assurance Program |
| LEAP | Livestock Export Accreditation Program |
| LiveCorp | Livestock Export Corporation. The industry body that represents Australian livestock exporters. |
| MENA | Middle East and North Africa |
| MLA | Meat & Livestock Australia. The industry body that represents Australian livestock producers. |
| NE Asia | North east Asia |
| NLIS | National Livestock Identification System |
| qPCR | Quantitative (= real-time) polymerase chain reaction |
| RFID | Radio frequency identification device |
| SA | South Australia |
| SE Asia | South east Asia |
| TEM | Thromboembolic meningitis (associated with *Histophilus somni* infection) |

| | |
|---|---|
| VIC | Victoria |
| WA | Western Australia |

# Chapter 1: General Introduction

Large numbers of cattle are exported each year (LiveCorp, 2012) and whilst average mortality rates are generally low (DAFF, 2014), even low mortality rates result in large numbers of cattle dying, which is of concern from an animal welfare perspective. The high value of each animal means that on-board losses also have a significant economic impact. The periodic occurrence of high mortality events generates ongoing public pressure on the live export industry to demonstrate that it is actively committed to minimizing mortality rates and improving animal welfare.

However, there are very few rigorous, credible, scientific studies describing the major causes of mortality in live export cattle. Two previous studies, 1 conducted between 1985 and 1987 (Hedlefs, 1988) and a second between 1998 and 2001 (Norris et al., 2003), identified heat stroke, trauma and/or lameness, and respiratory disease as the most common causes of death. Organisms that have been shown to be important in the development of bovine respiratory disease include the viruses Bovine coronavirus (*Betacoronavirus 1*) (BCoV), Bovine herpesvirus 1 (BoHV-1), Bovine viral diarrhea virus (BVDV), Bovine respiratory syncytial virus (BRSV), Bovine parainfluenza virus 3 (BPIV-3); and bacteria *Mannheimia haemolytica*, *Pasteurella multocida*, *Mycoplasma bovis*, and *Histophilus somni* (Fulton, 2009; Panciera and Confer, 2010; Saif, 2010). In an effort to reduce the risk of cattle developing respiratory disease during a voyage, cattle are often vaccinated against specific respiratory disease pathogens and/or treated with broad spectrum antibiotics prior to being loaded onto the vessel, and may be treated again before they reach their destination. However, there is little data to document unequivocally whether these practices are effective at reducing morbidity or mortality.

In 2006, several voyages to Israel experienced on-board mortality events that exceeded the reportable mortality level of 1% prescribed by the Australian Standards for the Export of Livestock (ASEL) (DAFF, 2011). The high mortalities (1.2-3.2%) experienced during these voyages were attributed to bovine respiratory disease (DAFF, 2014) and became the impetus for this study to further investigate on-board mortality events.

As part of the investigation into the causes of on-board mortalities the literature detailing the Australian live cattle export industry, management of cattle in the pre-export assembly period, risk factors for mortality during voyages, and common causes of death during voyages was reviewed and is summarised in Chapter 2.

Chapter 3 describes the development and validation of quantitative polymerase chain reaction (qPCR) assays for the detection of nucleic acids from 5 viruses (BCoV, BoHV-1, BRSV, BVDV, BPIV-3) and 4 bacteria (*M. haemolytica*, *P. multocida*, *M. bovis*, *H. somni* ) known to be important in the pathogenesis of bovine respiratory disease. Four duplex and 1 singleplex qPCR assays were developed; primer and probe sequences were based on a combination of published sequences and sequences developed during the study.

Chapter 4 describes the major causes of mortality in live export cattle on long-haul voyages, based on data and samples collected from animals that died during 20 research voyages. Samples included fixed tissue for histological examination and nasal swab and lung samples that were tested using the qPCR assays described in Chapter 3.

Chapter 5 provides a report on the nasal and seroprevalence of viruses and bacteria of interest in cattle in pre-export assembly feedlots. Nasal swab samples were collected from selected animals as they entered the assembly feedlot and tested using the qPCR assays described in Chapter 3. Results from qPCR testing and available epidemiological data were analysed to determine risk factors for pathogen prevalence prior to export which could

influence the development of disease during voyages. Risk factors for pathogen prevalence include the immune status of the animals in question, for example, whether or not they have circulating antibodies to relevant viral pathogens. Chapter 5 also describes serological testing of animals entering the assembly feedlots to determine their immune status. Serology for antibodies to BoHV-1, BRSV, BVDV, BPIV-3 was performed by ELISA testing.

Chapter 6 contains the results of analysis of voyage data collected by industry, daily mortality data collected from study voyages, and animal movement data available through the National Livestock Identification System database. Mortality trends and risk factors revealed by these analyses are discussed with reference to results described in Chapters 4 and 5 and previously published data.

A general discussion of the results and conclusions is provided in Chapter 7, with a focus on the implications of the findings from this study on the management of cattle prior to and during voyages to reduce the risk of mortality due to respiratory disease and other causes.

# Chapter 2: Literature review

## 2.1 Transport of live animals by sea

Between 2010 and 2012 Australia exported approximately 3.5 million live animals per year including 3000 cats, 7500 dogs, 64,000 goats, 740,000 cattle and 2.6 million sheep (DAFF, 2013a). Cats, dogs and goats generally travel by air while cattle and sheep are transported by sea.

Australia's international live cattle export trade began in the 1800s. In the 1840s shipments of 30-45 breeding cattle and bullocks were exported from New South Wales to New Zealand (Anonymous, 1840) and further exports in the 1860s were to supply meat for the military (Anonymous, 1887). In the 1870s cattle were exported to New Caledonia to satisfy local demand and for the production of preserved meat, which was shipped to the French military fighting in Europe (Anonymous, 1892). The first shipment of cattle to South East Asia (Hong Kong) occurred in 1885 (Anonymous, 1885). However, it was not until the 1970s and early 1980s, when economic development in some South East Asian (SE Asian) countries led to an increased demand for red meat, that there was significant growth in live cattle exports to this region (Farmer, 2011).

Since the late 1990s Australia has exported 600,000-800,000 live cattle each year to countries around the globe (LiveCorp, 2012). In recent years approximately 65% of cattle have been exported to countries in SE Asia and the Pacific with approximately 90% of these destined for Indonesia. Approximately 15% of cattle are exported to countries in the Middle East and North Africa (MENA), 10% to North East Asia (NE Asia; including China, Pakistan, Sri Lanka and Japan), and 10% to Turkey, the Russian Federation and Kazakhstan (LiveCorp, 2012).

Sea transport of live animals for slaughter is cheaper and easier than refrigerated transport of packaged meat (Phillips, 2008). In addition, live animals are preferred by SE Asian and

MENA markets for a variety of reasons including a preference for Halal slaughter to be performed by their own nationals rather than certified slaughtermen in Australia, a lack of infrastructure to transport and store refrigerated meat particularly for poorer consumers, and the potential for value-adding through fattening, slaughtering and processing in the destination country (Agra CEAS Consulting, 2008; Farmer, 2011).

Cattle are also exported live for use as breeding stock. Export of breeder animals accounts for around 25% of Australia's live export cattle (Agra CEAS Consulting, 2008) and between 2010 and 2012 approximately 40% of these were dairy cattle (LiveCorp, 2012). Over the last 10 years there has been an increasing demand from Asian countries, for example China, for in-calf dairy cattle to boost the national herd as these countries move toward a more Westernised diet. Beef and dairy breeding stock are also in demand in Turkey and the western Russian Federation.

The welfare of live export animals has been the focus of much media attention in recent years. In 2002, 614/1995 (31%) cattle died on the *MV Becrux* in the Persian Gulf with the majority of deaths attributed to heat stroke (Stinson, 2008). In 2003 the *MV Cormo Express* was prohibited from discharging its cargo of 57,973 sheep at Jeddah; at this point in the voyage the mortality rate was 0.9% (Stinson, 2008). These sheep were eventually discharged in Eritrea 80 days later (Stinson, 2008) with a final mortality rate of 9.83% (Greg Norman, *personal communication*).

More recently attention has shifted to the handling and slaughter of animals after they arrive at their destination. Investigations by the animal activist organisation Animals Australia have documented treatment of sheep in the Middle East and Pakistan, and cattle in Indonesia and the Middle East, in contravention of the Australian Model Code of Practice for the Welfare of Animals (Animals Australia, 2013). On the 30$^{th}$ May 2011 graphic footage, collected by Animals Australia, of the slaughter of cattle in Indonesia was shown

on the Australian Broadcasting Corporation's investigative journalism program Four Corners in an episode entitled 'A Bloody Business' (Australian Broadcasting Corporation, 2011). Public pressure generated by this program resulted in a temporary suspension of live cattle exports to Indonesia between the 8th of June and 6th of July 2011 and the commissioning of an Independent Review into Australia's Livestock Export Trade (Farmer Review) (Farmer, 2011).

Recommendations made in the Farmer Review led to the development and implementation of the Exporter Supply Chain Assurance System (ESCAS). The ESCAS allows all livestock, including feeder and slaughter cattle, to be traced through the supply chain from their home farm in Australia to slaughter in the importing country (DAFF, 2013b). Exporters must be able to demonstrate that handling of cattle in the supply chain will conform to the World Organisation for Animal Health (OIE) recommendations for animal welfare up to and including the point of slaughter (DAFF, 2013b). Although the welfare of live export animals is an important issue it is beyond the scope of this report so will not be discussed further.

## 2.2 Management of cattle prior to being loaded onto the vessel

The voyage itself is not the beginning (or the end) of the live export process. Prior to being loaded onto the vessel, animals are purchased from their property of origin or a saleyard, transported by road to quarantine facilities where they spend 24 hours to 30 days, and transported by road from the quarantine facilities to the port of loading.

Most cattle are bought directly off farm to comply with the property of origin residency requirements stipulated in the importing country's import protocol. Shipments comprise multiple properties with each property supplying up to 5% of the animals on the shipment.

Cattle may be purchased through saleyards but only if this does not contravene property of origin residency requirements. Sourcing of cattle may begin 1 week to months before the vessel sails, depending on the class of cattle being exported and the importing country's requirements.

Cattle must be assembled in an Australian Quarantine and Inspection Service (AQIS) registered premise (also known as an assembly depot) for a defined period of time to allow the animals to recover from the stress of transport and become accustomed to shipboard rations. Due to the variability and seasonality of the live export trade many registered premises are dual purpose; they operate as pre-export assembly depots when required and fattening feedlots for non-export animals at other times. All livestock entering a registered premises may only leave that facility for export or domestic slaughter.

Animals sourced for export must meet with various requirements as stipulated in the Australian Standards for the Export of Livestock (ASEL) (DAFF, 2011) and the importing country's protocol. "Protocolling" refers to the various checks and testing procedures that may be undertaken to ensure that animals comply with these requirements. Usually, protocolling is done while animals are being held in the assembly depot, although some procedures, e.g. pregnancy testing, may be done at the property of origin.

During protocolling animals are examined individually. Checks and procedures may include: scanning of individual electronic identification tags, application of visual eartags, collecting a blood sample for serological testing, recording the animals' liveweight, vaccination, administration of prophylactic antibiotics, treatment of minor ophthalmic conditions.

After protocolling animals are drafted into groups ("lines") according to one or more of the following: type (*Bos taurus* vs *Bos indicus*), sex, class (feeder, slaughter, breeder), weight, breed. Some lines of animals will receive additional treatments (e.g. prophylactic

antibiotics) in the lead up to loading, either as part of the protocol or at the exporter's discretion. These are usually animals that are considered to be at a higher risk of developing disease during the voyage, particularly respiratory disease.

During the assembly period cattle are kept in large pens typical of a fattening feedlot. Stocking densities must meet minimum ASEL requirements for square-metres per head: for a 500kg animal this is 4-9$m^2$ (± 0.04-0.09$m^2$/kg liveweight) depending on the number of days the animal is held in the registered premises (ASEL Standard 3.11) (DAFF, 2011). Pens have concrete or compacted dirt floors, water troughs and feed bunkers, and may be covered or uncovered. Cattle must be fed a minimum of 2.5% bodyweight per day (ASEL Standard 3.7e) (DAFF, 2011). The type of ration is not specified in ASEL and, although it may be similar to that fed during the voyage, it is rarely the same. Hay may be mixed in with the ration to stimulate gut activity and encourage shy feeders to eat. Animals are usually fed twice a day.

Cattle are usually checked 3 times a day by the depot stockpersons. Sick animals may be removed to a hospital pen or treated in the pen. Dead livestock must be collected and disposed of on a daily basis (ASEL Standard 3.16c) (DAFF, 2011). Dead animals are rarely necropsied to determine the cause of death.

Cattle may be held in the registered premises for 24 hours to 30 days depending on ASEL requirements (ASEL Standard 3.8A) (DAFF, 2011) and pre-export quarantine requirements stipulated in the importing country's protocol. Quarantine periods are generally shortest for slaughter cattle, longer for feeder cattle, and longest for breeder cattle.

Animals which do not meet protocol requirements may be rejected or carried-over. Reject stock are those that do not meet requirements for the current shipment and are unlikely to (or will not) meet requirements for future consignments. Rejects are isolated from other

consignment livestock, treated or euthanased if necessary, and removed from the depot, either by carcass disposal or domestic slaughter, in a timely manner. Carry-over stock are those that do not meet requirements for the current consignment but may do so for a future consignment. These animals are held separate to the consignment livestock and, if treatment is successful, may be considered for inclusion in a future consignment.

Cattle may be held off water for up to 48 hours prior to load-out to trucks (Australian Animal Welfare Standards and Guidelines for Land transport of Livestock, 2012; Standard B4.1 (DAFF, 2012)), although 12 hours is more common. Usually cattle are held off food for 6-8 hours before load-out.

During load-out cattle are loaded onto trucks by line and according to their final location on the ship. The road journey from the registered premises to port of loading must be less than 8 hours (ASEL Standard 3.0) (DAFF, 2011). In Western Australia, journey times from the registered premises to Fremantle port vary from 1.5-5 hours.

Animals are only permitted to leave the registered premises when both the animals to be exported and export vessel have passed the necessary AQIS and Australian Maritime Safety Authority inspections. If in an extraordinary circumstance loading cannot take place after cattle have already commenced their journey to the vessel, they can either return to the same facility or be taken to another registered premises en route if it is closer and/or offers a better welfare outcome.

Thus it can be seen that the pre-export period exposes animals to a number of risk factors for morbidity and mortality including long distance road transport, co-mingling of animals from different properties in the assembly depot, social stress associated with meeting new animals, change in environment, change in feed, transport to the port of loading, loading, and further disruption of social hierarchies as animals are drafted into pens of 6-30 animals

for the voyage. Added to this is the potential exposure of large number of animals of unknown health status to viruses and bacteria that, acting as primary and/or secondary infectious agents, are known to cause respiratory and other diseases.

## 2.3 Risk factors affecting mortality during voyages

### 2.3.1 Voyage length

Live export voyages are classified as long- or short-haul based on the number of days that the animals are at sea before they reach their destination: short-haul voyages are less than 10 days, long-haul voyages are greater than or equal to 10 days (ASEL Standard 5.5) (DAFF, 2011). Voyages to SE Asia and the Pacific are short-haul and those to all other destinations are long-haul. Long-haul voyages may be further sub-divided into 'short' long-haul voyages, for example to China, Japan and India with a voyage length of 10 days to 2 weeks, and 'long' long-haul voyages, for example to the MENA and Russian Federation where animals are on the vessel for an average of 3-4 weeks.

Mortality rates for short-haul voyages are generally lower than those for long-haul voyages (Norris et al., 2003). Overall, the vast majority of cattle on short-haul voyages are either *Bos indicus* feeder and slaughter cattle, or *Bos taurus* dairy breeder cattle. As discussed below, the low mortality rates seen on most of these voyages are likely to be due to a combination of the relatively short voyage length, special care given to valuable breeding animals, and the superior ability of *Bos indicus* cattle to cope with hot humid conditions.

### 2.3.2 Class of cattle (feeder, slaughter, breeder)

'Feeder' cattle are exported at a relatively light liveweight (250-350 kg) and then grown on in a feedlot in the importing country before slaughter. 'Slaughter' cattle are heavier cattle (350+ kg) that are slaughtered soon after they reach their destination. Exact liveweight cut-offs for feeder and slaughter cattle differ between importing countries. 'Breeder' cattle are animals that will be used for breeding in the importing country. Female breeder cattle may be non-pregnant or in the early stages of pregnancy; no more than 190 days pregnant, or approximately two-thirds the way through the gestation period, at the scheduled date of departure (ASEL Standard 1.10c) (DAFF, 2011).

Mortality rates for breeder cattle tend to be lower than for feeder and slaughter cattle. In 2012 the average value of dairy breeder cattle was $2294.96 per head, while that of all other cattle combined was $793.70 per head (LiveCorp, 2012). The higher value of breeder cattle means that they are often stocked at a density lower than that stipulated in ASEL (Standard 4.4) (DAFF, 2011), housed in more comfortable areas of the vessel, and provided with deeper bedding. In addition, breeder cattle tend to undertake shorter voyages than slaughter and feeder cattle. For example, in 2012 80% of breeder cattle were transported to NE Asia, SE Asia and New Zealand, 6.9% to the MENA, and 12.1% to the Russian Federation (LiveCorp, 2012).

### 2.3.3 Type of cattle (Bos taurus, Bos indicus)

On four study voyages Norris et al. (2003) reported lower death rates for *Bos indicus* compared to *Bos taurus* cattle and observed that *Bos indicus* cattle coped better with the hot, humid conditions on-board ship than *Bos taurus* cattle. Lower mortality rates in *Bos indicus* cattle was also noted in the investigation into the *MV Becrux* mortality incident in 2002 (Stinson, 2008).

*Bos taurus* and *Bos indicus* refer to subspecies of the Genus Bos in the Family Bovidae (Orrell, 2013). *Bos taurus* cattle are generally found in temperate climates and *Bos indicus* cattle in tropical and subtropical zones. *Bos indicus* cattle have a number of physiological adaptations that allow them to thrive in hot and humid conditions including a lower metabolic rate and increased ability to dissipate heat. In addition, cattle that have evolved in hot climates have acquired genes that protect cells from the deleterious effects of prolonged high temperatures (reviewed in (Hansen, 2004)).

The metabolic rate of *Bos indicus* cattle is approximately 80-85% that of *Bos taurus* cattle (Vercoe, 1970; Worstell and Brody, 1953). Therefore, the metabolic heat load, which accounts for approximately one-third of the heat load in a hot radiant environment (Finch, 1986), is lower in *Bos indicus* cattle. This is thought to be due to the relatively smaller size of the digestive tract of *Bos indicus* cattle (Swett et al., 1961).

*Bos indicus* cattle have a superior ability to transfer heat from the body core to the skin surface (Finch, 1986) and to dissipate heat through evaporative cooling (Blackshaw and Blackshaw, 1994). The short, smooth hair coat of *Bos indicus* cattle allows greater heat loss than the long, thick, woolly hair coat of *Bos taurus* cattle, particularly in humid conditions when water vapour may become trapped in the *Bos taurus* hair coat (Allen et al., 1970; Finch, 1985). *Bos indicus* cattle have a higher sweat gland density (Allen, 1962; Ferguson and Dowling, 1955; Nay and Heyman, 1956), larger sweat gland size (Nay and Heyman, 1956; Pan, 1963), and more cutaneous arteriovenous anastomoses (Findlay and Yang, 1948, 1950), all of which facilitate heat loss through evaporative cooling.

Due to the prevailing climatic conditions in Australia, cattle from southern ports tend to be primarily of the *Bos taurus* subspecies while those from the northern ports are primarily *Bos indicus* (DAFWA, 2009). Therefore mortality rates tend to be higher on voyages that load out of southern ports (Norris et al., 2003). Commonly exported *Bos taurus* breeds

include Angus, Herefords, Murray Greys and their crosses; *Bos indicus* breeds include Brahmans, heat-adapted Shorthorns and their crosses.

### 2.3.4 Importing country

Most countries in SE Asia, the Pacific and the MENA prefer *Bos indicus* feeder cattle because they perform better in local conditions. Although *Bos indicus* animals have inferior meat quality compared to *Bos taurus* cattle (Elzo et al., 2012; Johnson et al., 1990; Pringle et al., 1997; Shackelford et al., 1991; Wheeler et al., 2010; Wythes et al., 1989) these less desirable characteristic are also shared by endemic breeds and the method of butchering and food preparation in the importing countries offsets any quality advantage that *Bos taurus* cattle may have.

Premiums are paid for *Bos taurus* slaughter cattle in some MENA countries and *Bos taurus* feeder cattle are more suited to environmental conditions in Turkey and the Russian Federation. Therefore, voyages with the highest mortality rates tend to be those with a high *Bos taurus* content and with destinations in the MENA and the Russian Federation.

### 2.3.5 Season

The time of year that animals are exported can also influence mortality rates. Increased mortality rates on long-haul shipments loaded out of southern ports during the Australian winter and arriving in the Middle Eastern summer have been reported previously for both cattle (Norris et al., 2003) and sheep (Higgs et al., 1991; Richards et al., 1991). Richards et al. (1991) postulated that winter grazing on abundant pasture resulted in a metabolic state where sheep were adjusted to laying down body fat and so were less able to utilise adipose reserves for energy when required, for example during periods of inappetence prior to and during export, compared to animals grazing poor pasture at other times of year. Climatic factors are also likely to play a role; cattle on long-haul voyages in summer and winter pass from one climatic extreme to another, e.g. winter cold in southern Australia to summer

heat in the Middle East. Environmental conditions on the boat are relatively warm due to the heat load of the animals being carried, so cattle do not acclimatise to the changing weather conditions during the voyage.

Therefore it can be seen that mortality rates during voyages may be influenced by a number of factors including the type and class of cattle, port of loading, length of voyage, port of discharge, and the time of year that the voyage takes place.

## 2.4 Causes of mortality in live export cattle

There have been two previous studies investigating the causes of mortality in cattle during sea transport from Australia. Hedlefs (1988) collected samples from 10 voyages carrying beef cattle from Queensland (Brisbane) to Japan between August 1985 and June 1987. Heat stroke was found to be the most common cause of death in this study, accounting for 50/92 (54%) deaths, followed by distal limb abrasions acquired by slipping on abrasive pen floor surfaces (21/92 animals, 23%). The causes of mortality for the remaining 21 deaths were not described.

Approximately 10 years later Norris et al. (2003) investigated causes of death in 4 voyages from ports in Victoria (Portland), South Australia (Adelaide), and Western Australia (Fremantle, Port Hedland, Broome) to the Middle East (Libya, Jordan, Egypt) between December 1998 and April 2001. Of the 199 deaths that occurred during the study voyages the cause of mortality could be determined for 180 animals. Heat stroke and trauma/lameness were the two most common causes of death, accounting for 58/180 (32%) and 49/180 (27%) deaths for which a diagnosis could be made. Thirty-four (19%) animals were found to have died from respiratory disease, 22 (12%) from enteric diseases, 9 from septicaemia, and 8 from miscellaneous conditions. Detailed descriptions of the

three most common causes of death – heat stroke, trauma/lameness, respiratory disease – are given below.

### 2.4.1 Heat stroke

Heat stress occurs when a combination of environmental and animal factors lead to an increase in body heat content which is beyond the animals' normal physiological range ((Young, 1993) as cited by (Beatty et al., 2006)). When ambient temperature is less than skin temperature heat dissipation occurs primarily through non-evaporative cooling (passive heat loss) via radiation and convection (Cabanac, 1975). As heat stress progresses the rate of non-evaporative cooling decreases and is replaced by evaporative cooling from the outer body surface (e.g., by sweating), and evaporative cooling from the respiratory tract (e.g., by panting) (Mortola and Frappell, 2000).

Sources of environmental heat on-board vessels may include other animals, radiated heat from walls or ceilings, or heat added to ventilation airstreams by fans and motors (MAMIC, 2001). Animal factors include metabolic heat generated by rumination, heat generated by muscular movement, for example to maintain a standing posture and during panting, and inhibition of heat dissipation in fat cattle and those with long hair coats (Radostits et al., 2000; Yousef and Johnson, 1985).

The physiological responses of cattle to short periods of heat stress include an increased respiratory rate, decreased feed intake, increased water intake, and imbalances in blood gasses and plasma electrolytes (Blackshaw and Blackshaw, 1994; Gaughan et al., 1999; Sanchez et al., 1994). In a study of *Bos taurus* and *Bos indicus* cattle exposed to controlled environmental conditions over a period of 21 days designed to mimic conditions during a typical voyage to the Middle East, Beatty et al. (2006) noted an increased core body temperature, increased respiratory rate, decreased feed intake, and increased water intake in response to sustained high temperature and humidity. Clinical signs of heat stress

include open mouthed panting, inappetence, drooling, reluctance or inability to rise, reduced rumen motility, increased licking of coat, and general dullness including neurological signs with staring and glazed eyes (Beatty, 2005).

During heat stress an increased metabolic rate and decreased feed intake lead to a loss of body weight and muscle strength. A drop in central venous pressure occurs secondary to a combination of peripheral vasodilation to facilitate heat dissipation, fluid loss through evaporative cooling, and redirection of blood to the respiratory organs. This drop in central venous pressure combined with an increase in blood temperature results in an increased heart rate and increased blood coagulability which may be severe enough to cause myocardial weakness, physical collapse, cerebral hypoxia and depression of nervous system activity including the respiratory centre, disseminated intravascular coagulation and, ultimately, death ((Hales, 1988) as cited by (Hedlefs, 1988), (Radostits et al., 2000)).

Cattle dying from heat stroke may be noted to be panting heavily immediately prior to death or found dead with no previous clinical signs. Necropsy findings consistent with a diagnosis of heat stroke include a core body temperature ≥43°C (measured using a deep temperature probe), sunken eyes, muscles glowing pink and dry rather than red-brown and moist, heart tightly contracted with epicardial ecchymoses, and severe acute diffuse pulmonary congestion and oedema causing the lungs and mucosae of the trachea and bronchi to be dark red (Norris et al., 2003). There are no constant or specific histopathological changes associated with heat stroke (Radostits et al., 2000).

### 2.4.2 Lameness

Most of the deaths due to lameness are the result of the animal being euthanased because its injuries are severely compromising its welfare or because it would be rejected on arrival (Hedlefs, 1988; Norris et al., 2003). Lame and injured animals may also be more likely to die from heat stroke (Norris et al., 2003). Heavy animals and animals with ‘soft feet’, for

example dairy breeds and animals from southern areas of Australia, are more prone to becoming lame than lighter animals and pastoral cattle from the northern areas of Australia (Banney et al., 2009; Norris et al., 2003).

Lameness may begin prior to loading or during the voyage. During the assembly period poorly designed or maintained yards or trucks combined with rough handling can lead to foot and leg injuries that do not heal once the animal is on the vessel. For example, pre-loading processing and treatments may lead to toe wear and trauma if carried out on concrete yards (Banney et al., 2009). Animals with deformed hooves may have an increased risk of developing lameness (Norris et al., 2003).

During the voyage lameness develops secondary to injuries and abrasive floor surfaces. Injuries are more common during rough weather, with reduced stocking densities, and in heavy animals (>380 kg) (Banney et al., 2009; Hedlefs, 1988). Selection of material for pen flooring is a balance between considerations of function, durability, and ease of cleaning. Provision 23.6 of the Marine Order 43, which specifies the requirements of vessel conditions for the carriage of livestock, states that “The deck within pens, passageways and ramps between decks must have a surface that provides a satisfactory non-slip foothold for the cattle” (Australian Maritime Safety Authority, 2012). This surface must also be able to withstand corrosion associated with regular cleaning with high pressure sea water and disinfection with a 4% soda ash solution to meet Australian quarantine requirements (Commonwealth of Australia, Quarantine Act 1908).

Historically, welded metal meshes were used as flooring material on livestock vessels. This material provides a satisfactory non-slip surface but does not provide a comfortable surface for cattle to lie down on, is difficult to clean, and can cause injuries if rusted/broken (Banney et al., 2009; Hedlefs, 1988; Norris et al., 2003). Modern flooring surfaces comprise a gravel aggregate embedded in an epoxy/bitumen base (Banney et al., 2009). This surface

provides good protection against slipping and is relatively easy to clean. Larger aggregates provide better protection against slipping but are also more abrasive (Phillips and Morris, 2001) and can cause significant wear and tear on cattle, resulting in lameness and infection (Banney et al., 2009).

Cook and Norlund (2009) provide a comprehensive review of the effect of different types of flooring on lameness in housed dairy cattle. Covering traditional roughened or grooved concrete flooring with rubber matting provides more secure footing (Flower et al., 2007; Rushen and de Passille, 2006), better cushioning (Rushen and de Passille, 2006), and a more comfortable surface for cattle to lie down on (Platz et al., 2007) than uncovered concrete. Rubber matting has been found to result in decreased treatments for lameness (Eicher et al., 2013; Vanegas et al., 2006) and improved comfort for lame animals (Flower et al., 2007; Ouweltjes et al., 2009; Telezhenko and Bergsten, 2005; Telezhenko et al., 2007). However, rubber matting is more expensive than epoxy/bitumen based surfaces, almost impossible to clean to the standards required by Australian quarantine, may become slippery when decks are washed down during the voyage, and its durability in the face of regular high pressure hosing with seawater is not known (Banney et al., 2009; Hedlefs, 1988).

During the voyage, cases of lameness usually begin with abrasion of the decubital (pressure) points including the rostral or caudal sole, or abaxial surface of the hoof (Norris et al., 2003). Pain caused by the abrasion makes affected animals reluctant to stand and so they spend more time in recumbency, resulting in maceration and abrasion of the lateral surface of the hocks or elbows. When the animal does attempt to stand it places more weight on its fetlocks and knees, leading to abrasion of these areas. Ongoing maceration and bacterial contamination through contact with manure means that these lesions seldom heal without treatment (Banney et al., 2009).

The first line of treatment for lameness cases on-board vessels is systemic antimicrobials and anti-inflammatories. The cost of pharmaceuticals for treatment of leg injuries can be as much as $80 per animal per voyage (Banney et al., 2009). Animals may also be sedated for wound cleaning and bandaging. This type of treatment is time consuming and may not lead to a full recovery. Commercial shoes are of little value because they quickly become saturated with urine and faeces and fall off after 1-2 days (Hedlefs, 1988).

Sedation for wound cleaning and bandaging carries its own mortality risk. Hyperthermia is known to occur following administration of tranquilizing drugs to sheep in hot weather (Radostits et al., 2000). Xylazine is commonly used for sedation of cattle on-board vessels and hyperthermia secondary to a reduced ability to thermoregulate is a recognised side-effect of deep sedation with xylazine, particularly when animals are in an environment where overheating could occur.

### 2.4.3 Respiratory disease

Respiratory disease associated with long distance transport of animals was first reported in the scientific literature in 1925, although the disease had been recognised by veterinarians for at least 20 years previously (Hepburn, 1925). Hepburn (1925) describes respiratory disease, diarrhoea and toxaemia in cattle transported by truck, rail and ship from Ireland and Orkney to Scotland, and by truck and rail within Scotland. Bacteriological examination of severely affected lungs produced a pure culture of *Bacillus bovisepticus* (now *Mannheimia haemolytica*) although Hepburn postulated that "any circumstance whatever which lowers the general tone or vitality of the [transported animals' health]…must be looked upon as a contributory cause." ((Hepburn, 1925) pg 202). Proposed contributory causes included transportation of animals in poor body condition and cows in advanced stages of pregnancy, fatigue caused by long distance walking or trucking to markets or the port of embarkation, exposure to "contagion" at livestock markets, insufficient time for

rest and recovery before shipping, bruising and trauma to limbs etc. during transport, and conditions on trains and ships including poor ventilation resulting in a hot, “stuffy and vitiated atmosphere”, and high stocking densities preventing animals from lying down and resting (Hepburn, 1925). Provision of dry, comfortable, clean bed, fresh air, good nursing, and a tempting diet was considered the most effective treatment (Hepburn, 1925). It is interesting to note that the basic principles for the pathogenesis of transport-associated respiratory disease as described by Hepburn (1925) are still relevant today, almost 90 years later.

Risk factors for the development of BRD include environmental, animal and pathogen components. Environmental stressors including mustering, withholding food and water prior to transportation, exposure to noise and vibration during transportation, exposure to a new environment, and co-mingling with unfamiliar animals activate the stress response (Manteca, 2008; Phillips, 2008; Sainsbury and Sainsbury, 1988; Warriss, 2004), causing the release of glucocorticoids and/or catecholamines which can reduce immunity (Elenkov et al., 2000). Decreased efficiency of the immune system leads to increased viral shedding in subclinically affected animals and proliferation of potentially pathogenic commensal bacteria (Manteca, 2008; Panciera and Confer, 2010). Inhibition of respiratory clearance mechanisms and direct tissue damage by viral organisms allows proliferation and invasion of secondary bacterial organisms, often resulting in a terminal bacterial pneumonia (Panciera and Confer, 2010; Taylor et al., 2010).

Viruses implicated in the pathogenesis of BRD include Bovine herpesvirus 1 (BoHV-1), Bovine respiratory syncytial virus (BRSV), Bovine viral diarrhoea virus (BVDV), Bovine parainfluenza virus 3 (BPIV-3), Bovine coronavirus (BCoV), bovine rhinoviruses and bovine adenoviruses (reviewed in (Fulton, 2009; Panciera and Confer, 2010)). Bovine rhinoviruses and adenoviruses are thought to play only a minimal role in the pathogenesis of BRD, while

BoHV-1, BPIV-3 and BRSV are recognised as primary respiratory pathogens, and BVDV as a significant predisposing pathogen (Fulton, 2009; Panciera and Confer, 2010). Since its first isolation in association with respiratory disease in 1993 (Storz et al., 1996) BCoV has received attention as an emerging BRD pathogen, and is now thought to make a substantial contribution to the development of BRD in feedlot cattle (Saif, 2010; Storz et al., 2000a).

*Mannhaemia haemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma bovis* are considered to be the most important bacterial pathogens in BRD (reviewed in (Griffin et al., 2010; Taylor et al., 2010)). Additional bacterial agents isolated from bovine lung tissue include *Trueperella* (formerly *Arcanobacterium*) *pyogenes* (Watson and Scholes, 2010; Welsh et al., 2004) and *Bibersteinia* (formerly *Pasteurella*) *trehalosi* (Blackall et al., 2007; Watson and Scholes, 2010).

#### *2.4.3.1 Bovine herpesvirus 1*

Bovine herpesvirus 1 (BoHV-1) is a double stranded DNA virus that belongs to the family *Herpesviridae*, subfamily *Alphaherpesviridae*, genus *Varicellovirus*. The BoHV-1 virus can be divided into several subtypes based on genomic analysis and viral peptide patterns; BoHV-1.1 is associated with respiratory disease, BoHV-1.2 with reproductive disease, and BoHV-1.3 with neurological disease (reviewed in (Biswas et al., 2013)). Previous serological surveys of Australian cattle have found that BoHV-1.2 is common and BoHV-1.1, which is found in North American feedlots, does not occur in Australia (Dunn et al., 1995; St George, 1983).

Respiratory tract disease associated with BoHV-1 (then known as infectious bovine rhinotracheitis, IBR) was first described in the United States of America in the early 1950s (Miller, 1955; Schroeder and Moys, 1954). The causative agent was first isolated in 1956 (Madin et al., 1956) and assigned to the herpesvirus group in 1961 (Armstrong et al., 1961). In Australia, the first cases of rhinitis caused by BoHV-1 were described in 1964 during an

outbreak of vaginitis and rhinitis in Victorian dairy cattle (Snowdon, 1964b). However, Snowdon hypothesised that the syndrome of viral rhinitis had probably been present in Australian cattle for many years but due to the mild clinical signs and methods of husbandry practised most cases pass unnoticed (Snowdon, 1964b).

Infection with BoHV-1 leads to transient inhibition of the immune response including increased apoptosis of CD4+ T helper cells resulting in a reduced antibody response, impaired recognition and clearance of virus-infected cells by CD8+ cytotoxic T cells, and inhibition of interleukin-2 and interferon beta-mediated antiviral activity (reviewed in (Biswas et al., 2013; Jones and Chowdhury, 2010; Winkler et al., 1999). In addition, inflammation induced by viral infection causes direct tissue damage including destruction of tracheal cilia (Biswas et al., 2013).

Following acute infection of epithelial cells of the upper respiratory tract and tonsils, the BoHV-1 virus enters the nerve termini of the parasympathetic nervous system, undergoes retrograde transport to cell body, and establishes lifelong latency in the trigeminal ganglia (Ackermann et al., 1982; Narita et al., 1976). Following reactivation from latency, for example under the influence of exogenous or endogenous corticosteroids (Davies and Duncan, 1974; Snowdon, 1964a; Thiry et al., 1987), viral particles spread back towards the periphery leading to recrudescent shedding of BoHV-1 and potential transmission of the virus to in-contact cattle (reviewed in (Kramer and Enquist, 2013)).

#### *2.4.3.2 Bovine respiratory syncytial virus*

Bovine respiratory syncytial virus (BRSV) is a single negative stranded RNA virus that belongs to the family *Paramyxoviridae*, subfamily *Pneumovirinae*, genus *Pneumovirus*.

The association between a respiratory syncytial virus (RSV) and respiratory disease in cattle was first proposed in 1968 after antibodies to a human strain of RSV were demonstrated in sera from English cattle with respiratory illnesses of unknown cause (Doggett et al., 1968).

Shortly afterwards, BRSV was isolated from conjunctivo-nasal swabs and sera collected from cattle during an outbreak of respiratory disease in Swiss cattle in 1967 (Paccaud and Jacquier, 1970). In Australia, serum antibodies to BRSV were first reported in a study on diseases of cattle in Eastern Australian feedlots (Dunn et al., 2000; Dunn et al., 1995); 27% of cattle were seropositive at entry, rising to 71% at slaughter approximately 173 days later. Subsequently, serum antibodies to BRSV were detected in 100% (*n* = 20) of cattle with respiratory disease during an outbreak in a feedlot on the south coast of New South Wales (Hick et al., 2012). Bovine respiratory syncytial virus was not detected in nasal swabs from these animals (Hick et al., 2012).

Bovine respiratory syncytial virus causes disease though both direct tissue damage and modulation of the immune response. The following summary is drawn from comprehensive recent reviews on the immunology of bovine (Gershwin, 2012) and human (Tripp, 2013) respiratory syncytial virus.

The fusion (F) protein, a transmembrane glycoprotein found in the envelope of BRSV, interacts with toll-like receptor 4, leading to increased production of pro-inflammatory cytokines by respiratory epithelial cells, and inflammation-associated tissue damage. The F protein also causes fusion of cell membranes to facilitate movement of virus between cells, resulting in the pulmonary syncytia that are characteristic histological features of BRSV infection. In studies of human RSV, the major surface glycoprotein, or G protein, facilitates viral entry into cells and has pro-inflammatory effects via modulation of leukocyte chemotaxis. Bovine respiratory syncytial virus infection favours the induction of a less-aggressive CD4+ T helper 2 type response (anti-inflammatory) over the more vibrant T helper 1 type response (pro-inflammatory). This effect is achieved through suppression of interleukin-12 production by pulmonary dendritic cells, resulting in decreased T helper 1 cell proliferation, leading to a reduced cytotoxic T cell response.

Phylogenetic analyses have indicated that the recurrence of BRSV outbreaks within herds are due to reintroduction of BRSV into the herd, rather than re-activation of latent infection in carrier animals (Bidokhti et al., 2012; Larsen et al., 2000). Indeed, corticosteroid treatment of seropositive animals failed to reactivate infection, suggesting that latent infections are rare, if they occur at all (Van der Poel et al., 1997).

#### *2.4.3.3 Bovine parainfluenza virus 3*

Bovine parainfluenza virus 3 (BPIV-3) has a single stranded negative sense genome and belongs to the family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Respirovirus*. Based on sequence analysis of the haemagglutinin-neuraminidase gene, matrix gene and/or full genome sequences BPIV-3 isolates have been divided into 3 groups: BPIV-3a, BPIV-3b, BPIV-3c (de Almeida Vaucher et al., 2011; Horwood et al., 2008; Vecherov et al., 2003; Wen et al., 2012; Zhu et al., 2011). The occurrence of isolates from more than one group have been reported in Australia (BPIV-3a and BPIV-3b) (Horwood et al., 2008), China (BPIV-3a and BPIV-3c) (Wen et al., 2012; Zhu et al., 2011), and the Russian Federation (BPIV-3a and BPIV-3b) (Vecherov et al., 2003). In the case of the haemagglutinin-neuraminidase gene, different sequences may be related to different levels of pathogenicity through the role this gene plays in syncytium formation (Breker-Klassen et al., 1996).

A novel virus, designated mxyovirus SF-4, was first isolated in the United States in 1958, in 4-8 month old beef feedlot calves showing signs of respiratory disease (Reisinger et al., 1959). Shortly afterwards the virus was designated as a bovine strain of the parainfluenza-3 virus group, based on serological similarities with human parainfluenza-3 (Hoerlein et al., 1959). Serum antibodies to BPIV-3 were first reported in Australian cattle in 1969 (St George, 1969) and the virus does not appear to be important in BRD in Australian feedlot cattle (Dunn et al., 2000; Dunn et al., 1995).

In vitro, BPIV-3 infection is associated with cytopathic effects including loss of cilia, swelling and desquamation of epithelial cells and syncytium formation (Campbell et al., 1969) although the exact mechanisms behind these changes remain unknown (reviewed in (Ellis, 2010)). Bovine parainfluenza virus 3 has also been shown to inhibit both the innate and adaptive immune responses including causing suppression of interferon-beta production, depression of pulmonary alveolar macrophage function, and decreased proliferation of lymphoid cells (reviewed in (Ellis, 2010)).

#### *2.4.3.4 Bovine viral diarrhoea virus*

Bovine viral diarrhoea virus (BVDV) refers to a heterologous group of viruses with a single positive stranded RNA virus that belong to the family *Flaviviridae*, genus *Pestivirus*. The first subclassification of BVDVs was into 2 biotypes – cytopathic and non-cytopathic – based on cytopathic effects in cell culture (Gillespie et al., 1960; Lee and Gillespie, 1957). Since then, BVDV have been divided into two genotypes (species) based on phylogenetic and antigenic differences (Pellerin et al., 1994; Ridpath et al., 1994). Bovine viral diarrhoea virus 1 can be further subdivided into 12 subgenotypes, designated by the letters 'a' to 'l', and BVDV 2 into 2 subgenotypes (a and b) (Flores et al., 2002; Ridpath and Bolin, 1998; Vilcek et al., 2001). Genetic analyses of BVDV isolates from Australia indicate that BVDV-1c is the most prevalent genotype (96-98% samples tested), although BVDV-1a (2-3%) and BVDV-1b (0.3%) are also present (Mahony et al., 2005; Ridpath et al., 2010). Bovine viral diarrhoea virus 2 genotypes are not present in Australia (Peter Kirkland, *personal communication*). Antigenic differences between species are important since BVDV1 vaccines are not protective against BVDV2 strains (Bolin and Grooms, 2004; Fulton et al., 2003a). The practical importance of the subgenotypes is still under discussion (Ridpath and Fulton, 2009).

Bovine viral diarrhoea virus infections in cattle result in a number of manifestations including respiratory disease, digestive tract disease, mucosal disease, reproductive tract disease and foetal infections (reviewed in (Fulton, 2013; Ridpath, 2010)). Bovine virus diarrhoea was first recognised in 1946 (Olafson et al., 1946) and mucosal disease in 1951 ((Ramsey and Chivers, 1953), cited by (Ramsey and Chivers, 1957)). Respiratory signs, inclusive of nasal discharge and cough, were described as part of the clinical presentation in the acute form of bovine virus diarrhoea (Pritchard and Carlson, 1957).

Mucosal disease was first described in Australia in 1957 (Blood et al., 1957) and serum antibodies to BVDV were detected in Victorian herds with diarrhoea in 1962 (French, 1962). Bovine viral diarrhoea virus in association with respiratory disease in Australia was first reported in 1995, in eastern Australian feedlot cattle (Dunn et al., 1995).

In 2004 a novel putative pestivirus species (named “HoBi-like” virus, also known as BVDV-3 and atypical pestivirus) was detected in foetal bovine serum originating from Brazil (Schirrmeier et al., 2004). HoBi-like viruses were subsequently detected in foetal bovine serum or contaminated cell cultures originating from Brazil (Peletto et al., 2012; Stalder et al., 2005; Xia et al., 2011), Australia (Xia et al., 2011), Canada (Xia et al., 2011), Mexico (Xia et al., 2011) and the United States of America (Xia et al., 2011). Cattle naturally infected with HoBi-like virus have been detected in Brazil (Bianchi et al., 2011; Cortez et al., 2006), Thailand (Kampa et al., 2009; Stahl et al., 2007), and Italy (Decaro et al., 2011; Decaro et al., 2012a; Decaro et al., 2012b) and show a range of clinical presentations including respiratory disease, diarrhoea, and abortion.

Under experimental conditions BVDV has been shown to cause mild respiratory disease in calves in the absence of other organisms (Potgieter et al., 1985; Potgieter et al., 1984). Acute BVDV infection is associated with impaired mucociliary clearance in upper and lower respiratory tract and reduced function of cells of the innate and acquired immune systems,

in particular macrophages, neutrophils and lymphoid cells, which predisposes newly infected animals to secondary bacterial infections (Al-Haddawi et al., 2007; Fulton, 2013; Peterhans et al., 2003; Ridpath, 2010).

Infection of susceptible cows with non-cytopathic BVDV biotypes between day 25-90 of gestation may result in foetal infection and calves which are lifelong persistent shedders of BVDV (McClurkin et al., 1984). Persistently infected animals have viral antigen in their nasal secretions but do not produce antibodies to homologous BVDV species (Bolin, 1988; Bolin et al., 1985; Fulton et al., 2003b; McClurkin et al., 1984). Persistently infected animals can infect 70-100% of susceptible contacts in a pen (Fulton et al., 2005; Fulton et al., 2006) thereby playing a significant role in the pathogenesis of BRD in intensively housed cattle.

#### *2.4.3.5 Bovine coronavirus*

Bovine coronavirus (BCoV) is a positive-sense single stranded RNA virus in the order *Nidovirales*, family *Coronaviridae*. Based on serologic and genetic analysis the coronaviruses have been divided into three subgroups; BCoV belongs to phylogenetic subgroup 2a which is characterised by the presence of a gene encoding a haemagglutinin-esterase.

Bovine coronavirus is associated with enteric and respiratory disease syndromes in calves and adult cattle; calf diarrhoea and enzootic pneumonia in young animals, winter dysentery in adult cattle, and BRD in feedlot cattle (reviewed in (Saif, 2010)). A coronavirus-like agent was first isolated from cattle in 1972 from cases of neonatal calf diarrhoea (Stair et al., 1972). Since its first isolation in association with respiratory disease in 1993 (Storz et al., 1996) BCoV has been detected in beef and dairy cattle showing signs of respiratory disease in Canada (Gagea et al., 2006b; Martin et al., 1998), Finland (Autio et al., 2007; Hartel et al., 2004), Italy (Decaro et al., 2008a; Decaro et al., 2008b), Japan (Kanno et al., 2007; Tsunemitsu et al., 1991), Sweden (Hagglund et al., 2007), Turkey (Hasoksuz et al., 2005) and

the United States of America (Cho et al., 2001; Fulton et al., 2011; Hasoksuz et al., 2002a; Hasoksuz et al., 1999; Lathrop et al., 2000b; Storz et al., 2000b).

Bovine coronavirus was first detected in Australia in 2010 in an outbreak of respiratory disease in feedlot calves in southern New South Wales (Hick et al., 2012). The virus was detected in 10/30 nasal swabs from affected calves and 2/5 pneumonic lungs from necropsied animals (Hick et al., 2012).

The viral envelope of BCoV contains the haemagglutinin-esterase (HE) and spike (S) proteins which are important virulence factors. The HE protein contains a receptor destroying enzyme that inactivates the erythrocyte receptor for BCoV and increases infectivity (Vlasak et al., 1988). The S protein has a number of functions including neutralising epitopes and membrane fusion to facilitate spread of virus between cells (reviewed in (Clark, 1993; Saif, 2010)). Genomic variation in the S protein accounts for much of the variation in tissue tropism and host range among coronaviruses (Saif, 2010). However, with regards to enteric versus respiratory strains of BCoV, there appears to be more sequence diversion between BCoV strains across time than between co-temporal strains associated with different clinical syndromes (Hasoksuz et al., 2002b).

#### *2.4.3.6 Mannheimia haemolytica*

*Mannheimia haemolytica* (formerly *Pasteurella haemolytica*) is a member of the family *Pasteurellacae*. *Mannheimia haemolytica* consists of 12 capsular serotypes, designated A1, A2, A5, A6, A7, A8, A9, A12, A13, A14, A16 and A17 (Adlam, 1989; Younan and Fodar, 1995). Serotype A1 is the most commonly isolated serotype detected in pneumonic cattle lungs at necropsy, although A2, A5, A6 and A9 have also been reported (Al-Ghamdi et al., 2000; Frank, 1989; Purdy et al., 1997). Serotypes A2 and A4 may be recovered from the upper respiratory tract of healthy cattle but are seldom associated with respiratory disease (Frank, 1985, 1989; Frank and Smith, 1983). Through as yet unknown mechanisms, stress or

concurrent viral infections result in changes to the respiratory tract environment that favours multiplication and colonisation of serotype A1 (Briggs and Frank, 1992). Therefore *M. haemolytica* can cause respiratory disease as both a primary (Hodgins and Shewen, 2004) and secondary pathogen, although the latter is considered more common (reviewed in (Griffin, 2010; Rice et al., 2007)).

*Mannheimia haemolytica*, then known as *Bacillus bovisepticus*, was first reported in cattle with respiratory disease in the early 1920s; in calves in the United States (Smith, 1921), and in recently transported cattle in the United Kingdom (Hepburn, 1925). Subsequently, *M. haemolytica* has been detected in cattle around the world and is generally considered to be the most important bacterial pathogen in BRD (Griffin, 2010; Rice et al., 2007). *Mannheimia haemolytica* was recognised as a respiratory pathogen in Australian cattle as early as 1957 (Bain, 1957). In a previous study on Australian live export cattle, in 28/34 (82%) of animals that died due to respiratory disease necropsy and histological findings were consistent with a diagnosis of *M. haemolytica* or *Pasteurella multocida* infection (Norris et al., 2003).

*Mannheimia haemolytica* is an opportunistic pathogen and possess a number of virulence factors that allow it to evade the innate and adaptive immune responses, colonise the lung, and establish infection. Virulence factors include leukotoxin (LKT), lipopolysaccharide (LPS), adhesin, capsular polysaccharides, fimbriae, outer membrane iron-binding proteins, lipoproteins, and proteases (reviewed in (Griffin, 2010; Rice et al., 2007; Singh et al., 2011)).

Adhesin proteins allow bacteria to bind to the epithelial surface, facilitating initial colonisation and colony establishment (Clarke et al., 2001; Singh et al., 2011). Bacterial adherence is promoted by capsular polysaccharides (Morck et al., 1988) which have also been found to inhibit neutrophil phagocytosis and affect complement-mediated lysis of bacteria (Griffin, 2010; Singh et al., 2011). Proteases degrade opsonising antibodies, for example Immunoglobulin G, thus reducing phagocytosis and bacterial killing (Griffin, 2010;

Singh et al., 2011). The neuraminidase protein is thought to play a role in receptor-mediated adhesion (Singh et al., 2011). Outer membrane iron-binding proteins allow *M. haemolytica* to replicate in the low-iron host environment (Griffin, 2010; Ogunnariwo et al., 1997). Outer membrane proteins also play a role in bacterial adhesion, acting as chemotactic agents for neutrophils, inhibiting phagocytosis and complement mediated killing by neutrophils, and enhancing the production of proinflammatory cytokines and reactive oxygen species (Singh et al., 2011).

Leukotoxin and LPS are responsible for most of the destructive lesions associated with *M. haemolytica* infection (Panciera and Confer, 2010). Leukotoxin binds specifically to ruminant leukocytes and has a range of dose-dependent effects (Griffin, 2010; Rice et al., 2007; Singh et al., 2011). At low concentrations LKT activates macrophages and neutrophils, leading to the release of inflammatory cytokines and accumulation of inflammatory cells in the lung. Stimulation of mast cells results in histamine release and increased vascular permeability. At high concentrations LKT causes leukocyte apoptosis, and, at very high concentrations, transmembrane pore formation and oncotic cell death. Release of oxygen radicals and other toxic molecules from necrotic cells, in particular neutrophils, causes further damage to the pulmonary parenchyma (Rice et al., 2007; Singh et al., 2011).

Lipopolysaccharide, in particular the lipid A fraction, acts as an endotoxin, causing increased tumour-necrosis factor release, vascular damage, vasodilation, pyrexia and hypotensive shock (Rice et al., 2007; Singh et al., 2011). In addition, LPS forms complexes with LKT, enhancing cytotoxicity (Li and Clinkenbeard, 1999). Both LKT and LPS also affect the adaptive immune response through inhibition of mitogen-mediated lymphoid proliferation in the lung (LKT) and peripheral circulation (LPS) (Griffin, 2010; Rice et al., 2007).

Typically, infection of the lung with *M. haemolytica* results in a necrotizing bronchopneumonia, with prominent “oat cells” (necrotic neutrophils), copious fibrinous exudate, and thrombosis of lymphatic vessels (Caswell and Williams, 2007). These changes are largely due to the combined effects of LKT on neutrophils and platelet adhesion, and LPS-mediated vascular damage (Griffin, 2010; Rice et al., 2007).

#### *2.4.3.7 Pasteurella multocida*

*Pasteurella multocida* is a member of the family *Pasteurellacae* and consists of 5 capsular serogroups (A, B, D, E, F) and 16 serotypes (1-16) based primarily on lipopolysaccharide antigens (Carter, 1955; Heddleston et al., 1972). Serogroups B:2 and E:2 are associated with haemorrhagic septicaemia in cattle and buffalo (Carter and De Alwis, 1989; Shivachandra et al., 2011). Serogroup A is the most commonly isolated *P. multocida* in cases of bovine respiratory disease (80-90%) (Ewers et al., 2006; Frank, 1989; Katsuda et al., 2013), while serogroups D and F are isolated less frequently (approximately 5%) (Ewers et al., 2006). In a recent study no association was detected between *P. multocida* serogroup, isolated from nasal swabs from clinically normal and BRD-affected animals, and clinical status (Katsuda et al., 2013).

*Pasteurella multocida* exists as both a commensal bacteria and a primary or secondary agent involved with pneumonia in cattle (Frank, 1989). The isolation of *P. multocida* serogroup A from an Australian bovine was first reported in the late 1950s (Bain, 1957). Gross and histopathological lesions typical of *Pasteurella multocida* infection have been reported in Australian live export cattle (Norris et al., 2003), and *P. multocida* has been isolated from tracheal swabs and lung lesions in an outbreak of respiratory disease in an Australian feedlot (Hick et al., 2012).

A study on lung tissue or swabs from beef cattle at necropsy collected between 1994 and 2002 detected a trend towards an increased isolation of *P. multocida* and reduced isolation

of *M. haemolytica* as the principle bacterial pathogen associated with BRD (Welsh et al., 2004). There are a number of possible explanations for this trend including changes in bacterial virulence and antimicrobial resistance, changes in the efficacy of available vaccines and antibiotics, reduced age of cattle at feedlot entry, changes in the way sick cattle are identified and treated, and increased use of mass medication programs (Rice et al., 2007; Welsh et al., 2004).

Virulence factors possessed by *P. multocida* include LPS, capsular proteins, outer-membrane proteins, immunoglobulin binding proteins, fimbrae, proteases and lipases (reviewed in (Dabo et al., 2007; Harper et al., 2006; Panciera and Confer, 2010). Capsular proteins are associated with resistance to phagocytosis and complement-mediated lysis, they facilitate bacterial adhesion and survival, and utilize molecular mimicry of host proteins to evade the immune system. Outer-membrane proteins are involved with adherence and invasion as well as uptake of free iron. Lipopolysaccharide plays a role in the adhesion of bacteria to neutrophils, transmigration of bacteria through epithelial cells, and stimulation of expression of inflammatory cytokines, and causes widespread vascular alterations which eventually lead to endotoxic shock.

In cattle, respiratory infection with *P. multocida* leads to a suppurative bronchopneumonia, and much of the pathology can be attributed to the effects of LPS (Dabo et al., 2007; Harper et al., 2006). Typically, the bronchi, bronchioles and peribronchiolar alveoli are filled with an inflammatory cell infiltrate, which is composed of neutrophils and macrophages. The respiratory epithelium and inflammatory infiltrate are often necrotic. The interlobular lymphatics may be dilated by a fibrinous to fibrinopuruluent exudate (Caswell and Williams, 2007; Dabo et al., 2007).

### *2.4.3.8 Histophilus somni*

*Histophilus somni* (formerly *Haemophilus somnus*) is a member of the family *Pasteurellacae*. *Histophilus somni* is a commensal organism of the upper respiratory tract (Crandell et al., 1977) but is also commonly associated with a number of disease syndromes including fibrinopurulent pneumonia, thromboembolic meningitis (TEM), myocarditis and polyarthritis-serositis (reviewed in (Griffin et al., 2010)). Although TEM was the first clinical manifestation of disease associated with *H. somni* infection to be described (Brown et al., 1956), a number of subsequent reports suggest that case presentations of pneumonia, myocarditis and/or arthritis in the absence of TEM are becoming more common ((Harris and Janzen, 1989; Orr, 1992; Schuh and Harland, 1991; Van Donkersgoed et al., 1990).

Clinical signs of respiratory disease in association with *H. somni* infection were first reported in the late 1960s (Panciera et al., 1968) and since that time *H. somni* has become a common isolate from BRD cases (Fulton, 2009). *Histophilus somni* was first reported in Australian cattle in 1984 (Lancaster et al., 1984) although the author of the article suggested that, due to diagnostic test cross-reactivity, previously reported outbreaks of calf respiratory disease in Western Australia attributed to Actinobacillus actinoides (Allen, 1976) may have, in fact, been caused by *H. somni*. In recent years, *Histophilus somni* has come to be viewed as a BRD pathogen in feedlot cattle and cattle that have been recently transported (Read and Slattery, 2010). For example, *H. somni* was cultured from 1 out of 5 tracheal swabs collected from clinically affected cattle during an outbreak of respiratory disease in intensively managed cattle in New South Wales (Hick et al., 2012).

Virulence factors possessed by *H. somni* include lipooligosaccharide (LOS, endotoxin), outer-membrane proteins, immunoglobulin binding proteins, adhesins, exopolysaccharide, and histamine. The following summary of *H. somni* virulence factors is sourced from comprehensive recent reviews (Corbeil, 2007; Sandal and Inzana, 2010). Bacterial

attachment is facilitated by adhesins and exopolysaccharide, which functions as a scaffold to support biofilm formation and thus colonisation. Outer-membrane proteins inhibit antibody-mediated bacterial killing through binding to Immunoglobulin E, and take up iron to support colony growth. Immunoglobulin binding proteins form a fibrillar network on the surface of *H. somni* and are directly cytotoxic to macrophages, bind Immunoglobulin G2 and inhibit complement-mediated killing and T-cell mediated cellular immunity, and cause contraction of endothelial cells to facilitate haematogenous spread of the bacteria. Histamine production by *H. somni* is likely to account for early lesions of oedema, increased mucus secretion, bronchoconstriction and vasoconstriction.

Lipooligosaccharide promotes bacterial adherence and is the primary factor responsible for lesion formation. Apoptosis of endothelial cells and platelet activation by LOS leads to vasculitis, thrombosis, and oedema. Complement activation by LOS results in chemotaxis of inflammatory cells, and the suppurative and necrotic lesions associated with *H. somni* infection. Both outer-membrane proteins and LOS use antigenic variation to evade the immune response.

#### *2.4.3.9 Mycoplasma bovis*

*Mycoplasma bovis* is a member of the class *Mollicutes*, family *Mycoplasmatacae.* *Mycoplasma bovis* is an important cause of pneumonia, arthritis and tenosynovitis in feedlot cattle, and is also associated with mastitis in cows and otitis media in calves (reviewed in (Caswell et al., 2010; Maunsell et al., 2011)).

Pleuropneumonia-like organisms isolated from the lungs of 4-12 month old calves with shipping fever in Canada (Carter, 1954) were later confirmed as a *Mycoplasma* (Leach, 1967). However, the role of *M. bovis* in the development of BRD is still not well defined. Calves experimentally inoculated with *M. bovis* develop typical caseonecrotic lesions (Hermeyer et al., 2012; Rodriguez et al., 1996; Thomas et al., 1986) indicating that *M. bovis*

can be a primary pathogen. *Mycoplasma bovis* can be isolated from clinically normal animals (Caswell et al., 2010; Maunsell et al., 2011) and is most often isolated from cases of chronic pneumonia, suggesting a secondary role (Fulton, 2009).

*Mycoplasma* species were first reported in Australia in 1963, isolated from the joints of calves with arthritis in a research herd at the University of Queensland (Simmons and Johnston, 1963) and nasal swabs from apparently healthy cattle in a research herd at the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Victoria (Hudson and Etheridge, 1963). These isolates were later found to belong to serotypes 7 and 8 respectively (Leach, 1967). *Mycoplasma* serotype 5 (*M. bovimastitidis/M. agalacticae* subsp*. bovis/M. bovis*) was first detected in milk samples, but not nasal or lung samples, from Australian cattle in 1970 (Cottew, 1970) and has since been reported as an important cause of mastitis in dairy cattle in North Queensland and Victoria (Ghadersohi et al., 1999). *Mycoplasma bovis* was not detected in pneumonic lung or nasal swab samples from Australian beef feedlot cattle with respiratory disease (Dunn et al., 1995; Hick et al., 2012).

Although *Mycoplasmas* are the smallest self-replicating life forms on earth (Razin et al., 1998) and lack a cell wall, *M. bovis* possesses a number of virulence factors that allow it to survive and cause chronic lesions in the host. The membrane protein pMB67 is the primary antigen recognised during infection with *M. bovis* (Behrens et al., 1996) and also plays a role in adhesion (Caswell et al., 2010). Other membrane proteins associated with adhesion include the p26 protein (Sachse et al., 1993) and α-enolase (Song et al., 2012).

Variable surface proteins (Vsp) are a family of lipoproteins that undergo high-frequency phase and size variations (Lysnyansky et al., 1996), which may allow *M. bovis* to adhere to different host cell types (Sachse et al., 2000), adapt to different host microenvironments (Lysnyansky et al., 2001), and evade the immune system (Le Grand et al., 1996; Lysnyansky et al., 2001). In vitro the C-terminal fragment of VSP-L has been shown to impair mitogen-

induced lymphocyte proliferation (Vanden Bush and Rosenbusch, 2002). Some strains of *M. bovis* are able to form biofilms, which help them to resist dessication in the environment, and could play a role in immune system evasion and antimicrobial resistance (McAuliffe et al., 2006).

Through as yet unknown mechanisms *M. bovis* is able to suppress mitogen-induced proliferation of peripheral blood lymphocytes (Bennett and Jasper, 1977; Thomas et al., 1990) and inhibit activation of naïve lymphocytes (Vanden Bush and Rosenbusch, 2002). In vitro, *M. bovis* has been shown to induce lymphocyte apoptosis (Vanden Bush and Rosenbusch, 2002) and adhere to neutrophils and impair the neutrophil respiratory burst (Thomas et al., 1991) but the in vivo significance of these findings is unknown. Hydrogen peroxide produced by *M. bovis* may cause oxidative damage to lung tissue and contribute to the development of caseonecrotic lesions (Schott et al., 2014).

## 2.5 Conclusion

Mortality during live export is influenced by animal, environmental, management and husbandry factors in the pre-export and voyage phases of the live export supply chain. The purpose of this study was to investigate the possible causes of and risk factors for mortality in cattle on long-haul live export voyages, with the aim of developing recommendations to industry to improve animal welfare by minimising cattle morbidity and mortality during voyages.

# Chapter 3: Development of quantitative PCR assays for the detection of viruses and bacteria known to be associated with bovine respiratory disease

## 3.1 Summary

The objective of this study was to develop and validate quantitative polymerase chain reaction and reverse transcription polymerase chain reaction (qPCR) assays for the detection of viruses and bacteria known to be important in the pathogenesis of bovine respiratory disease (Bovine coronavirus (Betacoronavirus 1), Bovine herpesvirus 1, Bovine parainfluenza virus 3, Bovine respiratory syncytial virus, Bovine viral diarrhoea virus, *Mannheimia haemolytica*, *Pasteurella multocida*, *Mycoplasma bovis*, *Histophilus somni*) in swab and tissue samples. Where possible, assays were duplexed to reduce the number of tests required.

The limits of detection for each of the qPCR assays ranged from <10 to 200 copies of the viral/bacterial genome when tested with plasmid standards. It would be expected that these limits may be higher when testing genomic DNA or RNA.

## 3.2 Introduction

Bovine respiratory disease (BRD) is an important cause of death in live export cattle (Norris et al., 2003). Although necropsy findings in cases of BRD are usually consistent with a bacterial pneumonia, predisposing factors, including a number of viruses, play an important role in the pathogenesis of the disease (Panciera and Confer, 2010; Taylor et al., 2010). Viruses that have been shown to contribute to the development of BRD include BCoV, BoHV-1, BRSV, BVDV, BPIV-3 (Fulton, 2009; Panciera and Confer, 2010). The bacteria *M.*

*haemolytica*, *P. multocida*, *M. bovis*, *H. somni* are commonly isolated from cases of BRD (Griffin et al., 2010; Taylor et al., 2010).

Identification of aetiologic agents in cases of BRD, or where there is a clinical suspicion of BRD, may be performed through testing of samples including upper respiratory tract swabs (nasal, nasopharyngeal, tracheal), bronchoalveolar lavage fluid, or swabs and/or tissue collected from lung lesions (Fulton and Confer, 2012). Upper respiratory tract swabs may also be taken from apparently healthy animals, for example beef cattle entering fattening feedlots, to investigate the relationship between the presence/absence of viruses and/or bacteria in these samples and production parameters including (BRD) mortality risk (Allen et al., 1991; Cho et al., 2001; Frank and Smith, 1983; Fulton et al., 2005; Fulton et al., 2002a; Fulton et al., 2011; Hall et al., 1977; Hanzlicek et al., 2011; Hasoksuz et al., 2002a; Lathrop et al., 2000b; Lin et al., 2000; Magwood et al., 1969; Plummer et al., 2004; Thomas et al., 2006; Thomson et al., 1975; Van Donkersgoed et al., 1994; White et al., 2010; Wiggins et al., 2007; Yates et al., 1983).

The qPCR assays described in this chapter were developed for use on swab and tissue samples that were collected from cattle that were necropsied during live export voyages. Due to quarantine restrictions imposed by the Australian Government (Quarantine Act 1908), biological material collected during voyages could not be cultured. In addition, samples needed to be robust in the face of variable storage conditions on-board vessels and a significant time period (1-6 months) between sample collection and processing. Therefore, within the design of the study and conditions discussed above, qPCR assays were chosen as the most suitable technique for detection of viral and bacterial nucleic acids in samples collected during this study.

## 3.3 Materials and Methods

### 3.3.1 Samples

The most commonly tested samples were swabs of the nasal cavity collected from animals in the pre-export assembly feedlots, and nasal swabs and lung swabs and/or tissue (approximately 5mm$^2$) collected at necropsy from animals that died during voyages. Samples were collected into a 5 ml plastic screw top container filled with 2 ml of viral transport media (VTM; Hanks balanced salt solution, penicillin G (1,000 units/ml), streptomycin (25 mg/ml), and amphotericin B (0.1 mg/ml)) (Department of Agriculture and Food, Western Australia (DAFWA)) and frozen.

Viral samples used for test validation were obtained as follows. For BoHV-1, BPIV-3 and BVDV, isolates were recovered from DAFWA storage and the RNA extracted. For BCoV, a clinical sample from a Western Australian case of calf scours was used as a template for production of RNA transcripts of the gene targeted by the qPCR. Human respiratory syncytial virus was kindly provided by Dr Tim Mahoney (Department of Employment, Economic Development and Innovation, Queensland) as a positive control for the BRSV qPCR.

DNA samples from *M. haemolytica*, *P. multocida*, *M. bovis* and *H. somni* were kindly provided by Sam Hair from isolates held in the DAFWA bacteriology laboratory.

### 3.3.2 DNA/RNA extraction

Nucleic acid extraction from VTM aliquots was performed using a commercial kit (Ambion MagMax Viral RNA Extraction Kit, Life Technologies, Grand Island, NY) on a magnetic particle processor (MagMax Express 96 magnetic particle processor, Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Plates containing the eluate were stored at –80°C until required.

### 3.3.3 Quantitative PCR development

Primer and probe sequences were sourced from available sequences for BCoV (O'Dea, unpublished data), BoHV-1 (Lovato et al., 2003), BPIV-3 (Horwood and Mahony, 2011), BRSV (Mahony and Horwood, 2006), BVDV (Horwood and Mahony, 2011), *M. haemolytica* (Mahony and Horwood, 2006), *P. multocida* (Mahony and Horwood, 2006), and *H. somni* (Mahony and Horwood, 2006). Primer and probe sequences can be found in Table 3.1.

*Mycoplasma bovis* primer sequences were sourced from a previous study (Clothier et al., 2010) and the probe sequence was designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA) on available GenBank sequences for the Bovine ultra-violet (radiation) resistant (*uvrC*) gene. Primer and probe sequences can be found in Table 3.1.

To reduce the number of tests, required reactions were multiplexed as follows: BCoV and BPIV-3, BRSV and BVDV, *M. haemolytica* and *P. multocida*, *H. somni* and *M. bovis*. Bovine herpesvirus 1 was run as a single assay.

The final optimized multiplex qPCR assays were as follows: All assays consisted of a 20-µl reaction mix containing 2 µl of the nucleic acid sample, 4 µl of proprietary master mix, 0.4 µl of ROX (5-carboxy-X-rhodamine) dye solution, and 0.2 µl of RT mix (QuantiTect virus +ROX vial kit, Qiagen Inc., Valencia, CA). Primer and probe concentrations varied according to the assay. For BoHV-1, BoCV and BPIV-3, BRSV and BVDV, and *M. haemolytica* and *P. multocida*, 400 nM of each primer and 200 nM of probe were used. For *H. somni* and *M. bovis*, 100 nM of each *H. somni* primer and 50 nM of probe were used, and 400 nM of each *M. bovis* primer and 200 nM of probe.

Reactions were performed in an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA) using the conditions outlined in Table 3.2.

**Table 3.1.** Primer and probe sequences for qPCR assays.

| Organism | Target gene | Sequence type | 5' dye, 3' quencher | Sequence | Sequence Source |
|---|---|---|---|---|---|
| Viruses | | | | | |
| Bovine coronavirus | Transmembrane (M) gene | Primer (forward) | | CTG GAA GTT GGT GGA GTT TCA AC | O'Dea, *unpublished data* |
| | | Primer (reverse) | | AAT TAT CGG CCT AAC ATA CAT CCT TC | |
| | | Probe | 6FAM, MGBNFQ | TCA TAT CTA TAC ACA TCA AGT TGT T | |
| Bovine herpesvirus 1 | Glycoprotein C (gC) gene | Primer (forward) | | ATG TTA GCG CTC TGG AAC C | Lovato et al. (2003) |
| | | Primer (reverse) | | CTT TAC GGT CGA CGA CTC C | |
| | | Probe | dQuasar670, BHQ-2 | ACG GAC GTG CGC GAA AAG A | |
| Bovine parainfluenza virus 3 | Matrix (M) protein | Primer (forward) | | TGT CTT CCA CTA GAT AGA GGG ATA AAA TT | Horwood and Mahony (2011) |
| | | Primer (reverse) | | GCA ATG ATA ACA ATG CCA TGG A | |
| | | Probe | VIC, MGBNFQ | ACA GCA ATT GGA TCA ATA A | |
| Bovine respiratory syncytial virus | Fusion protein (F) gene | Primer (forward) | | TAC ACA GCT GCT GTT CAG TAC AAT GT | Mahony, *personal communication* |
| | | Primer (reverse) | | GAT GAT TGG AAC ATA GGA ACC CAT AT | |
| | | Probe | VIC, MGBNFQ | TAG AAA AAG ATG ATG ATC CTG CAT | |
| Bovine viral diarrhoea virus | 5' untranslated region (UTR) | Primer (forward) | | TGG ATG GCT TAA GCC CTG AGT A | Horwood and Mahony (2011) |
| | | Primer (reverse) | | CCT CGT CCA CGT GGC ATC | |
| | | Probe | 6FAM, MGBNFQ | AGT CGT CAG TGG TTC GA | |
| Bacteria | | | | | |
| *Histophilus somni* | 16S rRNA | Primer (forward) | | AGG AAG GCG ATT AGT TTA AGA GAT TAA TT | Mahony and Horwood (2006) |
| | | Primer (reverse) | | TCA CAC CTC ACT TAA GCT ACC ACC T | |
| | | Probe | 6FAM, TAMRA | ATT GAC GAT AAT CAC AGA AGA AGC ACC GGC | |
| *Mycoplamsa bovis* | Bovine ultra-violet resistant gene C (uvrC) | Primer (forward) | | TCT AAT TTT TTC ATC ATC GCT AAT GC | Clothier et al. (2010) |
| | | Primer (reverse) | | TCA GGC CTT TGC TAC AAT GAA C | Clothier et al. (2010) |
| | | Probe | VIC, MGBNFQ | ACT GCA TCA TAT CAC ATA CT | Developed in study |
| *Mannheimia haemolytica* | Leukotoxin A gene (lktA) | Primer (forward) | | AAG GCG ATG ATA TTC TCG ATG GT | Mahony and Horwood (2006) |
| | | Primer (reverse) | | TAC CAT CGC CTT TAC GGT GAA | |
| | | Probe | 6FAM, TAMRA | TAT CGA TGG CGG TAA AGG CAA CGA CCT A | |
| *Pasteurella multocida* | DNA adenine methylase (dam) virulence gene | Primer (forward) | | CGC AGG CAA TGA ATT CTC TTC | Mahony and Horwood (2006) |
| | | Primer (reverse) | | GGC GCT CCT CAG CTG TTT TT | |
| | | Probe | VIC, TAMRA | ACT GCA CCA ACA AAT GCT TGC TGA GTT AGC | |

**Table 3.2**. Cycling conditions for the qPCR assays.

| Step | Temperature (°C) | Time (min:sec) | Number of cycles |
|---|---|---|---|
| 1. Reverse transcription | 50 | 20:00 | 1 |
| 2. Initial activation | 95 | 5:00 | 1 |
| 3a. Denaturation | 95 | 0:15 | 45 |
| 3b. Annealing/extension | 60 | 0:45 | |

### 3.3.4 Construction of plasmid controls

Plasmid controls containing the primer sequences were constructed and titrated to provide exact copy numbers of the viral/bacterial gene, to allow testing of the limits of detection and construction of standard curves for the qPCR.

Nucleotide segments of various sizes (target sequences) were amplified using the primers outlined in Table 3.1 for *M. bovis* and *M. haemolytica*, and the primers outlined in Table 3.3 for *H. somni*, *P. multocida*, BoHV-1, BPIV-3, BRSV and BVDV.

Progmega PCR Master Mix (Promega, Fitchburg, WI) was used for amplification of bacterial target sequences. Reaction volumes of 20 µL consisted of 7 µL nuclease-free water, 10 µL 2X PCR Master Mix, 0.25 nM of each primer, and 2 µL of DNA template. Cycling conditions were as outlined in Table 3.4.

The Qiagen OneStep RT-PCR Kit (Qiagen Inc., Valencia, CA) was for amplification of target sequences for BPIV-3, BRSV and BVDV. Reaction volumes of 20 µL consisted of 9.9 µL nuclease-free water, 4 µL 5X PCR MasterMix, 0.8 µL dNTP mix (containing sodium salts of DNA nucleotides), 0.8 µL enzyme mix (containing reverse transcriptases and DNA polymerase), 0.25 nM of each primer, and 2 µL of DNA/RNA template. Cycling conditions were as outlined in Table 3.5.

**Table 3.3.** Primer data for validation sequences.

| Organism | Sequence type | Primer sequence | Target sequence length (base pairs) |
|---|---|---|---|
| Viruses | | | |
| Bovine coronavirus | Not required | See Table 3.1 | 25 |
| Bovine herpesvirus 1 | Primer (forward)<br>Primer (reverse) | CTT TAC GGT CGA CGA CTC<br>TGC GTT GTA GAA AGC AGG CG | 360 |
| Bovine parainfluenza virus 3 | Primer (forward)<br>Primer (reverse) | AGT TGC ACT TGC TCC TCA GT<br>TGC GTT AAC ATG GAG GCT GA | 376 |
| Bovine respiratory syncytial virus | Primer (forward)<br>Primer (reverse) | GTC ACT CAA CCA GCC TGT GA<br>ACT GGG CAT TTG GGC TAG TA | 309 |
| Bovine viral diarrhoea virus | Primer (forward) *<br>Primer (reverse) * | ATG CCC WTA GTA GGA CTA GCA<br>TCA ACT CCA TGT GCC ATG TAC | 290 |
| Bacteria | | | |
| *Histophilus somni* | Primer (forward)<br>Primer (reverse) | CAG CCA CAC TGG AAC TGA<br>GCC GTA CTC CCC AGG CG | 548 |
| *Mycoplamsa bovis* | Not required | See Table 3.1 | 20 |
| *Mannheimia haemolytica* | Not required | See Table 3.1 | 28 |
| *Pasteurella multocida* | Primer (forward)<br>Primer (reverse) | CAA TAC CAG TAC GTT TTA TTA TGC AAA<br>TCG CGT AAA CGG CGT ATC | 451 |

* Primer sequences Pan BVDV 324 (forward) and Pan BVDV 326 (reverse) sourced from Vilček et al. (1994). Other sequences were designed in study using commercially available software (Primer Express).

**Table 3.4**. Cycling conditions for PCR amplification of target sequences using the Progmega PCR Master Mix.

| Step | Temperature (°C) | Time (min:sec) | Number of cycles |
|---|---|---|---|
| 1. Initial activation | 95 | 5:00 | 1 |
| 2a. Denaturation | 95 | 0:30 | |
| 2b. Annealing | 45 | 0:30 | 35 |
| 2c. Extension | 72 | 0:45 | |
| 3. Final extension | 72 | 7:00 | 1 |
| 4. Hold | 14 | | |

**Table 3.5**. Cycling conditions for PCR amplification of target sequences using the Qiagen OneStep RT-PCR Kit. Annealing temperatures: [a] BRSV; [b] BVDV; [c] BPIV-3.

| Step | Temperature (°C) | Time (min:sec) | Number of cycles |
|---|---|---|---|
| 1. Reverse transcription | 50 | 30:00 | 1 |
| 2. Initial activation | 95 | 15:00 | 1 |
| 3a. Denaturation | 94 | 0:30 | |
| 3b. Annealing | 45[a], 48[b], 50[c] | 0:30 | 35 |
| 3c. Extension | 72 | 1:00 | |
| 4. Final extension | 72 | 7:00 | 1 |
| 5. Hold | 14 | | |

The HotStar Taq *Plus* Master Mix Kit (Qiagen Inc., Valencia, CA) was used for amplification of the BoHV-1 target sequence. Reaction volumes of 20 µL consisted of 5.5 µL nuclease-free water, 10 µL 2X HotStar Taq *Plus* Master Mix, 0.25 nM of each primer, and 2 µL of DNA template. A Touchdown thermocycling protocol was run on a Bio-RAD DNA engine® thermocycler using the cycling conditions were as outlined in Table 3.6.

**Table 3.6**. Cycling conditions for PCR amplification of target sequences using the HotStar Taq *Plus* Master Mix Kit. *Annealing temperature decreases by 2°C at every second cycle.

| Step | Temperature (°C) | Time (min:sec) | Number of cycles |
|---|---|---|---|
| 1. Initial activation | 96 | 3:00 | 1 |
| 2a. Denaturation | 95 | 1:00 | |
| 2b. Annealing | 48* | 0:30 | 35 |
| 2c. Extension | 72 | 1:00 | |
| 3. Hold | 4 | | |

Polymerase chain reaction products were electrophoresed in a 1.25% agarose gel (Certified TM Molecular Biology Agrose, Bio-Rad, Hercules, CA) containing 0.01% v/v SYBR® Safe DNA gel stain (Life Technologies, Grand Island, NY) at 90 volts for 30 minutes, and viewed with an ultraviolet transilluminator.

Bands of the predicted size were excised from the gel and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Fitchburg, WI) according to the manufacturer's protocol with the following modification: the DNA was eluted by adding 30 µL of nuclease-free water to the column membrane and incubated at room temperature for 5 minutes before centrifugation.

Purified DNA was then inserted into the pCR®II-TOPO vector (Invitrogen, Life Technologies, Grand Island, NY) using reagents supplied in the TOPO® Cloning reaction kit (Invitrogen, Life Technologies, Grand Island, NY) before transformation into TOP10 competent *E. coli* cells (Invitrogen, Life Technologies, Grand Island, NY) for blue/white colony screening.

Briefly, 2 µL of ligation reaction was added to 50 µL of cells before being incubated on ice for 30 minutes. Following this, the cells were heat shocked at 42 °C for 45 seconds before being placed on ice for a further 2 minutes. Then, 250 µL SOC media (Invitrogen, Life Technologies, Grand Island, NY) was added to a final volume of 300 µL, before cells were incubated at 37 °C for 1 hour on an orbital shaker. Cells were then plated on LB+Amp plates (Luria-Bertrani medium, Difco TM LB Agar, Miller (Luria-Bertani), Becton, Dickinson and Company, Franklin Lakes, NJ) with 100 µg/mL ampicillin (Sigma-Aldrich, St. Louis, MO)) supplemented with 80 µg/mL X-Gal (Sigma-Aldrich, St. Louis, MO) and grown for 24 hours at 37 °C. White colonies containing the recombinant plasmid were then re-streaked and grown for a further 24 hours at 37 °C before being screened by PCR for the presence of the insert. A colony containing the insert was picked and grown in 5 mL of LB+Amp media overnight at 37 °C. Extraction of plasmid DNA was performed using a Qiaprep spin kit (Qiagen) according to the manufacturer's instructions.

Plasmid DNA concentration was measured using a spectrophotometer (Tecan NanoQuant, ThermoFisher Scientific, Waltham, MA) and the number of genes present in the solution calculated. Following this, the plasmid DNA solution was diluted in sterile, nuclease-free water and titrated to give a range of 10-fold dilutions from $10^{12}$ – $10^{9}$ (depending on the number of genes in the neat solution for each organism) to $10^{1}$ viral gene copies/µL.

For RNA viruses, transcripts of the region of interest were produced in order to accurately assess the sensitivity of the qPCR assay reverse transcription and PCR steps. Following confirmation that the PCR products were cloned correctly into the pCR®II-TOPO vector downstream of the SP6 promotor region, transcripts were then produced using the MAXIscript® SP6 *in-vitro* transcription kit (Ambion, Austin, TX) according to the manufacturer's instructions. RNA was then quantified and standards diluted as for the DNA viruses and bacteria.

### 3.3.5 Test validation

To determine the sensitivity of the assays serial dilutions of plasmid or transcript standards were tested using the standard qPCR assay. For duplex assays $10^{10}$ plasmid standard solutions for each organism in the duplex were mixed, and the resulting solution diluted to $10^{1}$ gene copies/uL. Less than 40 cycles of amplification (Ct < 40) was used as the cut-off for positivity and the threshold was set at 0.1. A standard curve using 10-fold dilutions of positive control material was generated to allow determination of reaction efficiency and accuracy.

## 3.4 Results

The qPCR assays were shown to have detection limits of <10 up to 200 copies of the target gene (Table 3.7). Standard curves for each organism can be found in Figures 3.1-3.3 below.

**Table 3.7**. Standard curve plot data.

| Organism | Slope | y-intercept | Min. gene copies |
|---|---|---|---|
| Viruses | | | |
| Bovine coronavirus | -3.4 | 45 | 20 |
| Bovine parainfluenza virus 3 | -3.6 | 41 | <10 |
| Bovine respiratory syncytial virus | -3.5 | 48 | 100-200 |
| Bovine viral diarrhoea virus | -2.9 | 47 | 100-200 |
| Bovine herpesvirus 1 | -3.2 | 40 | <10 |
| Bacteria | | | |
| *Histophilus somni* | -3.6 | 40 | <10 |
| *Mycoplamsa bovis* | -3.4 | 40 | <10 |
| *Mannheimia haemolytica* | -3.4 | 38 | <10 |
| *Pasteurella multocida* | -2.9 | 41 | <10 |

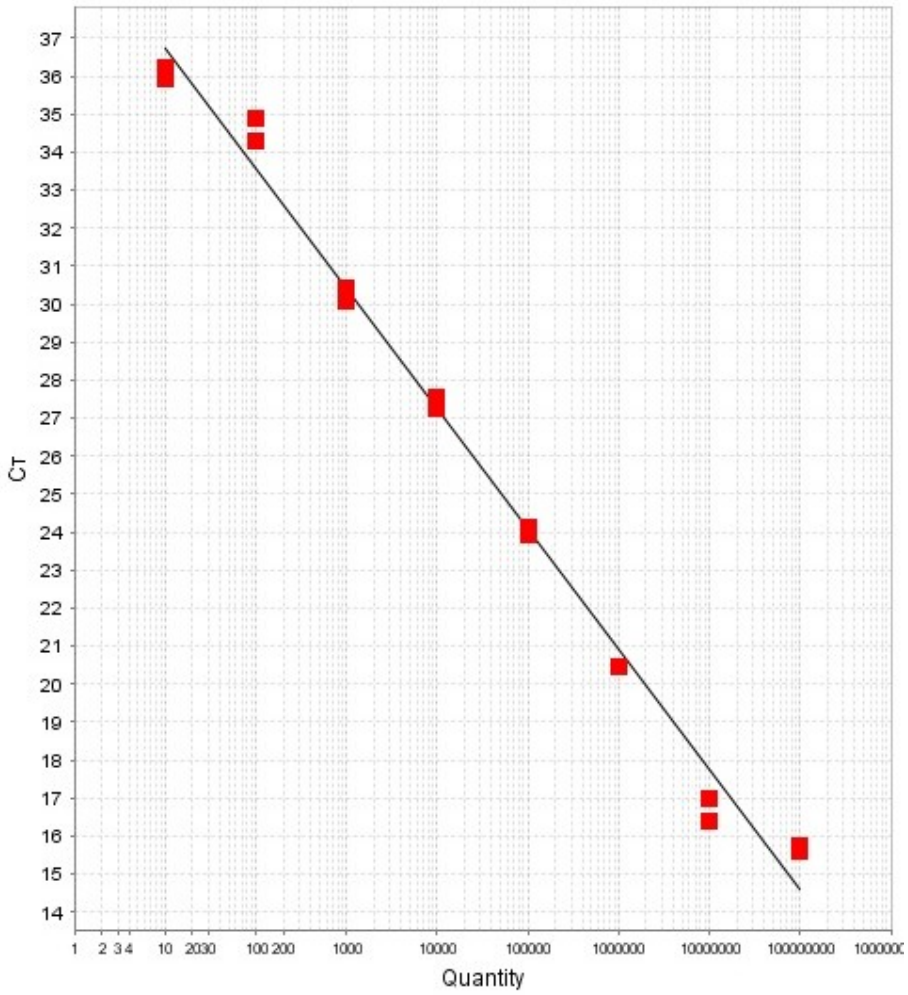


**Figure 3.1**. Standard curve for Bovine herpesvirus 1. CT, cycle threshold; Quantity, number of gene copies.

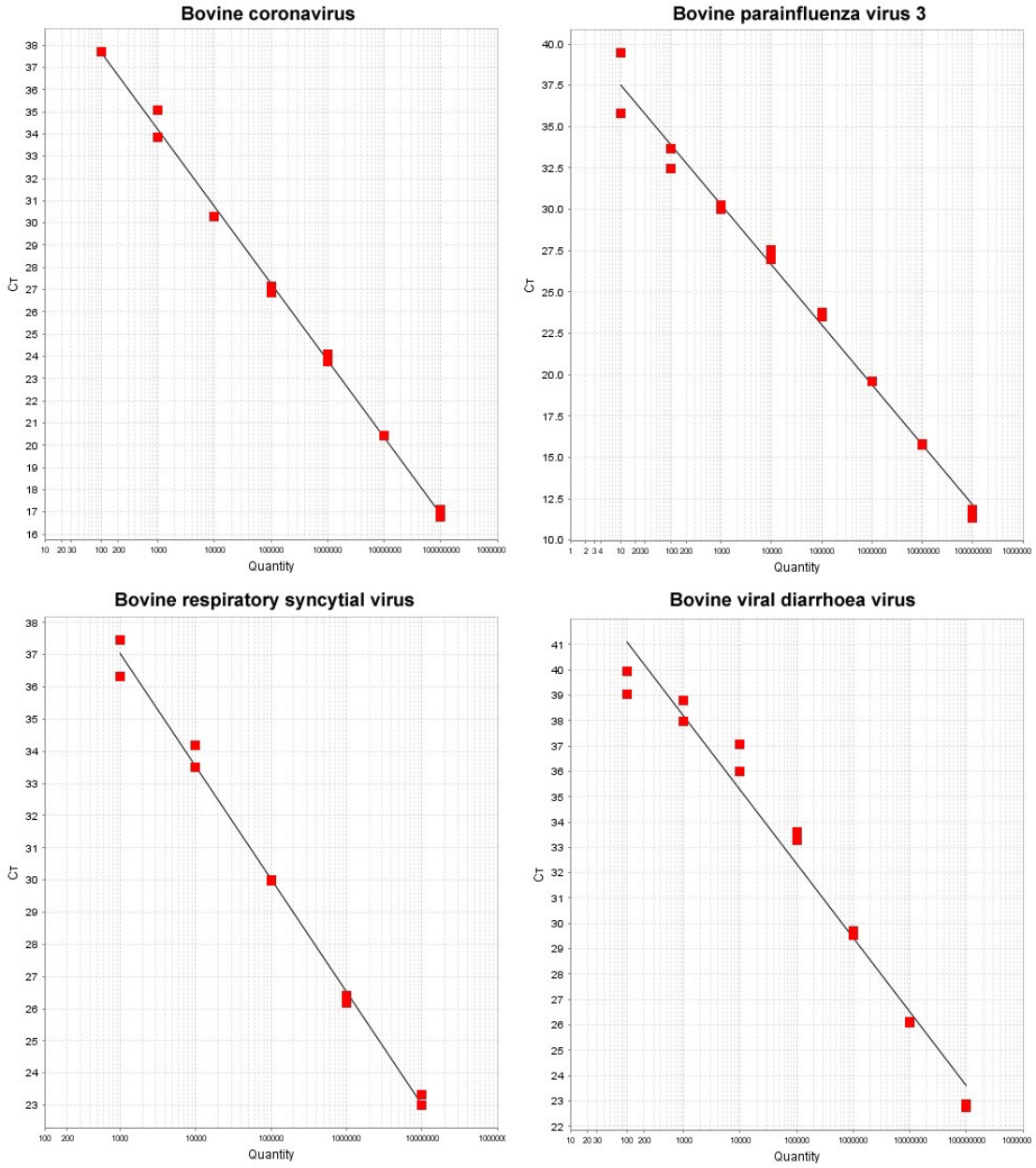


**Figure 3.2**. Standard curves for RNA viruses. CT, cycle threshold; Quantity, number of gene copies.

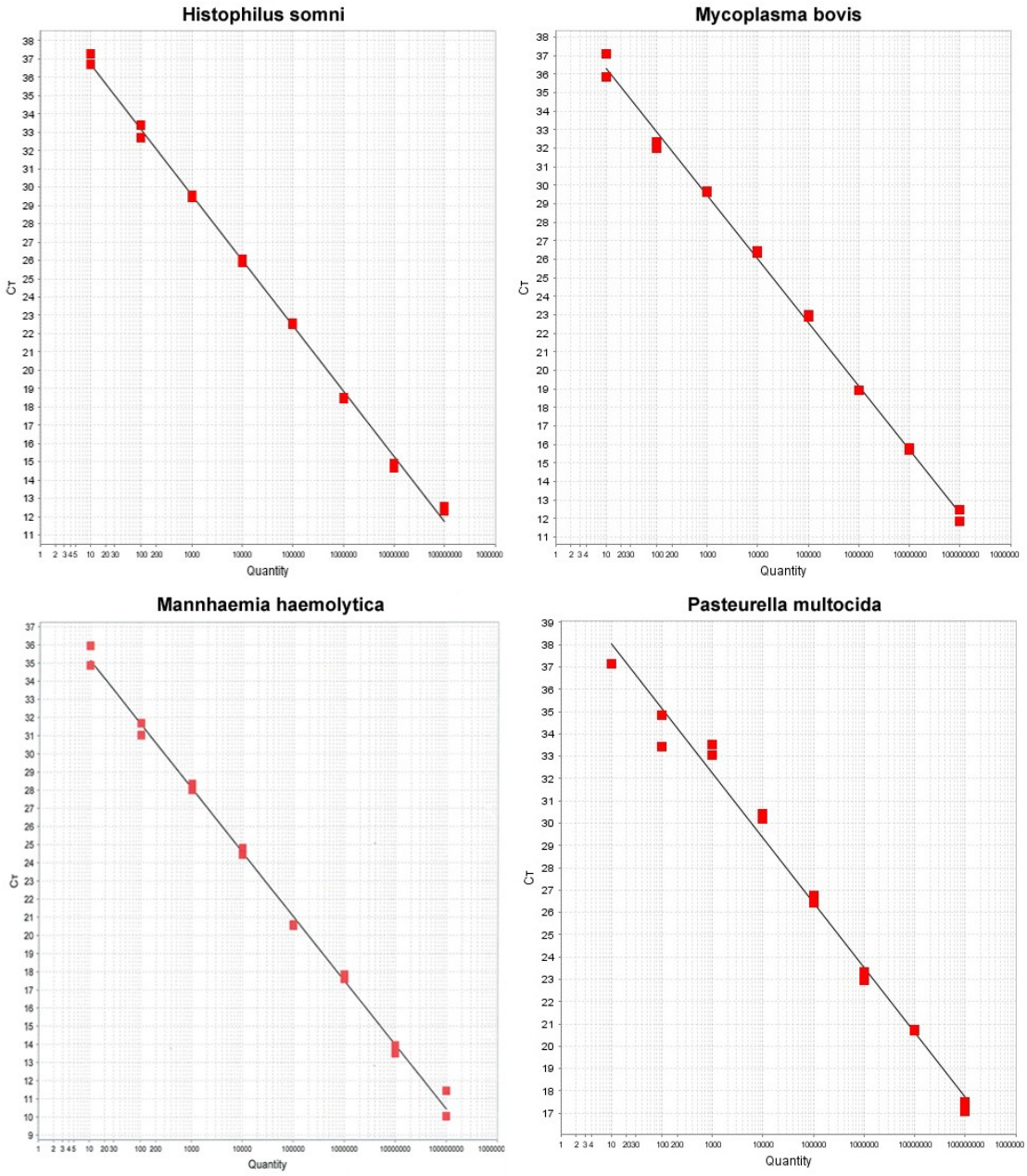


**Figure 3.3.** Standard curves for bacteria. CT, cycle number; Quantity, number of gene copies.

## 3.5 Discussion

Historically the preferred method of detection and identification of bacteria and viruses has been culture – on culture media in the case of bacteria, and in cell lines for viruses – followed by tests to identify the organism(s) grown. Results from such testing may take days to weeks to become available but multiple organisms can be detected from a single culture (Fulton and Confer, 2012). More recently polymerase chain reaction (PCR) based assays have been used to detect nucleic acids from organisms of interest. Molecular tests have a more rapid turn-around than culture and, since they are not dependent on the presence of live organisms, are more robust in the face of suboptimal sample handling and storage. However, due to the targeted nature of PCR-based tests, i.e. each PCR detects a specific nucleic acid sequence from a particular bacteria or virus, organism detection is limited by test selection (Fulton and Confer, 2012). In BRD studies PCR based assays have been used with (Fulton et al., 2011; Hanzlicek et al., 2011; White et al., 2010; Wiggins et al., 2007) or without (Decaro et al., 2008a; Rifatbegovic et al., 2007; Wiggins et al., 2007) prior culture.

It can also be difficult to determine the importance of infectious agents detected in tissue samples/swabs using qPCR alone. Because immunohistochemistry (IHC) or *in situ* hybridisation (ISH) can be used to localise an infectious agent within a tissue lesion, these techniques can facilitate a more accurate interpretation of the association between the infectious agent and disease than PCR alone (Fulton and Confer, 2012).

During the study design phase of this project both IHC and ISH were considered for detection of infectious agents in fixed tissue sections. However, for good ISH and IHC results it is generally recommended that tissue samples are fixed in formalin for no more than 24 hours before being embedded in paraffin. This is because prolonged fixation can decrease signal

intensity and adversely affect antibody binding (Kadkol et al., 1999; Taylor, 2011). Because samples collected during voyages could remain in unrefreshed fixative for weeks to months before returning to Australia for processing there was a risk that using IHC and/or ISH to identify and demonstrate the presence of infectious organisms in those samples would be unsuccessful.

Early in the project, in response to feedback from industry stakeholders, the project remit was expanded to include investigation of pre-export risk factors. Therefore available resources were redirected away from the development, optimisation and validation of IHC and ISH protocols in favour of qPCR test development as this would allow investigation of the presence of disease causing organisms both before and during voyages, which could not be done as easily using IHC or ISH. However, fixed tissues collected during this project are available to attempt IHC or ISH techniques to demonstrate viral and/or bacterial antigens/nucleic acids in lesional tissue in the future if required.

Multiplex qPCR refers to the simultaneous amplification of two or more DNA or cDNA targets in a single reaction and detection using a uniquely labelled fluorophore probe for each target (Vet et al., 1999). Performing assays in multiplex instead of singleplex has a number of benefits including reductions in time, cost, and the volume of template RNA/DNA required (reviewed in (Mackay, 2004)). In this study, development of a triplex assay to detect BoHV-1, BVDV and BPIV-3 proved unrewarding. This may have been due to interactions between primer pairs. Primer-dimer interactions were investigated using commercially available software (PrimerExpress software v2.0, Applied Biosystems, Foster City, CA) and although some interactions were present, they were not considered significant. A number of different qPCR mastermix kits were tried, e.g. an in-house general mastermix and a commercial multiplex

mastermix, but no difference in performance was observed. Therefore, due to time and cost constraints, duplex assays were developed instead.

During the test validation phase, attempts to amplify BoHV-1 DNA from control material were not successful, even though this sample tested positive using the diagnostic qPCR. Therefore, a Touchdown PCR protocol was used instead of a standard thermocycling protocol. Touchdown PCRs are typically used to improve specificity by limiting non-specific amplification but can also increase yields in suboptimal reactions (Hecker and Roux, 1996).

Also during the test validation phase, repeated attempts to clone target sequences generated by the qPCR assays into chemically competent E. coli were not successful. It was hypothesized that this may have been due to the relatively small size of the target sequences (17-30 base pairs, Table 3.1) resulting in failure of the target sequence to clone into the plasmid vector. Therefore, additional primers were designed that contained the qPCR target sequences listed in Table 3.1, but with an overall target sequence length of 290-548 base pairs (Table 3.3). Cloning and transformation using the larger target sequences was successful.

The reaction efficiency of optimized qPCR assays should be 90-105% which is equivalent to a standard curve slope of between -3.1 and -3.6. The reaction efficiencies for the majority of assays developed in this study were within the recommended range. The higher reaction efficiencies observed for BVDV and *P. multocida* assays may be due to pipetting error or coamplification of non-specific products, e.g. primer-dimers. However, the performance of all tests were considered acceptable for the requirements of the project.

Tests for BoHV-1, BPIV-3, *M. bovis*, *M. haemolytica*, *P. multocida* and *H. somni* showed a detection limit (analytical sensitivity) of less than 10 viral/bacterial gene copies, while tests for

BCoV, BRSV and BVDV showed detection limits of 20, and 100-200 copies respectively. The lower analytical sensitivity of BCoV, BRSV and BVDV assays may be due to interactions between primers pairs in the duplex. However, the analytical sensitivity of these two assays would still be higher than virus isolation or antigen capture ELISA for detection of viral genomes (Horner et al., 1995). Overall, these tests still provide a sensitive, rapid tool for detection of nucleic acids from viruses and bacteria in nasal and tissue swab samples which can be used to further assess respiratory pathogens in cattle.

# Chapter 4: Mortality of live export cattle on long-haul voyages: pathological changes and pathogens

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## 4.1 Summary

The cause of death in 215 cattle on 20 long-haul live export voyages from Australia to the Middle East, Russia, and China was investigated between 2010 and 2012 using gross, histological, and/or molecular pathology techniques. A quantitative polymerase chain reaction (qPCR) assay was used to detect nucleic acids from viruses and bacteria known to be associated with respiratory disease in cattle: Bovine coronavirus (Betacoronavirus 1), Bovine herpesvirus 1, Bovine viral diarrhoea virus, Bovine respiratory syncytial virus, Bovine parainfluenza virus 3, *Histophilus somni*, *Mycoplasma bovis*, *Mannheimia haemolytica*, and *Pasteurella multocida*.

The most commonly diagnosed cause of death was respiratory disease (107/180, 59.4%), followed by lameness (*n* = 22, 12.2%), ketosis (*n* = 12, 6.7%), septicaemia (*n* = 11, 6.1%), and enteric disease (*n* = 10, 5.6%). Two-thirds (130/195) of animals from which lung samples were collected had histological changes and/or positive qPCR results indicative of infectious lung disease: 93 out of 130 (72%) had evidence of bacterial infection, 4 (3%) had viral infection, 29 (22%) had mixed bacterial and viral infections, and for 4 (3%) the causative organism could not be identified. Bovine coronavirus was detected in up to 13% of cattle tested, and this finding is likely to have important implications for the management and treatment of respiratory disease in live export cattle.

Results from the current study indicate that although overall mortality during live export voyages is low, further research into risk factors for developing respiratory disease is required.

## 4.2 Introduction

Since the late 1990s, Australia has exported 600,000–985,000 live cattle each year to countries around the globe. Between 2010 and 2012, approximately 65% of cattle have been exported to countries in Southeast Asia and the Pacific with approximately 90% of these destined for Indonesia. Approximately 15% of cattle are exported to countries in the Middle East and North Africa, 10% to Asia (including China, Japan, Pakistan, and India), and 10% to countries near the Black Sea (Turkey and the Russian Federation) (LiveCorp, 2012).

Between 2000 and 2011, the average mortality percentage per voyage in cattle exported live from Australia was 0.14% (DAFF, 2014). However, due to the large numbers of animals exported each year and the relatively high value of each animal (approximately AUD $1,000 per head in 2012 (MLA, 2012a)), even low mortality percentages can have a significant economic impact.

Mortality rates and animal welfare are closely linked; increased mortality is often interpreted as an indicator of poor welfare (Knowles and Warriss, 2007). Increased public awareness and concern for animal welfare issues creates ongoing pressure on the live export industry to demonstrate that it is actively committed to minimizing mortality rates and improving animal welfare. Previous studies on cattle deaths during sea transport from Australia to the Middle East (Norris et al., 2003) and Australia to Japan (Hedlefs, 1988) identified heat stroke, trauma and/or lameness, and respiratory disease as the 3 most common causes of mortality. Heat stress is a recognized problem on export vessels for a number of reasons including stocking densities on board the ship, high ambient temperature and humidity particularly in the Equatorial region and in waters around the Arabian peninsula (Gulf of Aden, Red Sea, Gulf of

Oman, Persian Sea), and reliance on artificial ventilation especially on closed decks (Norris et al., 2003).

Bovine respiratory disease (BRD) has a multifactorial, polymicrobial etiology. Organisms known to be associated with BRD include viruses and bacteria: Bovine coronavirus (BCoV; Betacoronavirus 1), Bovine parainfluenza 3 virus (BPIV-3), Bovine herpesvirus 1 (BoHV-1), Bovine respiratory syncytial virus (BRSV), Bovine viral diarrhoea virus (BVDV), *Arcanobacterium pyogenes*, and *Bibersteinia* (formerly *Pasteurella*) *trehalose*, *Histophilus somni*, *Mycoplasma bovis*, *Mannheimia haemolytica*, and *Pasteurella multocida* (reviewed in (Panciera and Confer, 2010)). In an effort to reduce the risk of cattle developing BRD during a voyage, cattle are often vaccinated against specific respiratory disease pathogens and/or treated with broad spectrum antibiotics prior to being loaded onto the vessel, and may be treated again before they reach their destination. These treatments may be required by the importing country or, with regards to antibiotics, applied at the exporter's discretion to animals that they believe are at greater risk of developing BRD. During a voyage, treatments may be administered to animals showing clinical signs of disease or to animals considered at risk of developing disease (e.g., pen mates of sick animals). There is little data to document unequivocally whether any of these practices are effective at reducing morbidity or mortality.

The current study was initiated to determine the causes of mortality in live export cattle on long-haul voyages from Australia, based on necropsy reports and samples collected from study voyages. In addition, the study aimed to identify infectious agents involved in BRD in Australian live export cattle to inform on future preventative treatment and management strategies.

## 4.3 Materials and methods

Twenty eligible voyages were enrolled during the period March 2010 to September 2012. Eligible voyages were defined as those loading cattle in Australia for a long-haul voyage (≥10 days) to destinations including the Middle East (Israel, Saudi Arabia, Bahrain, Turkey), North Africa (Egypt, Libya), and the Russian Federation, and carrying at least 1,000 head of cattle.

### 4.3.1 Data and sample collection

For 19 out of 20 voyages, data and sample collection was performed by the Australian Quarantine and Inspection Service (AQIS; now DAFF Biosecurity) Accredited Veterinarian (AA Vet) accompanying the voyage. A standardized protocol was provided, and all participating AA Vets received training in the data and sample collection protocols. Necropsy equipment, containers for collecting samples, and paper or electronic templates for recording observations were all provided on board each ship. The AA Vets accompanying the voyages were encouraged to collect a defined set of data and samples from each animal that died, but this was not always possible due to the demands of other tasks and duties that they are routinely expected to perform during voyages. For 1 out of the 20 voyages, data and sample collection was performed by a member of the research team (SJ Moore).

Standardized data recording forms were used to collect animal and epidemiological data for each animal that died. Data included the animal's location on the ship, animal characteristics (including visual ear tag, electronic identification tag, breed, and weight), clinical signs displayed before death, risk factors or events that may have contributed to death, gross necropsy findings if available, and a preliminary cause of death.

Necropsy samples included fresh tissue samples collected into 10% buffered formalin at a maximum ratio of 1 part tissue to 10 parts formalin, and tissue samples (approximately 5 $mm^2$)

and/or swabs collected into a 5 ml plastic screw top container filled with 2 ml of viral transport media (VTM; Hanks balanced salt solution, penicillin G [1,000 units/ml], streptomycin [25 mg/ml], and amphotericin B [0.1 mg/ml]; Department of Agriculture and Food, Western Australia) and frozen.

The number and type of samples collected at necropsy depended on the animal's clinical signs prior to death and gross necropsy findings. Core samples collected from all animals were: lung (grossly normal and abnormal), trachea, heart, ileocecal junction, kidney, liver, and rumen into 10% buffered formalin, and nasal and lung swabs. When the animal showed clinical signs prior to death which were suggestive of a specific disease and which was confirmed at necropsy, then a range of additional samples were collected according to the suspected disease. When there was no obvious cause of morbidity/mortality and the cause of death could not be determined from gross necropsy findings then the core samples plus fixed skeletal muscle, reticulum, abomasum, small intestine, large intestine, pancreas, mesenteric lymph node, gall bladder, spleen, adrenal gland, and the brainstem and cervical spinal cord were collected into 10% buffered formalin.

Samples and data collection forms remained on the ship until the next time it berthed in Fremantle (Western Australia). Forms and samples were removed from the ship and taken directly to the quarantine approved premises at the Department of Agriculture and Food, Western Australia, for processing.

### 4.3.2 Processing of fixed tissues

Representative samples were taken from each fixed tissue and processed routinely for embedding in paraffin wax. Histological sections were cut at 5 µm, stained with hematoxylin and eosin, and examined for pathological changes using light microscopy.

### 4.3.3 Processing of samples in VTM

Tubes containing samples in VTM were thawed, vortexed, and allowed to settle for approximately 15 min. A 200-µl aliquot of VTM was taken and placed into a 96-well plate (Greiner Bio-One GmbH, Hannover, Germany). Both the original sample tubes and 96-well plates were stored at –80°C until required. Nucleic acid extraction from VTM aliquots was performed using a commercial kit (Ambion MagMax Viral RNA Extraction Kit, Life Technologies, Grand Island, NY) on a magnetic particle processor (MagMax Express 96 magnetic particle processor, Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Plates containing the eluate were stored at –80°C until required.

### 4.3.4 Detection of microbial nucleic acids

Quantitative polymerase chain reaction (qPCR) was used to detect nucleic acids from organisms commonly associated with BRD (Panciera and Confer, 2010). The qPCR was performed using a commercial kit (QuantiTect virus +ROX vial kit, Qiagen Inc., Valencia, CA) according to the manufacturer's instructions.

Primer and probe sequences were sourced from available sequences for BCoV (data not shown), BoHV-1 (Lovato et al., 2003), BPIV-3 (Horwood and Mahony, 2011), BRSV (Mahony and Horwood, 2006), BVDV (Horwood and Mahony, 2011), and the following 3 bacteria (Mahony and Horwood, 2006): *M. haemolytica*, *P. multocida*, and *H. somni*. *Mycoplasma bovis* primer sequences were sourced from a previous study (Clothier et al., 2010), and the probe sequence was designed using commercially available software (Primer Express software v2.0, Applied Biosystems, Foster City, CA). Primer and probe sequences can be found in Supplementary Table 4.1. To reduce the number of tests, required reactions were multiplexed as follows: BCoV and

BPIV-3, BRSV and BVDV, *M. haemolytica* and *P. multocida*, *H. somni* and *M. bovis*. *Bovine herpesvirus 1* was run as a single assay.

The final optimized multiplex qPCR assays were as follows. All assays consisted of a 20-µl reaction mix containing 2 µl of the nucleic acid sample, 4 µl of proprietary master mix, 0.4 µl of ROX (5-carboxy-X-rhodamine) dye solution, and 0.2 µl of RT mix (QuantiTect virus +ROX vial kit, Qiagen Inc., Valencia, CA). Primer and probe concentrations varied according to the assay. For BoHV-1, BCoV and BPIV-3, BRSV and BVDV, and *M. haemolytica* and *P. multocida*, 400 nM of each primer and 200 nM of probe were used. For *H. somni* and *M. bovis*, 100 nM of each *H. somni* primer and 50 nM of probe were used, and 400 nM of each *M. bovis* primer and 200 nM of probe. The cycling parameters were as follows: 50°C for 20 min, 95°C for 5 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 45 sec.

Each run contained duplicate samples of a positive control (either a virus isolate or a clinical extract that had previously been characterized), a negative control (an extract of cell culture–grade fetal bovine serum), and a blank (PCR-grade water). Runs were only considered valid if the positive control was amplified at the expected threshold cycle, and the negative and blank controls showed no amplification.

### 4.3.5 Interpretation of qPCR results

Positive samples were those with a characteristic sigmoidal curve similar to the positive control, crossing the threshold before 40 cycles. Negative samples were those with no characteristic sigmoidal curve. Samples crossing the threshold after 40 cycles were regarded as suspicious for containing the nucleic acid of interest and were retested before classifying as negative or positive.

### 4.3.6 Determining the cause of death

The final diagnosis for each animal was based on the combination of all available data: description of premortem clinical signs, gross necropsy findings, histology results, and qPCR results. Not all data was available for all animals. Respiratory disease was considered the cause of death if there was cranioventral consolidation of more than 30% of the lung (Gagea et al., 2006b) and/or the AA Vet had assigned a preliminary diagnosis of pneumonia/BRD and there were histological findings of moderate to severe pneumonia in lung tissue.

Heat stroke was considered the cause of death if the animal was noted to be panting heavily prior to death or was found dead with no previous clinical signs, the core body temperature (CBT) was ≥43°C (measured using a deep temperature probe), and necropsy findings were consistent with those described previously for heat stroke in live export cattle (Norris et al., 2003): eyes sunken, muscles glowing pink and dry rather than red-brown and moist, heart tightly contracted with epicardial ecchymoses, severe acute diffuse pulmonary congestion, and edema causing the lungs and mucosa of the trachea and bronchi to be dark red. Ambient environmental temperatures were not recorded.

### 4.3.7 Statistical analysis

The statistical software package R was used for data analyses (R Development Core Team, 2014). Mortality percentages were calculated as the cumulative incidence of deaths during the voyage with a denominator representing the total count of cattle loaded onto the ship at the port(s) of loading, and a numerator representing the count of deaths observed for the entire voyage (from first day of loading to last day of discharge). Voyage mortality rates were estimated using Poisson regression with models incorporating an offset based on total cattle-days at risk during the voyage to adjust for variation in numbers of cattle and voyage duration.

Poisson modeling was used to screen for associations between mortality rate and relevant explanatory variables (Supplementary Table 4.2). Due to sparseness of the data and the presence of confounding between many explanatory variables, Poisson models were run as univariable screening models only to assess for associations between explanatory variables and voyage mortality rate. Additional analyses were conducted using linear regression for continuous outcomes (core body temperature, necropsy sampling rate, severity of myocardial degeneration) and logistic regression for binary outcomes (pathogen prevalence in nasal or lung samples) to explore associations with various explanatory variables, including cause of death, that were present in the dataset. Spearman rank correlation test was used to test for correlations between the presence of various organisms, as detected by qPCR, in the nasal secretions and lung. A threshold of $P = 0.05$ was used for all statistical analyses.

## 4.4 Results

### 4.4.1 Voyage data

During the study period of March 2010 to June 2012, there were 85 voyages to the Middle East and Russian Federation of which 59 were carrying more than 1,000 head of cattle. Most voyages took place in the autumn (17/59, 29%) with approximately equal numbers during the spring ($n = 14$), summer ($n = 13$), and winter ($n = 15$). The average total voyage length for the 85 voyages during the study period was 25.3 ± 6.8 days (sailing plus discharge, expressed as mean ± standard error).

Necropsy samples were collected from 20 out of 59 (34%) eligible voyages during the study period (Table 4.1). The majority of study voyages took place in the southern hemisphere in autumn (*n* = 8 voyages) and winter (*n* = 5) with fewer voyages in summer (*n* = 4) and spring (*n* = 3). Seven ships were used to carry livestock with each ship making 1–5 voyages over the study period. The average total voyage length for the 20 eligible voyages was 27.4 ± 7.0 days.

Cattle were loaded out of 4 ports across 3 states: Fremantle and Broome in Western Australia, Portland in Victoria, and Adelaide in South Australia. The majority of cattle were loaded out of the southern port of Fremantle (Table 4.2). Cattle from southern ports (Portland, Adelaide, Fremantle) tend to be primarily of the *Bos taurus* subspecies while those from the northern port (Broome) are primarily *Bos indicus* (DAFWA, 2009).

Cattle were loaded out of a single port for 10 voyages while split loadings were used on the remaining 10 voyages. For split loadings, cattle were loaded at Broome, Portland, or Adelaide and then the ship sailed 4–5 days to Fremantle where additional cattle were loaded. All voyages out of Adelaide were split loaded while the remaining ports had a mixture of single and split loadings. The total number of cattle on a fully loaded ship varied from 3,994 to 19,990 head (Table 4.1).

With the exception of a single voyage, cattle were discharged at ports in 2 broad geographical zones: the Middle East and North Africa including Israel, Saudi Arabia, Bahrain, Libya, and Egypt, and Turkey and the western Russian Federation (Table 4.2). Turkey was the primary discharge country taking 54% of animals. Israel accounted for approximately 14%, the Russian Federation for 9%, and Saudi Arabia and Egypt for 7% each (Table 4.2).

**Table 4.1.** Voyage data. Voyage number 18 is not included in this table because it was not relevant to this phase of the study.

| Voyage code | Ship code | Voyage year | Season | Load port(s) | Discharge country(s) | Voyage length (days)* | Total cattle loaded | Mortality rate (%) | Sampling rate (%) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | A | 2010 | Autumn | Fremantle | Israel | 22 | 9,430 | 0.17 | 100.0 |
| 2 | A | 2010 | Autumn | Fremantle | Libya | 23 | 9,213 | 0.14 | 61.5 |
| 3 | B | 2010 | Winter | Broome | Egypt | 18 | 5,090 | 0.08 | 75.0 |
| 4 | C | 2010 | Spring | Fremantle | Saudi Arabia | 19 | 10,428 | 0.11 | 72.7 |
| 5 | C | 2010 | Summer | Fremantle | Turkey | 24 | 19,990 | 0.74 | 8.1 |
| 6 | D | 2010 | Summer | Portland | Russian Federation | 31 | 3,994 | 0.53 | 66.7 |
| 7 | E | 2010 | Summer | Fremantle | Turkey | 35 | 16,255 | 0.15 | 32.0 |
| 8 | C | 2011 | Autumn | Portland, Fremantle | Saudi Arabia, Turkey | 35 | 17,484 | 0.30 | 90.4 |
| 9 | F | 2011 | Autumn | Fremantle | Turkey | 42 | 10,237 | 0.59 | 8.3 |
| 10 | G | 2011 | Autumn | Portland, Fremantle | Russian Federation | 35 | 12,763 | 0.42 | 9.3 |
| 11 | E | 2011 | Winter | Portland, Fremantle | Turkey | 37 | 9,000 | 1.18 | 3.8 |
| 12 | C | 2011 | Winter | Broome, Fremantle | Egypt | 23 | 9,239 | 0.29 | 88.9 |
| 13 | B | 2011 | Winter | Broome, Fremantle | Bahrain | 20 | 1,350 | 0.44 | 83.3 |
| 14 | A | 2011 | Spring | Adelaide, Fremantle | Turkey | 34 | 4,274 | 0.35 | 26.7 |
| 15 | E | 2011 | Spring | Adelaide, Fremantle | Turkey | 31 | 12,256 | 0.50 | 27.9 |
| 16 | F | 2011 | Summer | Fremantle | Israel | 22 | 9,811 | 0.17 | 29.4 |
| 17 | A | 2012 | Autumn | Adelaide, Fremantle | Turkey | 34 | 7,811 | 0.50 | 28.2 |
| 19 | B | 2012 | Autumn | Portland | China | 18 | 5,880 | 0.14 | 25.0 |
| 20 | E | 2012 | Autumn | Adelaide, Fremantle | Turkey | 36 | 11,538 | 0.32 | 5.4 |
| 21 | A | 2012 | Winter | Adelaide, Fremantle | Israel | 30 | 8,209 | 0.26 | 19.0 |

* Voyage length includes sailing and discharge days.

**Table 4.2.** Loading and discharge data, including the type of loading (single vs. split) and the number of cattle loaded and discharged at each port.

| Load type | Load port (no. of cattle) | | | | |
|---|---|---|---|---|---|
| | **Fremantle** | **Portland** | **Adelaide** | **Broome** | **Total voyages** |
| Single port loading | 7 | 2 | | 1 | 9 |
| Split loading | 10 | 3 | 5 | 2 | 11 |
| Total cattle | 127,686 | 31,017 | 20,735 | 14,814 | |
| Discharge port | | | | | |
| Turkey | 76,732 | 9,949 | 19,158 | | 105,839 |
| Saudi Arabia | 13,434 | | | | 13,434 |
| Israel | 25,873 | | 1,577 | | 27,450 |
| Russian Federation | 1,569 | 15,188 | | | 16,757 |
| Egypt | 406 | | | 13,923 | 14,329 |
| Libya | 9,213 | | | | 9,213 |
| China | | 5,880 | | | 5,880 |
| Bahrain | 459 | | | 891 | 1,350 |
| Total head | | | | | 194,252 |

### 4.4.2 Mortality

The average voyage mortality percentage was 0.37% (range: 0.08–1.18%). With the exception of voyage 11, the mortality percentages for all study voyages were below the reportable level of 1% (DAFF, 2011) (Table 4.1). The average mortality rate derived from Poisson regression modeling was 0.125 deaths per thousand cattle-days (95% confidence interval [CI]: 0.121–0.139). Mortality rates were significantly associated with voyage ($P < 0.001$) and the risk of mortality was higher in summer and winter than in spring and autumn ($P < 0.001$).

No significant differences were detected between mortality rate for single versus split loadings ($P = 0.175$) or loadings out of southern (Fremantle, Adelaide, Portland) versus northern (Broome) ports ($P = 0.651$). The mortality rate for voyages loading out of eastern ports (Adelaide, Portland) was significantly higher than western ports (Fremantle, Broome; $P < 0.01$)

and this was because the mortality rate for voyages loading out of Portland was significantly higher than all other ports of loading ($P < 0.01$).

The mortality rate for voyages discharging in Turkey and the Russian Federation was significantly higher than for countries in the Middle East and North Africa (Bahrain, Egypt, Israel, Libya, Saudi Arabia) or China ($P < 0.001$). The mortality rate for split discharges was lower than for single port discharges (relative risk = 0.69, 95% CI: 0.58–0.82, $P < 0.001$).

### 4.4.3 Clinical presentation

Necropsy reports and/or fixed tissue samples were received from 215 of the 741 deaths (29%) that occurred on the study voyages. Animal data, a description of clinical signs, a description of necropsy findings, and/or fixed or frozen tissue were not received from the remaining 526 animals so these animals were not included in the study. Of the 215 animals, 135 (63%) were recorded as found dead and 57 (26%) as euthanased. The circumstances of death were not recorded for the remaining 23 (11%; Table 4.3).

Clinical signs recorded by the AA Vets were grouped into 8 clinical categories: no previous clinical signs, lameness, weakness and/or recumbency, ill-thrift and/or shy feeders, respiratory signs, neurological signs, misadventure, and miscellaneous. Of the 134 animals that were found dead and for which clinical data was available, 79 (59%) were recorded as having shown no previous clinical signs. Of the remaining 55 animals, 50 animals had been hospitalized, and the most common reasons for this were ill-thrift and/or shy feeders (32%), respiratory signs (22%), lameness (20%), and weakness and/or recumbency (16%; Table 4.3). Five were found dead with their head stuck under a pen rail (misadventure) and were assumed to have died from circulatory collapse and asphyxiation as a result of being cast.

**Table 4.3.** Clinical category and circumstance of death for animals from which epidemiological data and/or necropsy samples were received.

| Clinical category | Found dead | Euthanased | Not recorded | Total |
|---|---|---|---|---|
| Lameness | 10 | 19 | 2 | 31 |
| Recumbency/Weakness | 8 | 20 | 3 | 31 |
| Ill-thrift/Shy feeder | 16 | 5 | 2 | 23 |
| Respiratory signs | 11 | 2 | 2 | 15 |
| Neurological signs | 3 | 7 | | 10 |
| Misadventure | 5 | 3 | 0 | 8 |
| Sudden death | 1 | | | 1 |
| Urinary signs | 1 | | | 1 |
| Pink eye | | 1 | | 1 |
| No previous clinical signs | 79 | | | 79 |
| Column total | 134 | 57 | 9 | 200 |
| | | | | |
| No necropsy report | | | 13 | 13 |
| Not recorded | 1 | | 1 | 2 |
| Overall column total | 135 | 57 | 23 | 215 |

Fifty-seven animals were euthanased, 20 (35.1%) of these for weakness and/or recumbency and 19 (33.3%) for lameness that was not responsive to treatment (Table 4.3). Seven animals were recorded as having neurological signs prior to euthanasia. Two animals presented with respiratory signs that were not responsive to treatment. Of the 2 animals euthanased due to misadventure, 1 went down in the race during discharge and would not stand up again, and another was found trapped in a gate. Four animals that had been sedated for treatment of leg wounds failed to recover from the anaesthetic.

### 4.4.4 Gross pathology

Gross necropsies were performed on 197 of the 741 deaths (27%) that occurred on the study voyages, representing 92% of the 215 animals that formed the study population.

Core body temperature was measured using a deep probe thermometer in 97 animals across 14 voyages. Twelve animals were found to be hyperthermic (CBT ≥43°C) but only 2 animals were assigned a final diagnosis of primary hyperthermia (i.e., heat stroke). Hyperthermia secondary to moderate-to-severe respiratory disease was identified in 9 animals. One case was found dead with no previous clinical signs and no evidence of respiratory or other disease, including heat stroke, could be found. Overall, there was a significant association between CBT and time since death ($P < 0.001$) and this was found to be due to a drop in CBT at between 14 and 16 hr after death.

Gross pathology indicative of respiratory disease was confirmed in 12 (80%) of the 15 animals that were recorded as having clinical signs of respiratory disease prior to death. Two animals (13.3%) were found to have cardiovascular disease – 1 with traumatic reticuloperitonitis (hardware disease) and 1 with severe restrictive pericarditis. One animal had an impacted abomasum.

Respiratory disease was the most common necropsy diagnosis for animals presenting with no previous clinical signs (54/79, 68%), ill-thrift and/or shy feeders (13/23, 57%), or weakness and/or recumbency (13/31, 42%). Approximately 10% (13/31) of animals presenting with lameness and 12.5% (1/8) of those that died from misadventure were found to have concurrent respiratory disease at necropsy.

Data on the presence and gross distribution of lung lesions was available for 157 out of 197 cases. No significant lesions were seen in 35 out of 157 (22.3%) cases. Of the animals with grossly visible lung pathology, diffuse discoloration and/or consolidation was seen in the cranioventral lobes only in 85 out of 122 (69.7%) cases and all visible lung in 25 (20.5%) cases.

Multifocal lesions were present in 8 (6.6%) animals, focal lesions in 3 (2.5%) animals, and a single animal showed both cranioventral and multifocal lesions.

A gross necropsy diagnosis of inappetence was recorded for 7 out of 22 (31.8%) animals in the ill-thrift and/or shy feeders category and 5 out of 31 (16.1%) of animals presenting with weakness or recumbency. Inflammation of joints and muscle was found in 17 out of 30 (56.7%) animals presenting with lameness, and 3 of these also had histological lesions consistent with septicemia.

### 4.4.5 Histology

On average, necropsy samples were collected from 43.1% (range: 3.8–100%) of the animals that died on each study voyage. The sampling rate was found to vary with time since the start of the project ($P < 0.05$) and the total number of deaths that occurred during the voyage ($P < 0.05$). Sampling rates for voyages at the beginning of the project were generally higher than at the end. The AA Vet accompanying the voyage, project month, total voyage length, number of deaths, and mortality percentage had a significant effect on sampling rate in a univariate model (all $P < 0.05$), but not in a multivariate model.

Between 1 and 16 tissues were collected from each animal, with an average of 6 ± 3 tissues per animal. The most commonly collected tissues were lung (collected from 99% of necropsies), heart (80%), kidney (74%), liver (73%), trachea (67.5%), and rumen (46.2%). Approximately 50% of tissues were histologically normal, while in approximately 40% there were pathological changes (Additional Table 4.1). The remaining tissues were considered unsuitable for examination because the tissue structure(s) of interest was not present or the tissue was too autolysed.

The heart was collected from 158 out of 197 (80.2%) necropsies. Of the 16 cases that showed some pathology in the heart, mild to moderate, multifocal myocarditis was seen in 10 (62.5%). Acute myocarditis was observed in half of the cases, and 3 of these cases had lesions in other tissues consistent with a diagnosis of systemic infection with *H. somni*. The remaining 5 cases showed lymphoplasmacytic foci indicative of chronic myocarditis; sarcocysts (the asexual replication stage of *Sarcocystis* spp.) were observed in 3 of these cases.

The lung was collected for histological examination from 195 out of 197 (99%) necropsies. Of the 127 animals with histological evidence of respiratory disease, bronchopneumonia was seen in 104 (81.9%) and interstitial pneumonia in 19 (15%), with or without pleuritis. Histological lesions considered typical of specific organisms, and, where present, the morphology of intralesional bacteria, were used to determine the viruses and/or bacteria that were most likely to have caused the observed lung pathology (Table 4.4).

### 4.4.6 Molecular results

Lung tissue or swabs or both were collected for molecular pathology from 159 animals across 15 study voyages. Matched fixed and frozen lung samples were available for 151 cases.

The overall prevalence of the viruses of interest in lung samples was low; BCoV and BVDV were detected in approximately 10% of animals, BoHV-1 and BRSV were detected in less than 5% of cases, and BPIV-3 was not detected. Bacteria of interest were detected in 24% (*M. haemolytica*) to 60% (*M. bovis*) of animals tested (Table 4.4).

It was found that the prevalence of viruses was up to 4 times higher (all $P > 0.07$), and the prevalence of bacteria up to 26 times higher (all $P < 0.01$) in animals with histological evidence of respiratory disease than animals with histologically normal lungs. The only exception was

BRSV, for which the prevalence was 4 times higher in animals with normal lungs (Table 4.4), although the sample size was very small ($n$ = 6).

Of animals with histological features considered to be characteristic of infection with a particular organism, nucleic acid from the organism of interest was also detected by qPCR in 45% of cases for viruses (no significant correlation, $P$ = 0.076), and approximately 73% of cases for bacteria (statistically significant correlation, $P$ < 0.001).

The presence of *H. somni* in lung tissue was significantly correlated with the presence of *M. bovis*, *P. multocida*, and viral pathogens (all $P$ < 0.001), in particular BCoV and BVDV (both $P$ < 0.01). The presence of *M. bovis* and *M. haemolytica* were significantly correlated with the presence of *P. multocida* (both $P$ < 0.001).

Nasal swabs were collected at necropsy from 84 animals across 11 study voyages. Sterile swabs and VTM were included in the necropsy kit of all enrolled voyages but sampling of each case was at the discretion of the person performing the necropsy.

The viruses of interest were detected in the nasal swabs from up to 13% of animals, and bacteria from up to 49% of animals (Table 4.5). Matched nasal swab samples and lung histology results were available for 82 animals. Animals with *M. bovis*, *M. haemolytica*, or *P. multocida* in their nasal swabs had significantly higher odds of having histological evidence of respiratory disease and a final diagnosis of respiratory disease. Bovine coronavirus was also significantly associated with higher odds of a final diagnosis of respiratory disease (Table 4.5).

**Table 4.4.** Histological type of pneumonia versus prevalence of putative causative organisms detected using a qPCR assay on lung swab and/or tissue for animals from which matched fixed lung and lung swab and/or tissue was available. For result cells the first number is the number of animals positive.

| Organism | Histological diagnosis | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | ***M. bovis*** | ***M. bovis* and bacterial** | ***M. bovis* and viral +/– bacterial** | **Bacterial** | **Viral** | **Organism not determined** | **All pneumonia** | **No pneumonia** |
| BCoV | 0 | 2 | 1 | 7 | 0 | 1 | 11 | 4 |
| BoHV-1 | 0 | 0 | 1 | 1 | 1 | 0 | 3 | 0 |
| BRSV | 1 | 0 | 0 | 1 | 0 | 0 | 2 | 4 |
| BVDV | 2 | 4 | 1 | 7 | 1 | 0 | 15 | 2 |
| BPIV-3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| No. of cases | 14 | 26 | 6 | 46 | 5 | 5 | 102 | 49 |
| | | | | | | | | |
| *H. somni* | 3 | 19 | 5 | 12 | 1 | 0 | 40* | 6 |
| *M. bovis* | 8 | 22 | 5 | 15 | 0 | 0 | 50* | 1 |
| *M. haemolytica* | 1 | 9 | 1 | 18 | 0 | 0 | 29* | 2 |
| *P. multocida* | 3 | 15 | 1 | 15 | 0 | 0 | 34* | 3 |
| No. of cases | 14 | 25 | 5 | 32 | 4 | 4 | 84 | 44 |

* Organism prevalence significantly higher ($P < 0.01$) in pneumonic lungs (All pneumonia) than apparently normal lungs (No pneumonia).

**Table 4.5.** Prevalence of organisms of interest in nasal swab samples versus final diagnosis and histological diagnosis. For result cells, the first number is the number of positive animals.

| | Lung histology | | | | Final diagnosis | | | |
|---|---|---|---|---|---|---|---|---|
| | **Respiratory disease** | **No significant lesions** | **Overall** | **OR pneumonia: NSL** | **Respiratory disease** | **Other disease** | **Overall** | **OR respiratory: other disease** |
| No. of cases | 57 | 25 | 82 | | 42 | 42 | 84 | |
| BCoV | 9 | 2 | 11 | 2.16 | 9 | 2 | 11 | 5.45* |
| BoHV-1 | 9 | 1 | 10 | 4.50 | 7 | 3 | 10 | 2.60 |
| BRSV | 2 | 0 | 2 | NA | 2 | 0 | 2 | NA |
| BVDV | 4 | 1 | 5 | 1.81 | 4 | 1 | 5 | 4.32 |
| BPIV-3 | 1 | 0 | 1 | NA | 1 | 0 | 1 | NA |
| *H. somni* | 30 | 10 | 40 | 1.67 | 22 | 18 | 40 | 1.47 |
| *M. bovis* | 34 | 2 | 36 | 17.0* | 25 | 9 | 34 | 5.39* |
| *M. haemolytica* | 22 | 2 | 24 | 7.23* | 19 | 6 | 25 | 4.96* |
| *P. multocida* | 35 | 4 | 39 | 8.35* | 25 | 12 | 37 | 3.68* |

* *P* value for OR is <0.05.

Matched frozen nasal and lung samples were available for 79 cases. Of the 34 animals that were found to have *M. bovis* in their nasal swabs, *M. bovis* was detected in the lungs of 67.6% (Table 4.6). Coexistence of nasal shedding and presence of the organism of interest in the lung was also detected in at least half of the animals tested for BVDV (*n* = 5; 60%), *H. somni* (*n* = 39; 53.8%), and BRSV (*n* = 2; 50%).

**Table 4.6.** Detection of viruses and bacteria in nasal swab and/or lung tissue samples.

| | Nasal only | Lung only | Nasal + lung | Total | % nasal + lung |
|---|---|---|---|---|---|
| BCoV | 7 | 2 | 4 | 13 | 36.4 |
| BoHV-1 | 7 | 0 | 3 | 10 | 30 |
| BRSV | 1 | 1 | 1 | 3 | 50 |
| BVDV | 2 | 2 | 3 | 7 | 60 |
| BPIV-3 | 1 | 0 | 0 | 1 | 0 |
| | | | | | |
| *H. somni* | 18 | 4 | 21 | 43 | 53.8 |
| *M. bovis* | 11 | 5 | 23 | 39 | 67.6 |
| *M. haemolytica* | 14 | 7 | 9 | 30 | 39.1 |
| *P. multocida* | 20 | 3 | 17 | 40 | 45.9 |

Of the 9 animals from which swabs of grossly abnormal joints or cellulitic tissue were taken, 2 (22%) were positive for *M. bovis*. An additional 5 animals were positive for *M. bovis* on nasal or lung samples and had clinical and/or necropsy findings of lameness, but swabs of affected tissues were not taken. No significant association was found between an animal having at least 1 *M. bovis*–positive sample (i.e., nasal and/or lung and/or other tissue) and clinical signs or a final diagnosis of lameness.

### 4.4.7 Final diagnosis

Overall, causes of death were grouped into 8 categories: respiratory disease, lameness, misadventure, ketosis, septicemia, enteric disease, anesthetic complication, heat stroke, and

other (Table 4.7). Respiratory disease was the most common cause of death, accounting for nearly 50% of deaths overall (Table 4.7) and 59.1% of deaths for which a final diagnosis could be made. Of the 100 animals with a necropsy diagnosis of respiratory disease, respiratory disease was confirmed by histology in 86 cases, no significant lesions were seen in 8 cases, and the lung was not sampled in 6 cases. Histological evidence of concurrent respiratory disease was present in an average of 33% of animals for which respiratory disease was not considered the primary cause of death. Two-thirds (130/195) of animals from which lung samples were collected had histological and/or molecular results indicative of infectious lung disease: 93 out of 130 (72%) had evidence of bacterial infection, 4 (3%) had viral infection, 29 (22%) had mixed bacterial and viral infections, and, for 4 (3%), the causative organism could not be identified.

Seventeen of the 22 animals diagnosed with lameness were euthanased because their injuries were significantly compromising their welfare. Animals that were sedated for treatment of leg wounds and failed to recover from anesthetic accounted for an additional 4 lameness-related deaths. The proportion of deaths due to lameness and misadventure was significantly higher on vessel C when compared to other vessels ($P < 0.01$).

Most of the cases of ketosis occurred on a single shipment of pregnant dairy and beef breeder cattle sailing from Portland to the Russian Federation. Suspected predisposing risk factors for death identified by the AA Vet accompanying the voyage included a 2-week delay from the scheduled sail date, voyage length (31 days), suboptimal access to feed and water, and hot and humid conditions in the Gulf of Aden and Red Sea followed by cold conditions in the Mediterranean Sea.

**Table 4.7.** Final diagnosis versus voyage. The final diagnosis for each animal was based on the combination of all available data: description of premortem clinical signs, gross necropsy findings, histology results, and qPCR results. Not all data were available for all animals.

| Year/Voyage code | Respiratory disease | No diagnosis | Lameness | Ketosis | Septicemia | Enteric | Misadventure | Anesthetic complication | Heat stroke | Other | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2010 | | | | | | | | | | | |
| 1 | 9 | 3 | 3 | | | | | | 1 | | 16 |
| 2 | 6 | 1 | 1 | | | | | | | | 8 |
| 3 | 1 | | | 1 | | 1 | | | | | 3 |
| 4 | 4 | | | | | 2 | 2 | | | | 8 |
| 5 | 5 | 5 | | | 1 | | | 1 | | | 12 |
| 6 | | 5 | | 10 | 1 | 1 | | | | 1 | 18 |
| 7 | 6 | 1 | | | 1 | | | | | | 8 |
| 2011 | | | | | | | | | | | |
| 8 | 17 | 6 | 15 | | 3 | 2 | 4 | 1 | | 4 | 52 |
| 9 | 1 | 1 | | | 1 | 2 | | | | | 5 |
| 10 | | 2 | 1 | | | | | 1 | | | 4 |
| 11 | 2 | 2 | | | | | | | | | 4 |
| 12 | 20 | 1 | 1 | 1 | 2 | | 1 | 1 | | | 27 |
| 13 | 3 | 1 | | | 1 | | | | | | 5 |
| 14 | 1 | 2 | 1 | | | | | | | | 4 |
| 15 | 12 | 2 | | | 1 | 1 | | | | 1 | 17 |
| 16 | 5 | | | | | | | | | | 5 |
| 2012 | | | | | | | | | | | |
| 17 | 10 | | | | | | | | 1 | | 11 |
| 19 | | 1 | | | | 1 | | | | | 2 |
| 20 | 1 | 1 | | | | | | | | | 2 |
| 21 | 4 | | | | | | | | | | 4 |
| No. of cases total | 107 | 34 | 22 | 12 | 11 | 10 | 7 | 4 | 2 | 6 | 215 |
| % Deaths total | 49.8 | 15.8 | 10.2 | 5.6 | 5.1 | 4.7 | 3.3 | 1.9 | 0.9 | 2.8 | |
| % Resp. disease* | NA | 11.8 | 38.9 | 33.3 | 45.5 | 55.6 | 33.3 | 0 | 50 | 25 | |

* % Resp. disease = the percentage of animals for which respiratory disease was not considered the primary cause of death, but that had concurrent gross or histological evidence of respiratory disease; NA = not applicable since respiratory disease is the primary cause of death.

Miscellaneous causes of death included: severe urinary tract infection and/or obstruction requiring euthanasia (*n* = 1, voyage 6), *H. somni* myocarditis (*n* = 2) with encephalitis (n = 1) (voyage 8), severe pink eye requiring euthanasia (*n* = 1, voyage 8), and death due to pericarditis of unknown origin (*n* = 1, voyage 15). A final diagnosis could not be determined for 35 out of 215 (16.3%) cases. No significant association was found between "no diagnosis" cases and whether or not gross necropsy findings were recorded, whether or not the lung was sampled, or the number of tissues collected at necropsy (all $P > 0.1$).

Of the 79 animals that were found dead with no previous clinical signs, a gross necropsy diagnosis and histology samples were available for 68. The gross diagnosis was confirmed by histology in 56 out of 68 (82.4%) cases, no significant lesions were found on histology in 8 (11.8%) cases, and a different disease was diagnosed in 4 (5.9%) cases.

Of the 17 animals for which no significant lesions were found at necropsy, a final diagnosis based on histological samples alone could be made for 5 (29.4%). No significant lesions were found on histology for 8 (47.1%) cases, and the remaining 4 cases had histological lesions that were not severe enough to have been the cause of death.

## 4.5 Discussion

The current study involved the development of research protocols that were then applied by AA Vets on routine commercial voyages. This approach was aimed at providing a standardised collection of samples and data for the research project as well as longer term development of sustainable protocols for investigating morbidity and mortality under routine operating conditions.

The project enrolled approximately one-third of the eligible voyages that occurred during the time period and 20 voyages represents a substantive sample of export voyages. There were

potential biases in the enrollment of voyages and in the collection of postmortem material, for example, the decision to participate in the project was made by the exporter and decisions about whether to perform a postmortem and collection of samples were made by the AA Vet.

Adjusted mortality rates were significantly associated with voyage but the implications of this finding is difficult to explain because of the sparse data and confounding at multiple levels including with exporter, ship, AA Vet, and other unmeasured voyage-related factors (e.g., sea condition, climate, etc.). Adjusted mortality rates were higher for voyages in summer and winter than in spring and autumn. Increased mortality rates on long-haul shipments loaded out of southern ports in the Australian winter and arriving in the Middle East summer have been reported previously for both cattle (Norris et al., 2003) and sheep (Higgs et al., 1991; Richards et al., 1991). A previous study (Richards et al., 1991) postulated that winter grazing on abundant pasture resulted in a metabolic state where sheep were adjusted to laying down body fat and so were less able to utilize adipose reserves for energy when required (e.g., during periods of inappetence prior to and during export), compared to animals grazing poor pasture at other times of year. Climatic factors are also likely to play a role; cattle on voyages in summer and winter are going from one climatic extreme to another (e.g., summer heat in Australia to winter cold in the Russian Federation), with little time for physiological adaptation, which may predispose them to becoming sick or dying during the voyage.

Respiratory disease was the most common cause of mortality identified in the current study, accounting for 59.1% of the deaths for which a final diagnosis could be made. This prevalence is nearly 3 times that reported previously (Norris et al., 2003) (18.9%) but broadly similar to that reported in beef feedlots in Canada (46–65%) (Church and Radostits, 1981; Edwards, 1996; Gagea et al., 2006b; Janzen, 2003; Martin et al., 1982) and the United States (55–75%) (Jensen et al., 1976; Loneragan et al., 2001). Histological evidence of concurrent respiratory disease was also

present in an average of 33% of animals for which respiratory disease was not considered the primary cause of death.

One possible reason for the discrepancy between the previous study (Norris et al., 2003) and the current study could be sampling bias. In the previous study (Norris et al., 2003), necropsies were performed by designated research staff while in the current study necropsies were performed by the AA Vet accompanying the voyage, in addition to the duties that they are routinely expected to perform during voyages. Time pressures and varying levels of interest in the project, both between AA Vets and across time, meant that sampling was not consistent across all voyages. However, when only voyages on which at least 80% of deaths were necropsied were considered, deaths due to respiratory disease accounted for 48% of deaths, still more than twice that reported previously (Norris et al., 2003).

Quantitative PCR assay was used in the present study to detect potential respiratory pathogens in samples collected from the nose and lungs of cattle on board the vessels. The prevalence of viruses of interest (BCoV, BoHV-1, BRSV, BVDV, BPIV-3) was less than 15% in both nasal and lung samples. The prevalence of these viruses was higher in animals with clinical or histological evidence of respiratory disease than those without, with the exception of BRSV in lung samples. However, the only statistically significant association was between the prevalence of BCoV in nasal swabs and increased odds of a final diagnosis of respiratory disease (Table 4.5). In the pathogenesis of BRD, viruses play a primary role, causing damage to the respiratory tract and facilitating secondary invasion by bacteria that may go on to cause a fatal bacterial pneumonia (Panciera and Confer, 2010). Therefore the absence of a significant association between one particular virus and terminal BRD does not rule out the possibility that that virus may have contributed to the development of disease.

The current study reports a molecular diagnosis of BCoV in association with respiratory disease in Western Australian and South Australian cattle. In 2012, BCoV in association with BRD was

reported on a property on the south coast of New South Wales, Australia (Hick et al., 2012), and BCoV has been detected in fecal samples from scouring calves in Western Australia (M O'Dea, *personal observation*).

Bovine coronavirus was detected in 11 out of 102 (10.8%) lungs with histological evidence of respiratory disease. This is similar to that previously reported for cattle in a U.S. feedyard, where a conventional RT-PCR assay was applied to lung homogenates (21/194, 10.8%) (Fulton et al., 2009). Other studies using non–PCR-based techniques for BCoV detection in lung samples have reported detection rates of 1.4% in Danish cattle (Tegtmeier et al., 1999), 2% in Canadian cattle (Gagea et al., 2006b), and 69.2% in American cattle (Storz et al., 2000a). It is difficult to make direct comparisons between these studies due to differences in analytical methods, timing of data and sample collection, sample sizes, and environmental and animal factors. However, it would appear that, overall, BCoV detection rates in diseased lung tissue collected at necropsy are generally low. This is likely to reflect the role that BCoV is thought to play in the development of BRD; acting as a predisposing factor that allows commensal bacteria to infect the lungs, rather than a cause of terminal respiratory disease (Saif, 2010).

An association between BCoV in nasal swabs and clinical signs of respiratory disease has been found by some studies (Hasoksuz et al., 2002a; Storz et al., 2000b; Storz et al., 1996; Thomas et al., 2006) but not others (Cho et al., 2001; Hasoksuz et al., 2005). Nasal shedding of BCoV has also been linked to an increased risk of treatment for respiratory disease (Plummer et al., 2004), increased incidence of pulmonary lesions at slaughter (Lathrop et al., 2000a), and reduced weight gain (Thomas et al., 2006). In the study reported herein, animals with BCoV in their nasal swabs had higher odds of having histological evidence of respiratory disease (OR = 2.16, $P > 0.05$) and a final diagnosis of respiratory disease (OR = 5.45, $P < 0.05$) compared to animals that were BCoV negative. Therefore, although the detection rates of BCoV in lung samples and nasal swabs were

relatively low (10.8% and 13%, respectively), it is likely that BCoV is important in the development of BRD in Australian live export cattle.

Bovine viral diarrhoea virus was detected in the lungs of 15 out of 102 (15%) animals with histological evidence of pneumonia, and this was the highest detection rate of any of the viruses of interest. Previous studies on the presence of BVDV in lung tissues have reported detection rates of 6–21% in diseased lungs (Fulton et al., 2009; Fulton et al., 2002b; Gagea et al., 2006b; Tegtmeier et al., 1999). In feedlot cattle, the presence of BVDV in the lung has been found to be associated with earlier treatment for BRD, shorter treatment intervals when multiple treatments were required, and earlier death due to BRD than animals without BVDV in their lungs (Fulton et al., 2009; Gagea et al., 2006b).

In the current study, 7 out of 20 (35%) animals that tested positive on 1 or more samples were from consignments in which acutely or persistently infected animals had been identified by serum protein A antibody-capture ELISA (PACE) and removed from the consignment prior to loading. The detection of BVDV in animals on board the ship may be due to infection of naive animals by persistently infected animals during the assembly period or acutely infected animals that had insufficient viral loads to be detected by PACE. The present study suggests that a single test to remove persistently infected animals does not guarantee the removal of the virus from the consignment.

*Mycoplasma bovis* was detected in 51 out of 128 (40%) lung samples and in 34 out of 84 (40%) nasal swab samples. The presence of *M. bovis* in the lung and nasal secretions was significantly associated with higher odds of having histological evidence of respiratory disease and a final diagnosis of respiratory disease. The prevalence of *M. bovis* in lungs with histological evidence of respiratory disease was 60% (50/84). This is similar to that previously reported for beef feedlot cattle (51–88%) (Booker et al., 2008; Fulton et al., 2009; Gagea et al., 2006a). *Mycoplasma bovis* was most commonly found as a component of mixed bacterial infections, although all 6 animals

from which *M. bovis* was the only organism detected in the lung had histological evidence of respiratory disease.

No significant relationship was found between *M. bovis* and arthritis or lameness, although pneumonia and arthritis and/or tenosynovitis is a common presentation in animals with *M. bovis* (Caswell et al., 2010). This could be due to inadequate data collection, inadequate data on the presence of arthritis, difficulty in seeing lesions on board vessels due to darkness and manure, or the small number of samples that were taken from infected joints or adjacent tissues.

*Mannheimia haemolytica*, *P. multocida*, and *H. somni* have all been identified as important organisms in the development of BRD (Confer, 2009; Griffin et al., 2010). *Mannheimia haemolytica* and *P. multocida* were detected in 24% and 29% of lung samples and 30% and 44% of nasal swab samples, respectively. *Histophilus somni* was detected in 36% of lung and 48% of nasal swab samples (Tables 4.4, 4.5) and was the only bacterial organism that was not significantly associated with histological evidence of or a final diagnosis of respiratory disease (Table 4.5), although it was associated with increased severity of pneumonia.

Of particular interest was the identification of 3 cases with histological findings consistent with a diagnosis of myocarditis (*n* = 3), and pneumonia (*n* = 1) or thrombotic meningoencephalitis (TEM; *n* = 1) associated with *H. somni* infection. Myocardial abscesses were smaller and less severe than described previously (Harris and Janzen, 1989; Headley et al., 2013) although it is possible that additional lesions were present but not seen, or seen but not recorded and/or sampled by the AA Vet performing the necropsy. Although TEM was the first manifestation of disease associated with *H. somni* to be described (Brown et al., 1956), a number of subsequent reports suggest that case presentations of pneumonia, myocarditis, and/or arthritis in the absence of TEM are becoming more common (Harris and Janzen, 1989; Orr, 1992; Schuh and Harland, 1991; Van Donkersgoed et al., 1994).

Heat stroke was the most common cause of death in previous studies (Hedlefs, 1988; Norris et al., 2003) accounting for 50 out of 92 (54.3%) and 58 out of 180 (32.2%) of deaths for which a diagnosis could be made, respectively. Heat stroke was identified as a primary cause of death in only 2 animals in the present study (<1% of deaths) although hyperthermia secondary to other disease processes (e.g., severe bronchopneumonia and septicemia) was recorded in an additional 9 animals. The reduced incidence of deaths due to heat stroke in the study reported herein compared to previous studies could be due to a number of factors including misclassification, changes to the pre-export and on-board management of cattle, and differences in the type of cattle being exported.

The present study depended on collection of samples by AA Vets. AA Vets collecting the samples received training on identifying gross necropsy changes typical of heat stroke in cattle to reduce the risk of misdiagnosis. If none of these signs were evident at necropsy then the animal was not recorded as having died of heat stroke. AA Vets were encouraged to take CBTs and record the number of hours between death and necropsy to assist in making an informed and evidence-based diagnosis. However, CBT was measured in less than half of the animals in this study so it is possible that animals that died and whose CBT was not measured may have had primary or secondary heat stress.

Since the late 1990s, a combination of industry initiatives (Australian Live Export Standards (ALES), 1997 (Stinson, 2008)) and mandatory standards for animal health and welfare (Australian Standards for the Export of Livestock (ASEL), 2004 (DAFF, 2011)) have led to changes in the management of cattle prior to and during export. Changes include seasonal restrictions on geographical regions from which cattle can be sourced and maximum stocking densities based on season, voyage length, and type of animal (*Bos taurus* vs. *Bos indicus*). In 2003, the Livestock Export Heat Stress Risk Assessment Model (HotStuff) was implemented to help exporters to estimate and minimize the risk of heat stress mortalities occurring during a voyage (Maunsell

Australia, 2003). Ongoing public pressure ensures that exporters, AA Vets, and stockpersons are very aware that they are operating under and accountable to Commonwealth law. The results reported herein suggest that an increased awareness and consideration of animal welfare has achieved better conditions for live export cattle, including a reduction in deaths due to heat stroke.

The type of cattle sent for live export has changed over the last 25 years, mainly in response to import trade tariffs and consumer education. There is a trend towards shipping younger “feeder” cattle that are fattened at their destination, rather than older “slaughter” cattle that are killed soon after their arrival. Smaller animals have a larger surface area to volume ratio so they are better able to respond to changes in ambient temperature and humidity than larger animals. At the time of the Hedlefs (1988) study (1985–1987) and Norris et al. (2003) study (1998–2001) it is likely that the proportion of >600 kg slaughter animals in a shipment was higher than for the voyages in the current study. This difference in shipment composition may have contributed to the lower incidence of heat stress observed on the voyages reported herein.

Trauma, including lameness and misadventure, was the third most common cause of death. Deaths in the lameness category were generally the result of the animal being euthanized because its injuries were severely compromising its welfare. The incidence of trauma (13.5%) was lower than that reported previously (Hedlefs, 1988; Norris et al., 2003) (22.8% and 24.6%, respectively). This is likely due to a combination of improved vessel design and a change in the type of cattle being exported.

Lameness may begin prior to loading or during the voyage. During the assembly period, poorly designed or maintained yards or trucks combined with rough handling can lead to foot and leg injuries. During the voyage, lameness develops secondary to injuries and abrasive floor surfaces. Injuries are more common during rough weather, with reduced stocking densities, and in heavy animals (>380 kg) (Banney et al., 2009; Hedlefs, 1988). Ongoing maceration and bacterial

contamination through contact with manure means that these lesions seldom heal without treatment.

Risk factors for the development of lameness include both animal and management factors (Banney et al., 2009; Norris et al., 2003). Heavy animals and those with soft feet, particularly southern and dairy cattle, are more likely to become lame. Management factors include the type of pen flooring, type and amount of bedding, frequency of deck washing (to remove soiled bedding), and stocking densities. Recommendations for best practice for cattle selection and bedding management to reduce the incidence of lameness have been made (Banney et al., 2009), but morbidity and mortalities due to lameness are still relatively high, indicating that there is room for improvement.

Sedation for wound cleaning and bandaging carries its own mortality risk, as the 4 post-anaesthetic deaths show. Hyperthermia is known to occur following administration of tranquilizing drugs to sheep in hot weather (Radostits et al., 2000). Xylazine is commonly used for sedation of cattle on board vessels, and hyperthermia secondary to a reduced ability to thermoregulate is a recognised side-effect of deep sedation with xylazine, particularly when animals are in an environment where overheating could occur.

Enteric disease was uncommon. Conditions observed included bloat (*n* = 2), enteritis (*n* = 3), and gastrointestinal rupture (*n* = 2) or obstruction (n = 1). The most common disease involving an organ of the digestive system was fatty liver change secondary to inappetence (*n* = 3) associated with advanced pregnancy (*n* = 9). Animals are monitored daily during the pre-export period and voyage to identify and treat animals showing signs of inappetence (“shy feeders”). However, despite the best efforts of the AA Vets and on-board stockmen, a small proportion of animals fail to adapt to on-board conditions and rations.

The study reported herein builds on the previous study of cattle deaths during sea transport from Australia (Norris et al., 2003) by including a greater number of study voyages (20 versus 4) and the application of qPCR assays to detect bacteria and viruses of interest in necropsy samples. Heat stress appears to be less of a problem than previously reported and this is likely to be due to heat stress risk management strategies implemented by the export industry since the late 1990s and a change in the type of animals being exported. Lameness remains an important cause of morbidity and mortality, and further research is needed into ways to manage this problem.

Although overall mortality rates are low, respiratory disease is still an important cause of death. The current investigation provides baseline data on the bacteria and viruses involved in BRD in Australian live export cattle, which can be used to inform on future BRD prevention and management strategies. Further studies are already underway to investigate pre-loading risk factors including the prevalence of potential pathogens during the assembly period.

## 4.6 Author's contributions

SJM assisted with refinement of the study design, processed and examined fixed tissues for histological examination, processed and tested samples for qPCR testing, performed statistical analysis, and drafted the Chapter. MOD assisted with development of qPCR assays and testing of samples. NP assisted with the design of the study and provided advice on statistical anlaysis. MOH assisted with the design of the study, examination of fixed tissues and determination of Final diagnoses. All authors provided comment on the Chapter contents and read and approved the final draft of this Chapter.

## 4.7 Acknowledgements

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## 4.8 Supplementary Tables

**Supplementary Table 4.1.** See **Table 3.1.** Primer and probe sequences for qPCR assays.

**Supplementary Table 4.2.** Explanatory variables used in statistical analysis.

| Variable name | Details |
|---|---|
| VOYNUM | Numerical code for voyage, 1-21 |
| AAVET | A, B, C, D, E, F, G |
| VOYMTH | Month number, January=1 to December=12 |
| VOYYR | 2010, 2011, 2012 |
| CAT | Total cattle loaded |
| VOYSEASON | Summer (VOYMTH=12, 1, 2), Autumn (3-5), Winter (6-8), Spring (9-11) |
| VOYDAYS | Total voyage length in days including loading, sailing and discharge |
| SHIP | A, B, C, D, E, F, G |
| EXPORTER | A, B |
| LDPORT | Fremantle, Broome, Adelaide, Portland |
| LDTYPE | Single, split |
| LREG | Load region. South (Fremantle, Adelaide, Portland), North (Broome) |
| LREG1 | North-west (Broome), South-west (Fremantle), East (Adelaide, Portland) |
| DCTYPE | Single, split |
| DCCOUNTRY | Israel, Libya, Egypt, Saudi Arabia, Bahrain, Turkey, Russian Federation, China |
| DREG | Discharge region. Middle East and North Africa (MENA; Israel, Libya, Egypt, Saudi Arabia, Bahrain), Black Sea (Turkey, Russian Federation), China |
| FINALDNX | Respiratory disease, Lameness, Misadventure, Ketosis, Septicaemia, Enteric, Anaesthetic complication, Heat stroke, Other, No diagnosis |
| HISTPNEU | Histological evidence of pneumonia. Yes, No |

## 4.9 Additional Material

This table was not included the published manuscript but is presented here for completeness.

**Additional Table 4.1.** Tissues and disease processes (where present) for all tissues examined histologically. * See Chapter text.

| Body system | Tissue | n Sampled | % necropsies sampled | % abnormal | Disease processes (n Cases) |
|---|---|---|---|---|---|
| Respiratory | Lung | 195 | 99.0 | 65.1 | * |
| | Trachea | 133 | 67.5 | 45.1 | Tracheitis - acute (5), chronic (55) |
| | Pleura/Mediastinum | 3 | 1.5 | 100 | Steatitis (3) |
| Cardiovascular | Heart | 158 | 80.2 | 10.1 | * |
| Digestive | Reticulum | 6 | 3.0 | 50.0 | Reticulitis (3) |
| | Rumen | 91 | 46.2 | 16.5 | Rumenitis (14), hyperkeratosis (1) |
| | Omasum | 13 | 6.6 | 7.7 | Omasitis (1) |
| | Abomasum | 16 | 8.1 | 37.5 | Abomasitis (5), steatitis (1) |
| | Duodenum | 3 | 1.5 | 0 | |
| | Small intestine | 70 | 35.5 | 21.4 | Enteritis (14), enteropathy (1) |
| | Caecum | 6 | 3.0 | 16.7 | Typhlitis (1) |
| | Colon | 12 | 6.1 | 16.7 | Colitis (2) |
| | Liver | 144 | 73.1 | 44.4 | Cholangitis (23), Hepatitis - acute (9), chronic (8) |
| | Gall bladder | 5 | 2.5 | 20.0 | Cholecystitis (1) |
| | Pancreas | 3 | 1.5 | 0 | |
| | Omentum/Peritoneum | 5 | 2.5 | 80.0 | Steatitis (3), Peritonitis (1) |
| | Oral mucosa | 1 | 0.5 | 100 | Stomatitis (1) |

**Additional Table 4.1.** (continued) Tissues and disease processes (where present) for all tissues examined histologically.

| Body system | Tissue | n Sampled | % necropsies sampled | % abnormal | Disease processes (n Cases) |
|---|---|---|---|---|---|
| Urinary | Kidney | 146 | 74.1 | 69.9 | Interstitial nephritis/pyelitis/nephritis - acute (4), chronic (96). Miscellaneous (10). |
| | Urinary bladder | 1 | 0.5 | 0 | |
| Lymphoreticular | Spleen | 65 | 33.0 | 27.7 | Circulating neutrophilia (16), Follicular necrosis (1), Focal necrosis (1) |
| | Lymph node | 44 | 22.3 | 6.8 | Circulating neutrophilia (3) |
| Musculoskeletal | Skeletal muscle | 28 | 14.2 | 46.4 | Myocyte degeneration/necrosis (6), Cellulitis (9) |
| | Tissue from hock | 2 | 1.0 | 100 | Cellulitis (2) |
| | Tendon | 1 | 0.5 | 100 | Tendonitis (1) |
| | Skin haired | 1 | 0.5 | 100 | Vasculitis (1) |
| | Joint | 1 | 0.5 | 100 | Arthritis (1) |
| Endocrine | Adrenal | 15 | 7.6 | 40.0 | Adrenalitis (6) |
| Nervous system | Spinal cord | 4 | 2.0 | 25.0 | Wallerian degeneration (1) |
| | Whole brain | 3 | 1.5 | 33.3 | Encephalitis (1) |
| | Spinal cord and whole brain | 1 | 0.5 | 0 | |
| Reproductive | Placentome | 1 | 0.5 | 0 | |

# Chapter 5: Estimation of nasal shedding and seroprevalence of organisms known to be associated with bovine respiratory disease in Australian live export cattle

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## 5.1 Summary

The prevalence of organisms known to be associated with bovine respiratory disease was investigated in cattle prior to export. A quantitative PCR assay was used to detect nucleic acids from the following viruses and bacteria in nasal swab samples: *Bovine coronavirus* (BCoV, *Betacoronavirus 1*), *Bovine herpesvirus 1* (BoHV-1), *Bovine viral diarrhoea virus* (BVDV), *Bovine respiratory syncytial virus* (BRSV), *Bovine parainfluenza virus 3* (BPIV-3), *Histophilus somni*, *Mycoplasma bovis*, *Mannheimia haemolytica* and *Pasteurella multocida*. Between 2010 and 2012 nasal swabs were collected from 1484 apparently healthy cattle destined for export to the Middle East and Russian Federation. In addition, whole blood samples from 334 animals were tested for antibodies to BoHV-1, BRSV, BVDV and BPIV-3 using ELISA. The nasal prevalence of BCoV at the individual animal level was 40.1%. The nasal and seroprevalence of BoHV-1, BRSV, BVDV and BPIV-3 was 1.0% and 39%, 1.2% and 46%, 3.0% and 56%, and 1.4% and 87% respectively. The nasal prevalence of *H. somni*, *M. bovis*, *M. haemolytica* and *P. multocida* was 42%, 4.8%, 13.4%, and 26% respectively. Significant differences in nasal and seroprevalence were detected between groups of animals from different properties. The results of this study provide baseline data on the prevalence of organisms associated with BRD in Australian live export cattle in the pre-assembly period. These data could be used to develop strategies for BRD prevention and control prior to loading and during voyages.

## 5.2 Introduction

Respiratory disease associated with long distance transport of animals was first reported in the scientific literature in 1925, although the disease had been recognised by veterinarians for at least twenty years prior to this publication (Hepburn, 1925). Bovine respiratory disease (BRD) is an important cause of death in cattle exported live from Australia (Moore

et al., 2014; Norris et al., 2003). Morbidity and mortality due to BRD is the culmination of a combination of host, pathogen and environmental risk factors. As the pathogens involved in fatal BRD on live export voyages from Australia are similar to those associated with BRD in feedlot cattle (Moore et al., 2014), it is likely that the pathogenesis of BRD in these two cattle productions systems is similar.

Inconsistent access to feed and water, exposure to noise and vibration during transportation, exposure to a new environment, and co-mingling with unfamiliar animals activate the stress response in cattle (Manteca, 2008; Phillips, 2008; Sainsbury and Sainsbury, 1988; Warriss, 2004), causing the release of glucocorticoids and/or catecholamines which can reduce immunity (Elenkov et al., 2000). An impaired immune response predisposes to increased viral shedding in subclinically affected animals and subsequent proliferation of potentially pathogenic commensal bacteria (Manteca, 2008; Panciera and Confer, 2010). Inhibition of respiratory clearance mechanisms and direct tissue damage by viral infection facilitates proliferation and invasion of secondary bacterial organisms, often resulting in a fatal bacterial pneumonia (Panciera and Confer, 2010; Taylor et al., 2010).

The Australian Government requires animals destined for export to be held in a registered premises (also known as an “assembly depot”) for a minimum period of time prior to being loaded onto a vessel (Australian Standards for the Export of Livestock, Standard 3 (DAFF, 2011)). Time in the assembly depot allows the animals to recover from road transport, acclimatise to shipboard rations, and receive any health checks or treatments required by the importing country. However, co-mingling, handling and sorting of animals that have been recently transported from multiple farms also provides an ideal environment for transmission of viruses and bacteria between animals in the assembly depot.

Viruses implicated in the pathogenesis of BRD include bovine herpesvirus 1 (BoHV-1), bovine respiratory syncytial virus (BRSV), bovine viral diarrhoea virus (BVDV), bovine parainfluenza virus 3 (BPIV-3), bovine coronavirus (BCoV), bovine rhinoviruses and bovine adenoviruses. Bovine rhinoviruses and adenoviruses probably have only a minimal role in the pathogenesis of BRD, while BoHV-1, BPIV-3 and BRSV are recognised as primary respiratory pathogens, and BVDV as a significant predisposing pathogen (reviewed in (Fulton, 2009; Panciera and Confer, 2010)). Since its first isolation in association with respiratory disease in 1993 (Storz et al., 1996) BCoV has received attention as an emerging BRD pathogen, and is now thought to make a substantial contribution to the development of BRD in feedlot cattle (Saif, 2010; Storz et al., 2000a).

*Mannhaemia haemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma bovis* are considered to be the most important bacteria involved in BRD (reviewed in (Griffin et al., 2010; Taylor et al., 2010)). Additional bacterial agents isolated from pneumonic bovine lung tissue include *Trueperella* (formerly *Arcanobacterium*) *pyogenes* (Watson and Scholes, 2010; Welsh et al., 2004) and *Bibersteinia* (formerly *Pasteurella*) *trehalosi* (Blackall et al., 2007; Watson and Scholes, 2010).

This study reports the pre-export prevalence of viruses and bacteria associated with BRD in Australian live export cattle and identifies a number of factors that influence the prevalence of viruses and bacteria in nasal secretions. The seroprevalence of BoHV-1, BRSV, BVDV, and BPIV-3 were also determined. The baseline data generated by this study will be used to develop pathogen management strategies, both on-farm and at the assembly depot, with a view to reducing the impact of important organisms on morbidity and mortality during live export voyages.

## 5.3 Materials and Methods

### 5.3.1 Study population

Nasal swab samples were collected from 1484 cattle on 4 voyages between December 2010 and May 2012. The number of cattle sampled represented 1-5% of the total cattle on the voyage (Table 5.1). The percentage of animals sampled from each cohort ranged from 1.3–12.5% (mean = 5.0, median = 4.9, SE = 1.18).

Cattle were sampled at 8 assembly depots across 3 states (Table 5.2): 6 depots in Western Australia (depot codes 1-6) all loading out of Fremantle, 1 in Victoria (depot code 7, load port = Portland), and 1 in South Australia (depot code 8, load port = Adelaide). Animals were grouped into 14 cohorts based on class (feeder, slaughter, breeder) and load-out port (Fremantle, Portland, Adelaide) (Table 5.2).

Selection of voyages for sampling was opportunistic and based on the depot induction timetable and location of the assembly depots. Cohorts of cattle were selected to provide a representative subset of the final composition of the shipment (with regards to load port, class, type and sex) from as many depots and properties as possible, and to collect enough samples to be able to perform statistical comparisons between explanatory variables, while minimising the impact of study sampling on processing of cattle.

Data collected from each animal at sampling included the radio-frequency identification (RFID), property identification code (PIC), breed, sex and weight. With the exception of RFID, not all data were available for all animals.

**Table 5.1**. Voyage data for voyages from which nasal swabs and/or serological samples were collected.

| | Voyage Code | | | |
|---|---|---|---|---|
| | **5** | **8** | **17** | **18** |
| **Voyage year** | 2010 | 2011 | 2012 | 2012 |
| **Season** | Summer | Autumn | Autumn | Autumn |
| **Load port(s)** | Fremantle | Portland, Fremantle | Adelaide, Fremantle | Portland, Fremantle |
| **Discharge country(s)** | Turkey | Saudi Arabia, Turkey | Turkey | Russian Federation |
| **Voyage length (days) *** | 24 | 35 | 34 | 37 |
| **Total cattle loaded** | 19990 | 17449 | 7811 | 9068 |
| **Number cattle sampled (nasal swabs)** | 200 | 413 | 393 | 100 |
| **Percent cattle sampled (nasal swabs)** | 1.0 | 2.4 | 5.0 | 1.1 |
| **Number cattle sampled (blood)** | 0 | 0 | 334 | 0 |
| **Percent cattle sampled (blood)** | 0 | 0 | 4.3 | 0 |
| **Mortality rate (%)** | 0.74 | 0.30 | 0.50 | 0.36 |

* Voyage length includes sailing and discharge days

**Table 5.2**. Voyage, assembly depot and animal data for cohorts from which nasal swabs +/- blood were collected.

| Voyage code | Voyage month | Cohort code | Depot state | Depot code | Days before loading | Class | Number tested |
|---|---|---|---|---|---|---|---|
| 5 | Dec 2010 - Jan 2011 | 1 | WA | 4 | NA | Feeder[a] | 10 |
| | | 2 | WA | 4 | 9 | Feeder | 200 |
| | | 3 | WA | 1 | 5 | Slaughter | 200 |
| 8 | Mar - Apr 2011 | 4 | VIC | 7 | 28 | Feeder | 102 |
| | | 5 | WA | 2 | 27 | Feeder | 112 |
| | | 6 | WA | 4 | 21 | Slaughter | 99 |
| | | 7 | WA | 3 | 19 | Slaughter | 100 |
| 17 | Apr - May 2012 | 8a | WA | 5 | 16 | Feeder[b] | 25* |
| | | 9 | SA | 8 | 9 | Feeder | 92* |
| | | 10 | SA | 8 | 8 | Slaughter | 20 |
| | | 11 | WA | 6 | 13 | Feeder | 75* |
| | | 12a | WA | 5 | 12 | Feeder[d] | 143* |
| | | 8b | WA | 5 | 9 | Feeder[c] | 18 |
| | | 13 | WA | 5 | 5 | Slaughter | 80 |
| | | 12b | WA | 5 | 3 | Feeder[e] | 108 |
| 18 | Apr - May 2012 | 14 | WA | 1 | 28 | Breeder | 100 |

Depot state: WA, Western Australia; VIC, Victoria; SA, South Australia. Class: [a] Sick cattle, not loaded; [b] Carryover cattle, first round of testing, [c] group re-test 7 days later; [d] Newly received cattle, first round of testing, [e] group re-test 9 days later. * Matched serum samples collected for serology.

For voyages 5, 8 and 17 the consignments comprised a mixture of feeder and slaughter cattle. Voyage 18 was a shipment of breeder cattle to the Russian Federation. Overall the class breakdown was 60% feeder, 34% slaughter and 6% breeder. Eighty-four percent of cattle were *Bos taurus* and their crosses and 16% were *Bos indicus*. Eighty-seven percent of cattle were male (65% steer, 23% bull; 62% feeder, 38% slaughter) and 13% were female (57% breeder, 43% feeder).

### 5.3.2 Nasal swab samples

Nasal swabs were collected from live export cattle during induction processing up to 24 hours after they entered the pre-export assembly depot. All nasal swab sampling was performed by a single member of the project team (SJM) with the exception of sampling of slaughter cattle at depot 7 (cohort 10) that was undertaken by an AQIS-accredited third-party veterinarian.

For nasal swab collection the animal was restrained in a head bail and a 20 cm cotton swab was inserted approximately 10 cm into the nasal cavity. The swab was rotated across the nasal mucosa to collect a sample of the nasal secretions. During both insertion and removal care was taken to prevent the swab being contaminated by dirt etc. on the nostrils. The swab was immediately placed into a 5 mL plastic tube filled with 1-2 mL viral transport media (VTM; Hanks balanced salt solution, penicillin G [1,000 units/ml], streptomycin [25 mg/ml], and amphotericin B [0.1 mg/ml]; Department of Agriculture and Food, Western Australia) and kept chilled until transport back to the laboratory.

To investigate whether the prevalence of viral and bacterial shedding changed with time, 2 cohorts of animals were sampled 7 days (cohorts 8a and 8b) and 9 days (cohorts 12a and 12b) apart. Cohorts 8a and 8b were carryover animals from a previous shipment, and cohorts 12a and 12b were newly received animals (Table 5.2). It was not possible to select the same individuals for sampling at the first and second sampling sessions, but animals

that were sampled at the second session were selected from pens containing animals that had been sampled at the first session.

Nasal swab samples were processed and nucleic acids from the organisms of interest – BCoV, BoHV-1, BVDV, BRSV, BPIV-3, *H. somni*, *M. bovis*, *M. haemolytica*, *P. multocida* – were detected using quantitative polymerase chain reaction (qPCR) assays as described previously (Moore et al., 2014).

### 5.3.3 Serological samples

Serological samples were collected from animals from 1 voyage (voyage 17) (Table 5.1). Serum and nasal swab samples collected from the same animal at a single time-point (depot entry) were available for 334 feeder cattle: a single cohort of 91 animals from South Australia; and 3 cohorts from Western Australia, a total of 243 animals (Table 5.2). Cohort 8a comprised animals that had been carried over from a previous shipment. Cohorts 9, 11, and 12a were newly received animals.

Whole blood was collected at entry to the pre-export assembly depot as part of the pre-export health checks required by the importing country. While the animal was restrained in a crush, blood was collected into sterile plain vacutainers by venipuncture of the coccygeal vein.

Serum samples were sent to the Department of Agriculture and Food, Western Australia, for commercial Bluetongue and Bovine leukaemia virus antibody testing. After serum aliquots had been taken for commercial testing, another aliquot of serum was taken for serology as part of the study reported herein.

Aliquots of 200 µl of serum were tested for the presence of circulating antibodies to the viruses of interest using commercially available enzyme-linked immunosorbant assay (ELISA) kits according to the manufacturer's instructions for the following viruses: BoHV-1

(Infectious Bovine Rhinotracheitis (IBR) gB X2 Ab Test, IDEXX, Montpellier, France), BRSV (Bovine Respiratory Syncytial Virus (BRSV) IgG Antibody Test Kit, IDEXX, Montpellier, France), BVDV (Bovine Viral Diarrhoea Virus (BVDV) Antibody Test Kit, IDEXX, Montpellier, France), BPIV-3 (Parainfluenza-3 Virus (PI3) Antibody Test Kit, IDEXX, Montpellier, France). It was not possible to test for antibodies to BCoV since a validated commercial test is not available. Samples with optical density reading above the level set by the kit manufacturer were considered positive, below negative, and in-between were inconclusive. Inconclusive samples were not retested, and animals with inconclusive test results were not included in the analysis.

### 5.3.4 Statistical analysis

Sample size estimates were based on power analyses conducted using Win Episcope 2.0 (www.clive.ed.ac.uk), suggesting that 20-100 samples per property of origin would be sufficient to allow description of prevalence and preliminary assessment of association between risk factors and pathogen prevalence while adjusting for property level clustering. The final approach involved sampling 20-25 animals per property and up to 4-5 properties per cohort.

The statistical software package 'R' was used for descriptive statistics (R Development Core Team, 2014) and Stata (version 13, www.stata.com) for regression analyses. Ninety-five percent confidence intervals for descriptive prevalence estimates were generated using the Wilson approach and expressed as percentages (Brown et al., 2001; Newcombe, 1998). Overall prevalence estimates were generated as an average of the individual cohort prevalence estimates, and as an overall individual animal prevalence.

Negative binomial regression with adjustment for clustering at the property of origin (PIC) level was used to investigate associations between the apparent prevalence of viruses and bacteria of interest and available explanatory variables: voyage, sampling month, sampling

year, assembly depot, assembly depot state (Western Australia, Victoria, South Australia), sex, type (*Bos taurus*, *Bos indicus*), class (feeder, slaughter, breeder) and live-weight (in kilograms). Data on animal breed was only available for a limited subset of animals and was not used in the analysis. Because of the sparseness of the data and presence of confounding between many explanatory variables, regression models were run as univariate screening models only to assess for associations between explanatory variables and apparent pathogen prevalence. Spearman's rank correlation test was used to test for correlations between the presence of various organisms, as detected by qPCR, in the nasal swab samples. A threshold of $P = 0.05$ was used for all statistical analyses.

## 5.4 Results

### 5.4.1 Nasal prevalence

One or more of the viruses or bacteria of interest were detected in the nasal swabs from 1150/1484 (77%) of cattle. The remaining 334/1484 (23%) animals were negative for all organisms tested. Pathogen prevalences for all viruses and bacteria were found to vary significantly between cohorts ($P < 0.05$).

Bovine coronavirus was the most commonly detected virus, found in cattle from all voyages and in all but 2 of the cohorts tested (cohort number 8b and 10). Forty percent of animals were positive for BCoV RNA in nasal swabs, with an average cohort level prevalence of 32% (Table 5.3). The remaining viruses were present in cattle from 2 (BoHV-1), 3 (BVDV, BPIV-3) or 4 (BRSV) voyages but generally at low prevalences. Exceptions included the prevalence of BRSV for voyage 18 (10%), and the prevalence of BVDV for 2 cohorts in voyage 17 (cohort 9 = 12%, cohort 11 = 10%) (Table 5.3).

**Table 5.3**. Cohort and individual level pathogen prevalences based on qPCR testing of nasal swab samples. See Table 5.2 for sampling and animal data for each cohort. SE, standard error.

| Cohort code | BCoV (%) | BoHV-1 (%) | BRSV (%) | BVDV (%) | BPIV-3 (%) | *H. somni* (%) | *M. bovis* (%) | *M. haem.* (%) | *P. mult.* (%) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 70 | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 60 |
| 2 | 94 | 0 | 1 | 5 | 1 | 7 | 0 | 44 | 61 |
| 3 | 59 | 0 | 0 | 6 | 1 | 27 | 0 | 5 | 22 |
| 4 | 57 | 0 | 0 | 1 | 0 | 5 | 0 | 5 | 20 |
| 5 | 2 | 0 | 0 | 0 | 0 | 10 | 0 | 5 | 45 |
| 6 | 57 | 0 | 0 | 1 | 1 | 49 | 5 | 36 | 33 |
| 7 | 6 | 4 | 1 | 0 | 1 | 58 | 0 | 7 | 15 |
| 8a | 8 | 8 | 0 | 12 | 0 | 92 | 24 | 0 | 28 |
| 9 | 45 | 5 | 0 | 4 | 4 | 64 | 27 | 7 | 47 |
| 10 | 0 | 0 | 0 | 10 | 0 | 10 | 0 | 5 | 10 |
| 11 | 9 | 3 | 0 | 3 | 3 | 71 | 0 | 0 | 1 |
| 12a | 22 | 0 | 0 | 5 | 3 | 69 | 8 | 6 | 13 |
| 8b | 0 | 6 | 0 | 6 | 0 | 72 | 17 | 6 | 28 |
| 13 | 5 | 0 | 0 | 1 | 5 | 21 | 4 | 0 | 9 |
| 12b | 26 | 1 | 4 | 1 | 2 | 72 | 18 | 24 | 9 |
| 14 | 47 | 0 | 10 | 0 | 0 | 87 | 0 | 6 | 4 |
| Ave. cohort prevalence (SE) | 31.6 (7.6) | 1.7 (0.7) | 1 (0.2) | 3.4 (0.9) | 1.3 (0.4) | 45.3 (7.6) | 6.4 (2.4) | 9.7 (3.4) | 25.3 (4.7) |
| Ave. individual prevalence (SE) | 40.1 (1.3) | 1.0 (0.3) | 1.2 (0.3) | 3 (0.4) | 1.4 (0.3) | 42 (1.3) | 4.8 (0.6) | 13.4 (0.9) | 26 (1.1) |

*Histophilus somni* and *P. multocida* were detected in nasal swabs from cattle across all voyages and cohorts with average detection rates of 42% and 26% respectively at the individual animal level (Table 5.3). Eight cohorts across 3 voyages included cattle that were *M. bovis* positive, with a maximum cohort prevalence of 27% (Table 5.3). *Mannheimia haemolytica* was detected in cattle from most cohorts and across all voyages, with an average prevalence of 13% at the individual level and 10% at the cohort level (Table 5.3).

Multiple organisms were detected in nasal swab samples from many animals in this study. Overall, a single bacteria only was detected in 503/1150 (43.7%) animals, while one or more viruses and bacteria were detected in 434 (37.7%) animals, and one or more viruses in 213 (18.5%) animals. Bovine coronavirus was present in 92% of single viral detections and 92% of mixed viral-bacterial detections. The presence of BCoV was significantly correlated with the presence of BRSV, *M. haemolytica*, *P. multocida* and *H. somni* (all $P < 0.01$). Bovine viral diarrhoea virus was present in 8% of mixed infections but was not significantly correlated with any other viruses or bacteria (all $P > 0.1$). The presence of BRSV was significantly correlated with the presence of *H. somni* ($P < 0.001$). Bovine herpesvirus 1, BVDV and BPIV-3 were not significantly correlated with any other organism.

*Histophilus somni* was present in 77% of single bacterial detections and 54% of mixed viral-bacterial detections. The presence of *H. somni* was significantly correlated with the presence of BCoV, BRSV, *M. bovis* (all $P < 0.001$) and *P. multocida* ($P < 0.05$). *P. multocida* was present in 33% of single bacterial detections and 51% of mixed viral-bacterial detections. The presence of *P. multocida* was significantly correlated with the presence of BCoV ($P < 0.001$), *M. haemolytica* ($P < 0.001$) *and H. somni* ($P < 0.05$).

Of the resampled cohorts significant decreases in pathogen prevalence were observed for BCoV and *H. somni* in the carryover cohorts (cohorts 8a and 8b; both $P < 0.01$). For the newly received cohorts (12a and 12b) the prevalence of *M. haemolytica* ($P < 0.001$), BRSV

(P < 0.01) and *M. bovis* (P < 0.05) was higher at the second testing session, and BVDV was borderline significantly lower (P = 0.057).

### 5.4.2 Analysis of nasal prevalence versus explanatory variables

The total number animals that tested positive for BCoV, BoHV-1, BRSV, BVDV and BPIV-3 was 594, 15, 17, 44 and 21 respectively. For *H. somni*, *M. bovis*, *M. haemolytica* and *P. multocida* the total number of positive animals was 622, 72, 199 and 387 respectively.

Negative binomial regression with PIC as a fixed effect showed a number of associations between pathogen prevalence for BCoV, *H. somni*, *M. haemolytica* and *P. multocida*, and available explanatory variables (Supplementary Table 5.1). Datasets for BoHV-1, BRSV, BVDV, BPIV-3 and *M. bovis* were not suitable for univariate analysis due to the low number of animals testing positive for these organisms.

There were significant differences between voyages, sampling month, and sampling season for all organisms (all $P < 0.01$) on which univariate analysis was performed. Sampling year was significant for *M. haemolytica* and *P. multocida* (both $P < 0.01$), and assembly depot for BCoV and *P. multocida* (both $P < 0.001$). Results for sampling year, sampling season and sampling month should be interpreted with caution due to a high degree of confounding between these variables and voyage. Assembly depot state, sex, type, class and weight were not significant for any organisms (all $P > 0.1$) (Supplementary Table 5.1). Multivariate analyses were attempted but were not successful due to the sparsity of data and confounding between explanatory variables.

### 5.4.3 Seroprevalence

Overall, 88% of animals were seropositive for BPIV-3, 58% for BVDV, 50% for BRSV and 43% for BoHV-1 (Table 5.4). There were significant differences in seroprevalence between PICs (all $P < 0.001$) and cohorts for all viruses. For newly received cattle the average seropositive rate was 87% for BPIV-3, 56% for BVDV, 46% for BoHV-1, and 39% for BRSV.

**Table 5.4.** Summary data showing the number of animals returning conclusive seroprevalence test results and the seroprevalence estimates (percentage positive and 95% confidence interval) for four sampled cohorts each tested for presence of antibodies to four BRD viruses. n, number of cattle with unequivocal results (animals with inconclusive results are not included in this count); seroprevalence, mean (95% confidence interval.

| | **Cohort** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **8a** | | **9** | | **11** | | **12a** | |
| | n | Seroprevalence | n | Seroprevalence | n | Seroprevalence | n | Seroprevalence |
| BoHV-1 | 25 | 92 (75 - 97.8) | 85 | 57.6 (47 - 67.6) | 74 | 79.7 (69.2 - 87.3) | 140 | 10 (6.1 - 16.1) |
| BRSV | 25 | 100 (86.7 - 100) | 90 | 37.8 (28.5 - 48.1) | 75 | 13.3 (7.4 - 22.8) | 143 | 68.5 (60.5 - 75.6) |
| BVDV | 25 | 84 (65.3 - 93.6) | 84 | 60.7 (50 - 70.5) | 71 | 76.1 (65 - 84.5) | 142 | 47.2 (39.2 - 55.4) |
| BPIV-3 | 25 | 100 (86.7 - 100) | 91 | 79.1 (69.7 - 86.2) | 75 | 78.7 (68.1 - 86.4) | 143 | 97.2 (93 - 98.9) |

Carryover animals had significantly higher seropositive rates for all viruses when compared to newly received animals ($P < 0.01$).

Of the 330 animals for which serological results were available for all 4 viruses of interest (i.e. excluding animals with inconclusive results for any virus) 12 (4%) were seronegative, 47 (14%) were seropositive for antibodies to a single virus, 89 (27%) to 2 viruses, 130 (39%) to 3 viruses, and 52 (16%) to all 4 viruses (Table 5.5).

**Table 5.5**. Combinations of seropositive results to single or multiple viruses. Animals with inconclusive results for one or more viruses are not included in this table.

| Virus(es) | No. positive (% total) |
|---|---|
| BRSV, BVDV, BPIV-3 | 66 (20) |
| BoHV-1, BRSV, BVDV, BPIV-3 | 52 (16) |
| BoHV-1, BVDV, BPIV-3 | 44 (13) |
| BRSV, BPIV-3 | 38 (12) |
| BPIV-3 | 34 (10) |
| BoHV-1, BPIV-3 | 25 (8) |
| BoHV-1, BRSV, BPIV-3 | 20 (6) |
| BVDV, BPIV-3 | 16 (5) |
| Negative | 12 (4) |
| BoHV-1, BVDV | 10 (3) |
| BVDV | 10 (3) |
| BoHV-1 | 3 (1) |
| **Total** | **330** |

The results from the matched nasal swab and serum samples for each animal were combined to determine that animal's shedding and serological status for each virus at a single time-point. Animals were placed into 1 of 4 groups: seropositive and nasal swab negative, seronegative and nasal swab negative, seropositive and nasal swab positive, seronegative and nasal swab positive (Table 5.6).

**Table 5.6.** Serological status and nasal swab results for each virus for newly received animals. Pos., positive; neg., negative.

| | Serological and nasal shedding status | | | |
|---|---|---|---|---|
| | **Seroneg. Nasal pos.** | **Seroneg. Nasal neg.** | **Seropos. Nasal pos.** | **Seropos. Nasal neg.** |
| BoHV-1 | 0.7 | 58.5 | 1.3 | 39.5 |
| BRSV | 0.0 | 53.9 | 0.0 | 46.1 |
| BVDV | 3.4 | 38.7 | 1.0 | 56.9 |
| BPIV-3 | 2.6 | 10.0 | 0.6 | 86.7 |

For all viruses, the proportion of animals that were nasal swab negative and seropositive was significantly higher (all $P < 0.01$), and the proportion nasal swab negative and seronegative was significantly lower (all $P < 0.05$) for carryover animals compared to newly received animals.

The percentage of animals that were nasal swab positive and seronegative was higher in the carryover cohort (cohort 8a, 3/25 animals, 12%) than in the 3 cohorts of newly received cattle (cohorts 9, 11 and 12, 2.7-4.9%). Animals in cohort 8a had been carried over from a previous shipment for reasons including a positive serum BVDV protein A antibody-capture ELISA (PACE) antigen test.

## 5.5 Discussion

This is the first time that a systematic evaluation of the prevalence of organisms known to be associated with BRD, based on qPCR testing of nasal swab samples, has been reported for Australian cattle. In addition, this is the first time that the seroprevalence of viruses known to be associated with BRD has been determined for Australian live export cattle, although there have been a number of previous serosurveys for these viruses in Australian cattle (Dunn et al., 2000; Dunn et al., 1995; Durham and Paine, 1997; Littlejohns and

Horner, 1990; St George, 1969, 1983; St George et al., 1967; Taylor et al., 2006; Zyambo et al., 1973). Comparison of the nasal and seroprevalences determined in this study to previously published work, combined with available data on BRD-related risk factors, allows hypotheses to be made about the role of these organisms in the development of BRD in live export cattle.

The major constraints on the number of samples and range of animals (type, class, sex) that could be collected were finances, time/labour, and industry tolerance. Due to the large numbers of animals that must be processed and the economic incentives to minimise the length of time that the animals spend in the assembly depot prior to load-out, animals are protocolled through multiple depots on the same day. Depots may be hundreds or thousands of kilometres apart so it was not possible to sample cattle at all depots on all days.

Nasal swab samples were collected from 1484/55,902 (2.6%) and matched serum samples from 334 (0.6%) of cattle across the 4 study voyages. This means that, particularly in the case of serological results, the prevalences reported for sampled animals may not necessarily represent the prevalence of these organisms across the entire shipment. The collection of paired sera and calculation of seroconversion rates would have increased the power of this study. It was not possible to access the same animals for a second sampling occasion during the assembly period and there are no facilities on-board ship to re-bleed these animals.

The individual animal prevalence of BCoV was similar to that reported previously for cattle on the south coast of New South Wales with clinical BRD (Hick et al., 2012), and within the range of previously reported values for clinically normal beef feedlot cattle 0-85% (Cho et al., 2001; Fulton et al., 2011; Hasoksuz et al., 2002a; Lathrop et al., 2000b; Lin et al., 2000; Plummer et al., 2004; Thomas et al., 2006). An association between nasal shedding of BCoV

and clinical signs of respiratory disease has been reported in some studies (Hasoksuz et al., 2002a; Storz et al., 2000b; Storz et al., 1996; Thomas et al., 2006) but not others (Cho et al., 2001; Hasoksuz et al., 2005). The presence of BCoV in nasal secretions has been linked to increased odds of death due to BRD during live export voyages (Moore et al., 2014), and an increased risk of treatment for respiratory disease (Fulton et al., 2011; Lathrop et al., 2000a; Plummer et al., 2004), reduced weight gain (Thomas et al., 2006) and increased incidence of pulmonary lesions at slaughter (Lathrop et al., 2000a) in feedlot cattle.

Serological testing for BCoV antibodies was not performed as part of the study reported here due to a lack of a commercially available test kit. However, animals with BCoV in their nasal swabs tend to have low serological titres for BCoV antibodies (Hasoksuz et al., 2002a; Plummer et al., 2004; Thomas et al., 2006) and low antibody titres at feedlot entry are associated with an increased likelihood of developing clinical respiratory disease (Lathrop et al., 2000a; Plummer et al., 2004; Thomas et al., 2006) and requiring treatment for respiratory disease (Fulton et al., 2011; Plummer et al., 2004). It is therefore likely that live export cattle that are shedding BCoV on entry to the assembly depot will have low antibody titres and will thus be more likely to develop and require treatment for respiratory disease during voyages than animals without BCoV in their nasal swabs.

Bovine viral diarrhoea virus was detected in nasal swabs from 44 out of 1484 (3%) animals, with the highest prevalences in a cohort of Western Australian carry-over cattle (12%) and south Australian slaughter cattle (10%). To the authors' knowledge BVDV has not been previously detected in individual animal nasal swabs from naturally exposed cattle including beef and dairy cattle with clinical BRD (Decaro et al., 2008a; Storz et al., 2000b), and beef calves at feedlot entry (Fulton et al., 2005; Fulton et al., 2002a). Bovine viral diarrhoea virus was detected in nasal swabs and lung samples from animals that died

during voyages but was not significantly associated with fatal respiratory disease (Moore et al., 2014).

The seroprevalence of BVDV was 56% which is within the range of previously reported values for Australian cattle (45-77%) (Dunn et al., 1995; Durham and Paine, 1997; Littlejohns and Horner, 1990; St George et al., 1967; Taylor et al., 2006), and similar to the "approximately 60%" reported for Western Australian live export cattle in 1985 (Littlejohns and Horner, 1990). Seroconversion during the feeding period is linked to an increased likelihood of requiring treatment for BRD (O'Connor et al., 2001).

Thirteen out of 334 animals were nasal swab positive and seronegative for BVDV, and 3 out of 334 animals were nasal swab positive and seropositive. These animals were either transiently infected and captured immediately prior to, or soon after, mounting an immune response, or they were persistently infected (PI). Persistently infected animals have viral antigen in their nasal secretions and are mostly seronegative, although they may be seropositive if they have been exposed to a heterologous BVDV strain (Bolin, 1988; Bolin et al., 1985; Fulton et al., 2003b; McClurkin et al., 1984).

In feedlots PI animals can infect 70-100% of susceptible contacts in a pen (Fulton et al., 2005; Fulton et al., 2006). Acute BVDV infection is associated with impaired function of cells of the innate and acquired immune systems, in particular lymphoid cells, which predisposes newly infected animals to secondary bacterial infections (Al-Haddawi et al., 2007; Fulton, 2013; Peterhans et al., 2003; Ridpath, 2010). The risk of initial treatment for BRD was reported to be 43% greater in cattle exposed to PI cattle than unexposed cattle (Loneragan et al., 2005), although another study did not find an association between exposure to a PI animal and an increase in BRD prevalence (O'Connor et al., 2005). Consideration should be given to the management of potential PI animals, particularly carryover animals, during the assembly period to minimise the risk of BVDV infection and

subsequent development of BRD, bearing in mind that the application of a single PACE test to remove PI animals does not guarantee the removal of the virus from a live export consignment (Moore et al., 2014).

Bovine herpesvirus 1 prevalence was similar to that observed in cattle at feedlot entry in the U.S. (0-1.37%) (Fulton et al., 2002b; Storz et al., 2000b). Bovine herpesvirus 1 is known to play an important role in the development of BRD through both direct tissue damage and immunosuppressive effects that allow secondary bacterial infections to cause respiratory disease (reviewed in (Biswas et al., 2013; Jones and Chowdhury, 2010)). However, BoHV-1 in nasal or lung samples collected from cattle on long haul voyages is not significantly associated with mortality due to BRD (Moore et al., 2014), and the presence of BoHV-1 in nasal swabs is not associated with subsequent illness or performance in American feedlot cattle (Fulton et al., 2002b).

Thirty-nine percent (122/309) of newly received animals were seropositive for antibodies to BoHV-1. This is higher than that found in a 1967 study on Western Australian cattle (29%) but within the range of previously reported seroprevalences for Australian beef cattle (13-85%) (Dunn et al., 2000; Dunn et al., 1995; Durham and Paine, 1997; St George et al., 1967; Zyambo et al., 1973), and similar to seroprevalences reported for cattle elsewhere in the world (mean 38%, 95% confidence interval 28.8 – 46.9%) (Algirdas et al., 2008; Assie et al., 2009; Choi et al., 1982; Corbett et al., 1989; Davison et al., 1999; Fulton et al., 2000; Ghirotti et al., 1991; Ghram and Minocha, 1990; Guarino et al., 2008; Jetteur et al., 1988; Kampa et al., 2009; Mahin et al., 1985; Mahmoud and Allam, 2013; Martin et al., 1990; Martin et al., 1999; Njiro et al., 2011; Pernthaner et al., 1990; Sakhaee et al., 2009; Shirvani et al., 2012; Suzan et al., 1983; Thompson et al., 2006; Yavru et al.; Yesilbag and Gungor, 2008).

The 61% of animals that were seronegative in the study reported herein were susceptible to infection by pen-mates shedding BoHV-1. No significant increase in BoHV-1 nasal prevalence was recorded for newly received animals sampled at feedlot entry and again 9 days later. Therefore it is likely that BoHV-1 does not play a primary role in the development of BRD during voyages.

Bovine respiratory syncytial virus is recognised as a common primary pathogen in respiratory disease in calves less than 6 months of age (Brodersen, 2010; Sacco et al., 2013) and has also been reported in association with respiratory disease outbreaks in adult dairy cattle (Bidokhti et al., 2012; Ellis et al., 1996; Elvander, 1996). Bovine respiratory syncytial virus has not previously been detected in nasal swabs from beef feedlot cattle over the age of 4 months with (Decaro et al., 2008a; Hick et al., 2012; Storz et al., 2000b; Van der Poel et al., 1997) or without (Fulton et al., 2005; Fulton et al., 2002a) clinical signs of respiratory disease. However, BRSV was present in nasal swabs from 4 out of 616 (0.6%) 3 week old, clinically normal, Scottish beef and dairy calves (Hotchkiss et al., 2010) and 67-100% of 2-6 month old calves after experimental infection with BRSV (Tjornehoj et al., 2003). In this study the significant increase in BRSV nasal prevalence between depot entry and re-resting 9 days later (cohorts 12a and 12b) is likely to indicate transmission of BRSV to naïve animals during this period.

The seroprevalence of BRSV in the study reported here was 46%. This is higher than that reported from a serosurvey of Australian feedlot cattle (27%) (Dunn et al., 1995) and lower than that detected in cattle in an outbreak of respiratory disease in New South Wales (100%) (Hick et al., 2012). In other countries around the world the average seroprevalence BRSV in cattle older than 6 months is 57% (95% confidence interval 48 – 67%) (Algirdas et al., 2008; Alm et al., 2009; Assie et al., 2009; Beaudeau et al., 2010; Bidokhti et al., 2012; Burgu et al., 1990; Collins et al., 1988; Elazhary et al., 1980; Figueroa-Chavez et al., 2012;

Fulton et al., 2005; Fulton et al., 2000; Ganaba et al., 1995; Grubbs et al., 2001; Mahin et al., 1985; Mahmoud and Allam, 2013; Martin et al., 1990; Martin et al., 1999; Moteane et al., 1978; Obando et al., 1999; Pernthaner et al., 1990; Rossi and Kiesel, 1974; Saa et al., 2012; Sakhaee et al., 2009; Shirvani et al., 2012; Solis-Calderon et al., 2007; Suzan et al., 1983; Van Vuuren, 1990; Yavru et al., 2005; Yesilbag and Gungor, 2008). Seroconversion during the feeding period has been linked to an increased risk of treatment for BRD (Caldow et al., 1988; Thomas et al., 2006).

The seroprevalence for BPIV-3 (87%) was the highest out of the four viruses. This, combined with a low nasal prevalence (1.4%) and lack of evidence for an association between BPIV-3 and respiratory disease in Australian live export cattle (Moore et al., 2014) or Australian (Dunn et al., 2000; Dunn et al., 1995; Van Vuuren, 1990) or North American (Fulton et al., 2002a) feedlot cattle, suggests that BPIV-3 plays a secondary role, if any, in the development of BRD in Australian live export cattle.

In all previous studies on the prevalence of bacteria in nasal swab samples, bacterial culture was used for isolation and identification of bacteria, while in our study culture was not performed prior to PCR detection of bacterial DNA. Bacterial culture has been found to be less sensitive than PCR for identifying bacteria of interest in lung swabs (Tegtmeier et al., 2000) and milk (Fulton and Confer, 2012; Mahmmod et al., 2013). Therefore it is likely that some of the differences in prevalence observed between our study and previous studies are due to diagnostic sensitivity and sample location.

*Histophilus somni* is a commensal organism of the upper respiratory tract (Crandell et al., 1977) but is also commonly associated with a number of disease syndromes including fibrinopurulent pneumonia, myocarditis and polyarthritis-serositis (Griffin et al., 2010). The individual animal and cohort level prevalences for *H. somni* were both approximately 42%. Previous reports of the prevalence of *H. somni* in nasal swabs range from 0-9% (Allen et al.,

1991; Corbeil et al., 1986; Fulton et al., 2002a; Hall et al., 1977; Saunders and Janzen, 1980; Van Donkersgoed et al., 1994). There is currently no *H. somni* vaccination registered for use in Australia (Australian Pesticides and Veterinary Medicines Authority) so the relatively high prevalence of *H. somni* reported here is not due to detection of shedding of an attenuated vaccine strain. Nasal isolation rates at feedlot entry have not been associated with subsequent respiratory disease in feedlot cattle (Allen et al., 1991; Fulton et al., 2002a). Although the prevalence of *H. somni* in live export cattle that died during voyages was relatively high (48%) this bacteria was not significantly associated with either histological evidence of pneumonia or death due to BRD (Moore et al., 2014).

The prevalence of *M. haemolytica* was 13% at the individual level and 10% at the cohort level. Previously reported prevalences for *M. haemolytica* in nasal swabs from clinically normal feedlot cattle range from approximately 2% to 33% (Allen et al., 1991; Frank and Smith, 1983; Fulton et al., 2002a; Magwood et al., 1969; Timsit et al., 2013; Yates et al., 1983). *Mannheimia haemolytica* is found as a commensal organism of the nasopharynx and tonsils of healthy cattle and can cause respiratory disease as both a primary (Hodgins and Shewen, 2004) and secondary pathogen, although the latter is considered more common (reviewed in (Griffin, 2010; Rice et al., 2007)). An association between a higher prevalence of *M. haemolytica* in nasal swab samples and clinical respiratory disease has been found in some studies (Allen et al., 1991; Timsit et al., 2013) but not others (Frank and Smith, 1983; Fulton et al., 2002a; Magwood et al., 1969).

In a cohort of recently received animals the prevalence of *M. haemolytica* increased significantly between depot entry (6%) and resampling 9 days later (24%). This increase in prevalence is likely to be due to the proliferation of commensal bacteria following inhibition of the immune system secondary to environmental stressors, for example transportation, co-mingling, and inter-animal transmission of viruses and bacteria (Griffin,

2010; Rice et al., 2007). However, rhythmic variation in the presence/absence of *M. haemolytica* in the nasal secretions of healthy calves has been reported previously (Magwood et al., 1969).

*Pasteurella multocida* was detected in nasal swabs from 26% of cattle with a cohort level prevalence of 27%. This is within the range of previously reported prevalences for clinically normal beef cattle (6-61%) (Allen et al., 1991; Fulton et al., 2002a; Magwood et al., 1969; Van Donkersgoed et al., 1994). Most studies have not detected an association between nasal isolation rates of *P. multocida* and respiratory disease (Fulton et al., 2002a; Magwood et al., 1969; Van Donkersgoed et al., 1994) but Allen et al. (1991) found that *P. multocida* was isolated more frequently from the nasopharynx of animals with clinical signs of respiratory disease than those without. However, a study on lung tissue or swabs from beef cattle at necropsy collected between 1994 and 2002 detected a trend towards an increased isolation of *P. multocida* and reduced isolation of *M. haemolytica* as the principle bacterial pathogen associated with BRD (Welsh et al., 2004). Similarly, *P. multocida* was isolated more frequently than *M. haemolytica* in lung and nasal swab samples collected from cattle that died during live export voyages (Moore et al., 2014). There are a number of possible explanations for this trend including changes in bacterial virulence and antimicrobial resistance, changes in the efficacy of available vaccines and antibiotics, reduced age of cattle at feedlot entry, changes in the way sick cattle are identified and treated, and increased use of mass medication programs (Rice et al., 2007; Welsh et al., 2004).

*Mycoplasma bovis* is an important cause of pneumonia, arthritis and tenosynovitis in feedlot cattle (Caswell et al., 2010). *Mycoplasma bovis* was detected in nasal swabs from 5% of cattle in the study reported here. Previously reported prevalences of *M. bovis* in apparently healthy feedlot calves range from 0-43% (Allen et al., 1991; Hanzlicek et al., 2011; White et al., 2010; Wiggins et al., 2007; Yates et al., 1983). The role of *M. bovis* in the

development of BRD is still not well defined. A positive nasal swab result for *M. bovis* was associated with an increased odds of fever in one study (Wiggins et al., 2007) but had no effect on morbidity or mortality risk in another (Hanzlicek et al., 2011). The significant increase in the prevalence in *M. bovis* in newly received cattle observed between depot entry (8%) and resampling 9 days later (18%) may be due to proliferation of *M. bovis* in individual animals from undetectable to detectable levels, or indicate that inter-animal transmission of *M. bovis* is occurring during the assembly period. This, combined with the finding that *M. bovis* is significantly associated with mortality due to respiratory disease during voyages (Moore et al., 2014) suggests that *M. bovis* is an important pathogen in live export cattle.

Significant associations were detected between the presence of BCoV and BRSV, *M. haemolytica*, *P. multocida* and *H. somni* in nasal swab samples. Storz et al. (2000b) isolated a number of organisms – including BCoV, BRSV, *M. haemolytica*, and *P. multocida* – from 4-8 month old beef feedlot calves with clinical BRD. Correlations between organisms are not reported, but the researchers hypothesised that bacterial infections, in particular *M. haemolytica*, played a role in enhancing shipping fever in the cattle in their study.(Storz et al., 2000b) Co-shedding of BCoV and BRSV has not been reported previously (Decaro et al., 2008b; Storz et al., 2000b).

Of the epidemiological factors for which data were available, property of origin had the most significant effect on both nasal and seroprevalence. When differences in nasal prevalence between properties was taken into account, no significant differences were detected between animals from different states or of different sex, type (*Bos indicus* vs *Bos taurus*), class (feeder, slaughter, breeder), or weight categories. Co-mingling of cattle from multiple sources has been linked to an increased incidence of BRD in feedlot cattle (Sanderson et al., 2008; Taylor et al., 2010). This may result from the exposure of large

numbers of animals of unknown health status to a variety of viral and bacterial organisms (Sanderson et al., 2008; Taylor et al., 2010). The majority of animals on long-haul voyages are sourced directly from their property of origin to meet import protocol requirements so co-mingling at the assembly depot presents an important BRD risk.

Given that there are significant differences in nasal and seroprevalence between properties, strategies to boost immunity prior to loading (e.g. vaccination) would be more effective if they were applied at the property of origin, rather than once the animals have entered the pre-export assembly depot. However, on-farm vaccination is associated with logistic and planning implications that are not compatible with the existing live export cattle sourcing process. Therefore, while exporters could encourage producers preparing cattle for sale to the live export market to consider on-farm vaccination, the development of single shot, fast acting, efficacious vaccines that can be administered at arrival at the assembly depot may prove to be more cost effective and feasible from a management perspective.

*Mycoplasma bovis*, *M. haemolytica*, or *P. multocida* were associated with increased odds of death due to BRD during voyages (Moore et al., 2014). The significant increase in prevalence of *M. bovis* and *M. haemolytica* between depot entry and re-sampling 9 days later may be the result of transmission of bacteria between animals, or proliferation of commensal bacteria secondary to stress associated with transport and the novel assembly depot environment. However, all 4 bacteria of interest can be found as commensal organisms in the nasal cavity of apparently healthy cattle. Therefore, further work is needed to determine the link between the prevalence of bacteria in the assembly depot and risk of mortality due to BRD during voyages.

The high prevalence of BCoV and the association between this virus and deaths due to BRD during voyages (Moore et al., 2014) make it likely that BCoV plays an important role in the

development of BRD in Australian live export cattle. Based on the results of this study, BCoV would appear to be the viral pathogen of most significance for future studies into feedlot vaccination protocols. The other viruses of interest – BoHV-1, BPIV-3, BRSV, BVDV – were present at low prevalences in the assembly depot and none of these viruses were significantly associated with BRD during voyages (Moore et al., 2014). However, it is possible that these 4 viruses may be contributing to the development of BRD through their adverse effects on the respiratory tissues and immune system (Panciera and Confer, 2010; Taylor et al., 2010).

The results of this study show a mixed level of nasal prevalence and immunity to organisms associated with BRD in animals entering pre-export feedlots. Therefore, the implementation of a vaccination strategy to raise antibody levels to the common viral BRD pathogens (e.g., BCoV) prior to export may mitigate the risk of development of BRD during voyages. In addition, informed selection of animals and the application of co-mingling strategies for animals from different properties has the potential to reduce exposure of naïve animals to BRD potential pathogens and thus reduce losses due to BRD morbidity and mortality during voyages.

## 5.6 Author's contributions

SJM led the design of the study, collected and tested samples, performed the statistical analysis, and drafted the manuscript. MO assisted with development of qPCR assays and testing of samples and helped to draft the manuscript. NP assisted with the design of the study, provided advice on statistical anlaysis, and helped to draft the manuscript. AJO assisted with the design of the study and drafting of the manuscript. All authors read and approved the final manuscript.

## 5.7 Acknowledgements

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## 5.8 Supplementary Tables

**Supplementary Table 5.1.** Results of negative binomial regression analysis. BCoV, Bovine coronavirus; IRR, incidence rate risk; NS, explanatory variable not suitable for analysis due to zero values.

| | | | BCoV | | | | *H. somni* | | | | *M. haemolytica* | | | | *P. multocida* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Variable** | **Category** | **nTested** | **Positive** | **IRR** | **95% CI** | **P value** | **Positive** | **IRR** | **95% CI** | **P value** | **Positive** | **IRR** | **95% CI** | **P value** | **Positive** | **IRR** | **95% CI** | **P value** |
| VoyageId | | | | | | | | | | | | | | | | | | |
| | 5 | 410 | 312 | 3.6 | (1.6 – 8.3) | 0.008 | 67 | Reference | | 0.001 | 97 | 14.8 | (5.3 – 41.5) | <0.0001 | 165 | 14.0 | (2.7 – 73.6) | 0.004 |
| | 8 | 413 | 122 | 1.4 | (0.8 – 2.5) | | 123 | 4.4 | (0.7 – 26.2) | | 54 | 4.6 | (1.8 – 11.8) | | 118 | 6.5 | (1.4 – 30.6) | |
| | 17 | 435 | 86 | Reference | | | 253 | 8.1 | (1.4 – 47.8) | | 15 | Reference | | | 79 | 4.5 | (0.9 – 21.5) | |
| | 18 | 100 | 47 | 2.3 | (1.1 – 4.7) | | 87 | 11.7 | (1.9 – 72.3) | | 6 | 1.8 | (0.4 – 7.5) | | 4 | Reference | | |
| SampMth | | | | | | | | | | | | | | | | | | |
| | 2 | 413 | 122 | 7.0 | (0.9 – 56.7) | 0.018 | 123 | 4.5 | (0.9 – 22.8) | <0.0001 | 54 | 14.9 | (3.2 – 265) | <0.0001 | 118 | 2.8 | (0.7 – 11.3) | 0.010 |
| | 3 | 435 | 129 | 7.3 | (0.9 – 58.7) | | 321 | 10.2 | (2 – 51.1) | | 20 | 4.8 | (1 – 86.1) | | 74 | 1.6 | (0.4 – 6.9) | |
| | 4 | 100 | 4 | Reference | | | 19 | 2.9 | (0.5 – 18.7) | | 1 | Reference | | | 9 | Reference | | |
| | 12 | 410 | 312 | 18.0 | (2.1 – 157.2) | | 67 | Reference | | | 97 | 31.7 | (6.9 – 562) | | 165 | 6.0 | (1.3 – 27.8) | |
| SampSeason | | | | | | | | | | | | | | | | | | |
| | Summer | 823 | 434 | 2.6 | (1.2 – 5.7) | 0.018 | 190 | Reference | | 0.047 | 151 | 5.4 | (2.5 – 11.6) | <0.0001 | 283 | 2.9 | (1.3 – 6.1) | 0.006 |
| | Autumn | 535 | 133 | Reference | | | 340 | 6.6 | (1 – 43.1) | | 21 | Reference | | | 83 | Reference | | |
| SampYr | | | | | | | | | | | | | | | | | | |
| | 2010 | 410 | 312 | 2.7 | (1.2 – 6.1) | 0.052 | 67 | Reference | | 0.134 | 97 | 12.5 | (5.1 – 30.6) | <0.0001 | 165 | 3.9 | (1.7 – 9) | 0.0049 |
| | 2011 | 413 | 122 | 1.1 | (0.6 – 1.8) | | 123 | 4.4 | (0.8 – 26.1) | | 54 | 3.9 | (1.8 – 8.6) | | 118 | 1.8 | (1 – 3.1) | |
| | 2012 | 535 | 133 | Reference | | | 340 | 9.0 | (1.5 – 52.3) | | 21 | Reference | | | 83 | Reference | | |
| AssDepot | | | | | | | | | | | | | | | | | | |
| | 1 | 300 | 164 | 30.6 | (3.2 – 292.5) | <0.0001 | 140 | 21.4 | (1.3 – 3.4) | 0.053 | 15 | | | NS | 48 | 6.7 | (0.1 – 402.1) | 0.0007 |
| | 2 | 112 | 2 | 1.0 | | | 11 | 2.4 | (0.1 – 52.9) | | 6 | | | | 50 | 66.3 | (1.3 – 3267.2) | |
| | 7 | 102 | 58 | 35.0 | (21.3 – 406.5) | | 5 | Reference | | | 5 | | | | 20 | 29.5 | (0.6 – 1554.9) | |
| | 3 | 100 | 6 | 3.3 | (0.3 – 43.4) | | 58 | 12.5 | (0.8 – 207.4) | | 7 | | | | 15 | 18.4 | (0.3 – 980.2) | |
| | 4 | 299 | 244 | 41.2 | (4.4 – 383.6) | | 63 | 9.5 | (0.6 – 152.6) | | 124 | | | | 154 | 63.0 | (1 – 3045.3) | |
| | 5 | 248 | 38 | 10.4 | (1.1 – 101.4) | | 139 | 15.8 | (1 – 252) | | 8 | | | | 33 | 24.0 | (0.5 – 1101.9) | |
| | 8 | 112 | 41 | 20.1 | (2.1 – 194.6) | | 61 | 13.8 | (0.8 – 224.4) | | 7 | | | | 45 | 58.0 | (1.2 – 2845.6) | |
| | 6 | 75 | 7 | 7.6 | (0.7 – 82.3) | | 53 | 15.3 | (0.9 – 249.1) | | 0 | | | | 1 | Reference | | |
| AssDepotState | | | | | | | | | | | | | | | | | | |
| | SA | 112 | 41 | Reference | | 0.697 | 61 | 14.6 | (0.5 – 421.4) | 0.294 | 7 | Reference | | 0.817 | 45 | 1.9 | (0.3 – 11.4) | 0.406 |
| | VIC | 102 | 58 | 1.8 | (0.4 – 9.1) | | 5 | Reference | | | 5 | 1.1 | (0.1 – 17.1) | | 20 | Reference | | |
| | WA | 1134 | 461 | 1.0 | (0.4 – 2.8) | | 464 | 13.7 | (0.5 – 374.3) | | 160 | 1.6 | (0.3 – 7.8) | | 301 | 1.0 | (0.2 – 5.5) | |
| Sex | | | | | | | | | | | | | | | | | | |
| | Bull | 310 | 82 | Reference | | 0.954 | 201 | 1.5 | (0.9 – 2.3) | 0.210 | 25 | 1.6 | (0.3 – 7.9) | 0.405 | 71 | 1.2 | (0.4 – 3.2) | 0.632 |
| | Heifer | 174 | 51 | 1.2 | (0.5 – 2.9) | | 90 | 1.5 | (0.7 – 3.1) | | 12 | Reference | | | 41 | Reference | | |
| | Steer | 853 | 434 | 1.0 | (0.7 – 1.6) | | 236 | Reference | | | 135 | 2.4 | (0.5 – 10.6) | | 252 | 1.4 | (0.6 – 3.8) | |
| Type | | | | | | | | | | | | | | | | | | |
| | Indicus | 230 | 77 | Reference | | 0.193 | 114 | 1.0 | (0.7 – 1.5) | 0.893 | 23 | Reference | | 0.765 | 53 | Reference | | 0.636 |
| | Taurus | 1117 | 483 | 1.3 | (0.9 – 1.9) | | 415 | Reference | | | 149 | 1.1 | (0.6 – 2.2) | | 313 | 1.1 | (0.7 – 1.9) | |
| Class | | | | | | | | | | | | | | | | | | |
| | Breeder | 100 | 47 | 1.8 | (0.6 – 5.2) | 0.529 | 87 | 2.0 | (7 – 23.5) | 0.275 | 6 | Reference | | 0.491 | 4 | Reference | | 0.107 |
| | Feeder | 749 | 330 | 1.0 | (0.5 – 2.2) | | 264 | 1.1 | (0.6 – 2.2) | | 113 | 2.0 | (0.2 – 17.7) | | 261 | 6.7 | (1 – 44.5) | |
| | Slaughter | 499 | 183 | Reference | | | 179 | Reference | | | 53 | 3.1 | (0.3 – 28.4) | | 101 | 4.8 | (0.7 – 33.4) | |
| Weight | | | | | | | | | | | | | | | | | | |
| | | | | 1.01 | (0.96 – 1.06) | 0.687 | | 0.85 | (0.65 – 1.11) | 0.225 | | 1.02 | (0.93 – 1.11) | 0.740 | | 1 | (0.93 – 1.08) | 0.978 |

# Chapter 6: Risk factors for voyage mortality in cattle exported live from Australia by sea

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## 6.1 Summary

This study was initiated to improve our understanding of mortality trends and risk factors for death in cattle exported live from Australia.

Mortality data for all voyages from Australia to all destinations between 1995 and 2012 were analysed retrospectively. Daily mortality trends were assessed for 20 long-haul voyages from Australia to the Middle East and Russian Federation between 2010 and 2012.

The overall voyage mortality percentage was 0.17% across the 13 million cattle exported on 6,447 voyages. Mortality rates decreased significantly after 2000 and stabilised at low levels from 2003. The mortality rate for voyages to the Middle East and North Africa (0.44%) was significantly higher than for voyages to South East Europe (0.28%), North East Asia (0.12%) and South East Asia (0.09%). Cattle exported from ports in southern Australia carry a higher mortality risk than those exported from northern ports for both long- and short-haul voyages. The daily mortality rate peaks at 3-4 weeks post departure.

The marked reduction in mortality rate since 2000 is due to a number of factors including industry initiatives, government legislation, and market demand that have resulted in changes to the selection of cattle for export and management of cattle prior to and during voyages. Routine collection of animal performance data combined with NLIS records and use of methods described in this paper have the potential to contribute to more effective management of mortality risks across the export chain.

## 6.2 Introduction

Australia's international live cattle export trade began in the 1800s. In the 1840s shipments of 30-45 breeding cattle and bullocks were exported from New South Wales to New Zealand (Anonymous, 1840) and further exports in the 1860s were to supply meat for the military (Anonymous, 1887). The first shipment of cattle to South East Asia (Hong Kong) occurred in 1885 (Anonymous, 1885). However, it was not until the 1970s and early 1980s, when economic development in some South East Asian (SE Asian) countries led to an increased demand for red meat, that there was significant growth in live cattle exports to this region (Farmer, 2011).

Voyage mortality percentages (number of cattle dead/number of cattle loaded) for most live export cattle voyages are below reportable levels: 0.5% for voyages less than 10 days duration, and 1% for voyages of 10 days or more (Australian Standards for Export of Livestock, Standard 5.2 (DAFF, 2011)). However, the sporadic occurrence of mortality incidents, i.e. where mortality is equal to or greater than the reportable level (DAFF, 2014), means that there is ongoing public pressure on the industry to demonstrate that it is actively committed to managing mortality risk and minimising deaths during voyages.

A previous study on mortality in Australian live export cattle involved the examination of ship Masters' reports for cattle voyages between 1995 and 2000 to determine mortality percentages and identify risk factors for death (Norris et al., 2003). For the period examined the average overall mortality percentage was 0.24% across 2,732 voyages. Important findings from this study included: significantly higher mortality on voyages to the Middle East than to SE Asia; significantly higher mortality in cattle exported from southern compared to northern ports; an increased risk of death for animals arriving in the Middle East summer compared to those arriving at other times of year (Norris et al., 2003).

The study reported here was initiated to investigate risk factors for mortality in live export cattle using data from Master's reports and additional sources, allowing more detailed analysis than was possible previously. Since the late 1990s industry initiatives, government legislation, and market demand have resulted in changes to the selection of cattle for export and management of cattle prior to and during voyages. This study provides an opportunity to compare the potential effects of these changes on mortality rates and risks.

## 6.3 Materials and Methods

### 6.3.1 Retrospective data: 1995-2012

Retrospective data for sea voyages between January 1995 and December 2012 were obtained from the Shipboard Mortality Database (SMDB) which is funded by Meat & Livestock Australia and administered by the Department of Agriculture and Food, Western Australia. The SMDB provides a repository of information, including livestock mortalities, on every voyage on which sheep, cattle, and/or goats are transported live by sea from Australia (Norris and Norman, 2013). For the purposes of this report only voyages carrying cattle, either as the only species or as a mixed sheep/cattle shipment, were included in the analysis.

Load regions were coded as described previously (Norris et al., 2003): northern ports are those north of 20° latitude south (Port Hedland, Broome, Wyndham, Darwin, Karumba, Weipa, Mourilyan, Townsville); southern ports are those south of 31° latitude south (Fremantle, Bunbury, Esperance, Thevenard, Adelaide, Portland, Geelong, Devonport, Port Kembla, Sydney, Newcastle); other ports are those located between 20° and 31° latitude south (Geraldton, Denham, Carnarvon, Dampier, Mackay, Gladstone, Brisbane).

Destination regions were coded based on destination port and country location using a similar approach to that described in earlier annual reports (Norris and Norman, 2013). Regions included Middle East and North Africa (MENA: Bahrain, Egypt, Israel, Jordan, Kuwait, Libya, Oman, Pakistan, Qatar, Saudi Arabia, Sudan, United Arab Emirates), South East Asia (SE Asia: Brunei, Indonesia, Malaysia, Philippines, Singapore, Vietnam), North East Asia (NE Asia: China, Japan, Russian ports on the Pacific facing coast of Russia, South Korea), South East Europe (SE Europe: Turkey and Russian ports on the Black Sea), Miscellaneous (East Timor, Mauritius, Mexico, New Caledonia, Russian ports on the west coast near Finland, Samoa, Solomon islands, Sri Lanka).

The primary outcome variable (voyage mortality rate) was based on a numerator measuring the count of deaths that occurred during loading, voyage and discharge for each voyage, and a denominator representing animal time at risk. Animal time at risk was calculated by multiplying the total cattle loaded by the sum of voyage days plus discharge days.

Preliminary descriptive analysis of voyage mortality rate was suggestive of overdispersion and consequently a negative binomial model was used to model associations between total mortality and explanatory variables of interest (voyage year, voyage month, load region, discharge region). Models incorporated an offset for animal days at risk and ship was included as a cluster variable to adjust for clustering of animals within each ship. Model output is reported as a voyage mortality rate and represents a cumulative incidence rate using the total number of cattle loaded at the beginning of the voyage as the population at risk of dying during a voyage.

Univariate models with significant overall effects for an explanatory variable were followed by pair-wise comparisons to assess for differences between individual levels of the explanatory variable. For variables with 4 or more levels, pair-wise comparisons were

reported with adjusted p-values using the False Discovery Rate approach to ensure that family-wise type-I error rates were maintained at or below 0.05 (Pike, 2011).

### 6.3.2 Daily mortality data

Daily mortality data were sourced from the Master's Reports for 20 research voyages enrolled in a study on the causes of mortality in live export cattle on long haul voyages (Moore et al., 2014). Raw data on the number of deaths per day, total count of cattle loaded and the duration in days for each voyage were used to generate a dataset with one row per animal. Each row included a variable measuring time at risk for that animal. For animals that survived, time at risk was equal to the length of the voyage in days and for animals that died it was equal to the voyage day when death occurred.

Voyages going to the Aegean Sea, Sea of Marmara and the Black Sea were all considered to be geographically proximate (SE Europe) and were assigned to 1 level of the binary variable called destination. The other level included voyages going to the Persian Gulf (only a single voyage), ports in the Red Sea and 1 voyage that went to a port in the lower Mediterranean Sea.

Daily mortality rate data were analysed using survival analysis. An attempt was made to build a multivariable model using Cox proportional hazards. All variables were considered for inclusion and were removed if they were not significant. The model reduced down to a single effect, coding for destination (0, 1). The findings were identical to those reported for the univariate screening. Destination was then allowed to interact with the natural logarithm of time at risk to assess for evidence of violation of proportional hazards. The interaction term was not significant ($P = 0.1$). Schoenfeld residuals were assessed and there was little evidence of violation of proportional hazards.

A flexible parametric survival model (Royston-Parmar model) was then applied in Stata (Royston and Lambert, 2011). An initial model was fitted that incorporated proportional

hazards assumptions (no interaction between destination and time). The approach involved fitting splines using five degrees of freedom and then generating predicted hazards for defined time periods (based on the range of time periods covered by both levels of the destination variable (out to day 35). Model output was used to describe mortality rates per day of voyage (daily mortality rate).

### 6.3.3 Cattle movement data

In Australia it is mandatory for cattle to be tagged with a radio frequency identification device (RFID) that is linked to the property identification code (PIC) in the National Livestock Identification System (NLIS) (MLA, 2012b). A property identification code may be used to identify properties, saleyards, feedlots, processors (abattoirs), pre-export assembly depots, and ports of export. When cattle move from one PIC to another the move must be recorded in the NLIS database within 7 days. The data in the NLIS was used to identify putative risk factors for cattle based on their physical location throughout their life prior to embarkation

The RFID code was used to extract movement histories from the NLIS database for Western Australian live export cattle on 3 voyages. The data contains the date the movement was recorded on the database, and the start and end locations of each move. As the record date can be up to 7 days after the actual movement, an algorithm was developed which ordered these data for each animal to ensure the logical sequence from property to property.

Property locations were ascertained by using a number of data sources, including the public brands database (Department of Agriculture and Food Western Australia, 2014. Available at: http://spatial.agric.wa.gov.au/brands/index.asp), Google Earth (Google, 2014. Available at: http://www.google.com/earth/) and saleyard postcode locations. Where a property could not be positively identified by a combination of the above methods the

location was approximated by using the centroid of the polygon containing the feature. The resulting dataset was then used to infer location (consolidated to the shire level and also to a corresponding climate zone), number of moves and distance travelled for each animal, for the whole of life and 90 days prior to export. These movements were viewed using a Geographic Information System to identify general patterns.

## 6.4 Results

### 6.4.1 Retrospective data

Between January 1995 to December 2012 cattle were transported from 29 ports in Australia to 124 ports in 30 countries around the globe (Table 6.1). South East Asia accounts for the majority of the market share; on average of 78% (range 53-97%) of cattle exported per year are destined for this market. Approximately 21% (range 3-47%) of cattle are exported to countries in the MENA, and 6% (1-18%) to NE Asia. The number of cattle exported to each market varies each year (Figure 6.1).

Across all voyages the average mortality percentage (number of cattle dead/number of cattle loaded) was 0.17%. On 2,976 out of 6,447 voyages (46.2%) there were no mortalities (Table 6.1). There was a significant correlation between the number of deaths during the voyage and discharge phases ($P < 0.001$, rho 0.342).

**Table 6.1.** Summary statistics for all voyages from Australia to all destinations between 1995 and 2012.

| Parameters | Units | MENA | SE Asia | NE Asia | SE Europe | Misc | Total |
|---|---|---|---|---|---|---|---|
| Voyages | N | 1,028 | 4,909 | 395 | 14 | 101 | 6,447 |
| Cattle loaded | N | 2,632,296 | 9,378,399 | 700,567 | 75,170 | 198,084 | 12,984,516 |
| Mortality overall | % of cattle loaded | 0.44 | 0.09 | 0.12 | 0.28 | 0.46 | 0.17 |
| Mortality range per voyage | % of cattle loaded | 0-41.5 | 0-4.8 | 0-2.6 | 0-0.87 | 0-74.7 | 0-74.7 |
| Average voyage duration | days | 17.5 | 6.6 | 17.0 | 28.8 | 18.6 | 9.2 |
| Average discharge period | days | 3.8 | 0.9 | 1.3 | 3.6 | 1.4 | 1.4 |
| Voyages with zero mortalities | N | 293 | 2,533 | 118 | 1 | 31 | 2,976 |

Four very high mortality voyages (mortality rate >26%) were removed from the dataset prior to analysis of voyage mortality rates (hereafter referred to as 'mortality rates'). Mortality percentages for these voyages were 3 to 9 times higher than the next highest voyage. These voyages were all investigated by the Australian government and were described as being special events caused by a combination of vessel mishaps, for example ventilation breakdown and/or adverse weather conditions (DAFF, 2014).

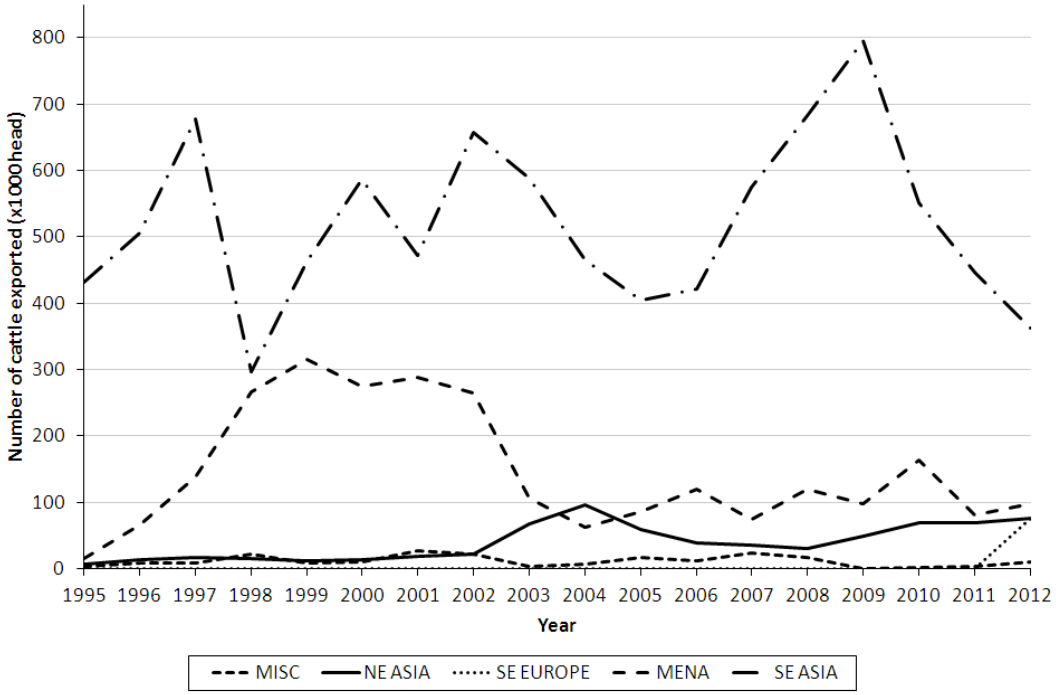


**Figure 6.1.** Total number of cattle exported from Australia between 1995 and 2012 by year and destination region.

The annual mortality rate (per 1,000 cattle days) rose sharply from 1996-1998, stabilised between 1998 and 2000, and decreased from 2000-2003 (Figure 6.2). The annual mortality rate for 2000 was significantly higher than for 2003 onwards, with the exception of 2007 ($P = 0.14$) (Figure 6.2). There was no difference in the annual mortality rate for 2002 and rates from 2003 to 2009 (all $P > 0.05$). However, there was a significant difference between 2002 and rates for 2010, 2011 and 2012 (all $P < 0.05$). The rate in 2009 was not different to 2010 ($P = 0.063$) or 2012 ($P = 0.14$) but was significantly higher than in 2011 ($P = 0.046$).

Monthly mortality rates grouped by year period (1995-2002, 2003-2009, 2010-2012) can be found in (Figure 6.3). For 1995-2002 there is a distinct peak in mortality rate in summer (December to March) but this seasonal fluctuation is less marked in later years.

Voyages loaded in southern ports had significantly higher mortality rates than voyages loaded in northern ($P < 0.001$) or other regions ($P = 0.001$). There was no difference between northern ports and other regions ($P = 0.7$). When only voyages that loaded in southern ports were considered, the mortality rate for SE Asia was found to be significantly higher than all other destinations except the Miscellaneous group ($P = 0.08$) (Figure 6.4).

Across all years the mortality rate for voyages to different regions were all significantly different to each other with the exception of the MENA and miscellaneous destinations ($P = 0.54$), SE Asia and SE Europe ($P = 0.09$), and NE Asia and SE Europe ($P = 0.09$). When results were analysed by year period mortality rates on voyages to SE Asia, MENA and miscellaneous destinations decreased with increasing year period (Figure 6.5). For NE Asia the trend was similar although there has been an increase in mortality between 2003-2009 and 2010-2012 (Figure 6.5).

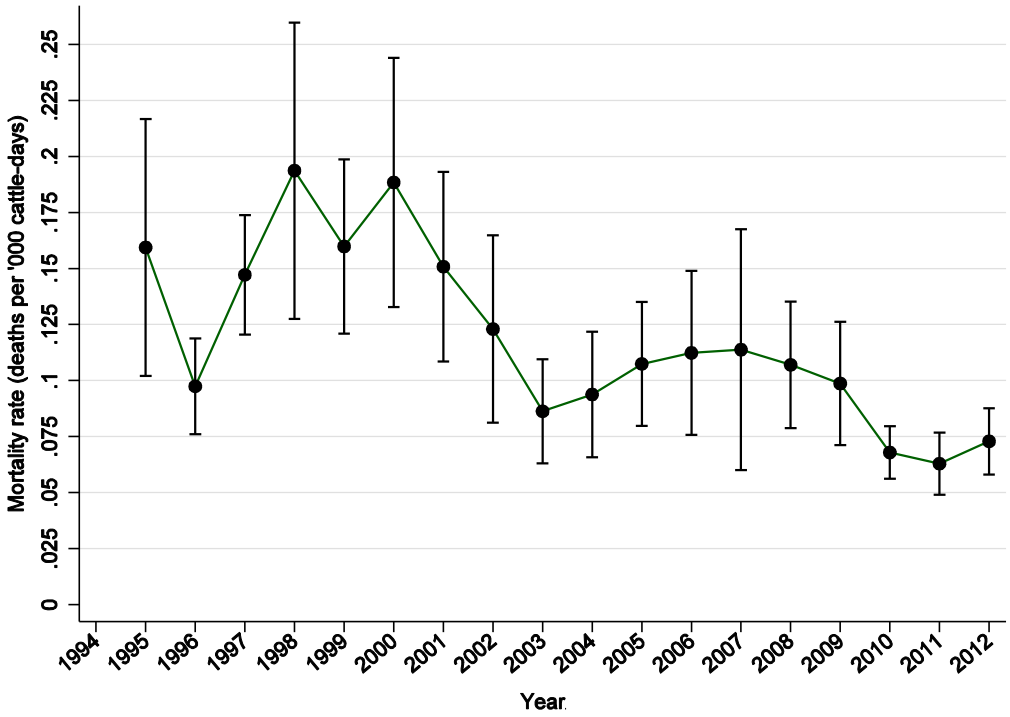


**Figure 6.2.** Average annual voyage mortality rate for live export cattle voyages from Australia between 1995 and 2012 by year. Bars represent 95% confidence intervals.

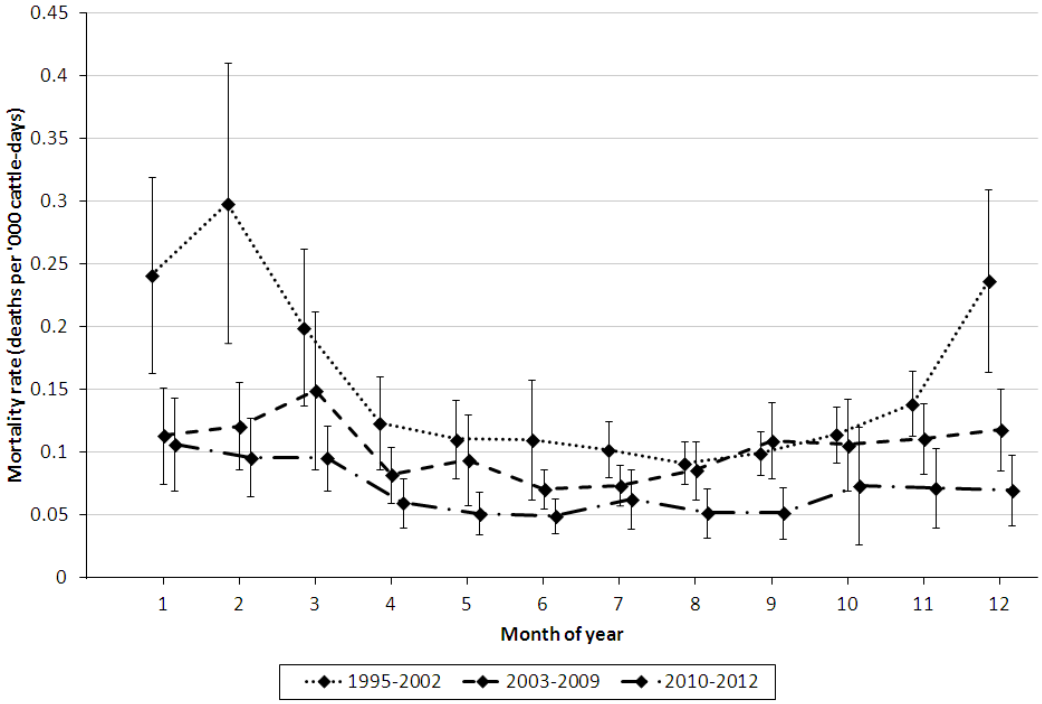


**Figure 6.3.** Average monthly voyage mortality rate for live export cattle voyages from Australia between 1995 and 2012 by year period. Bars represent 95% confidence intervals.

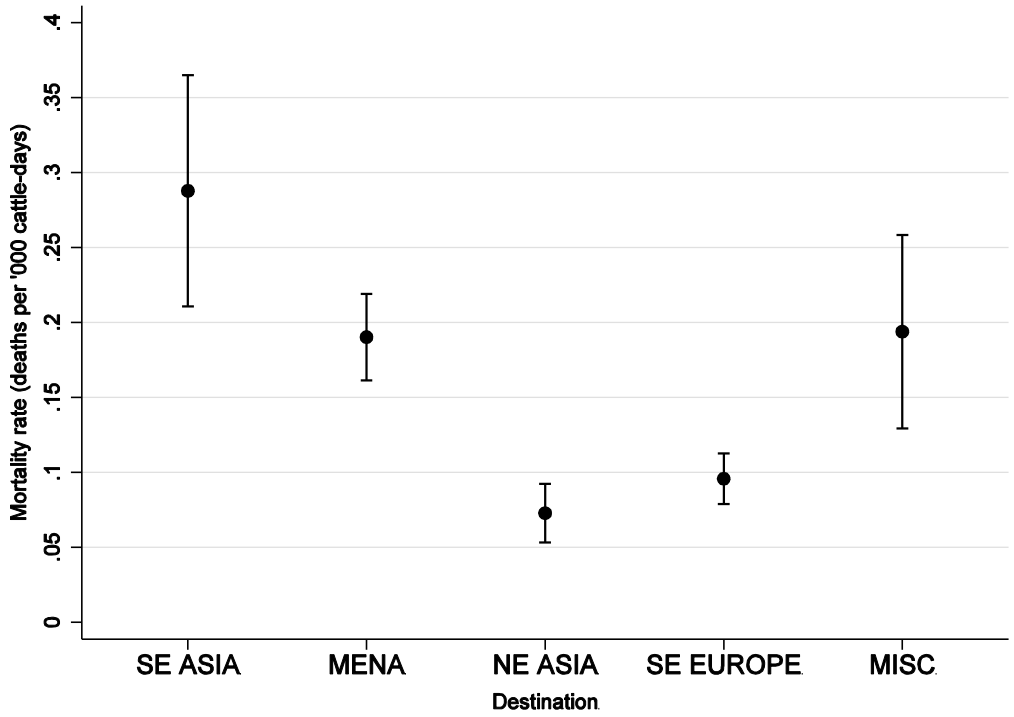


**Figure 6.4.** Voyage mortality rate by destination region for voyages loaded at ports south of 31° latitude south between 1995 and 2012. Bars represent 95% confidence intervals.

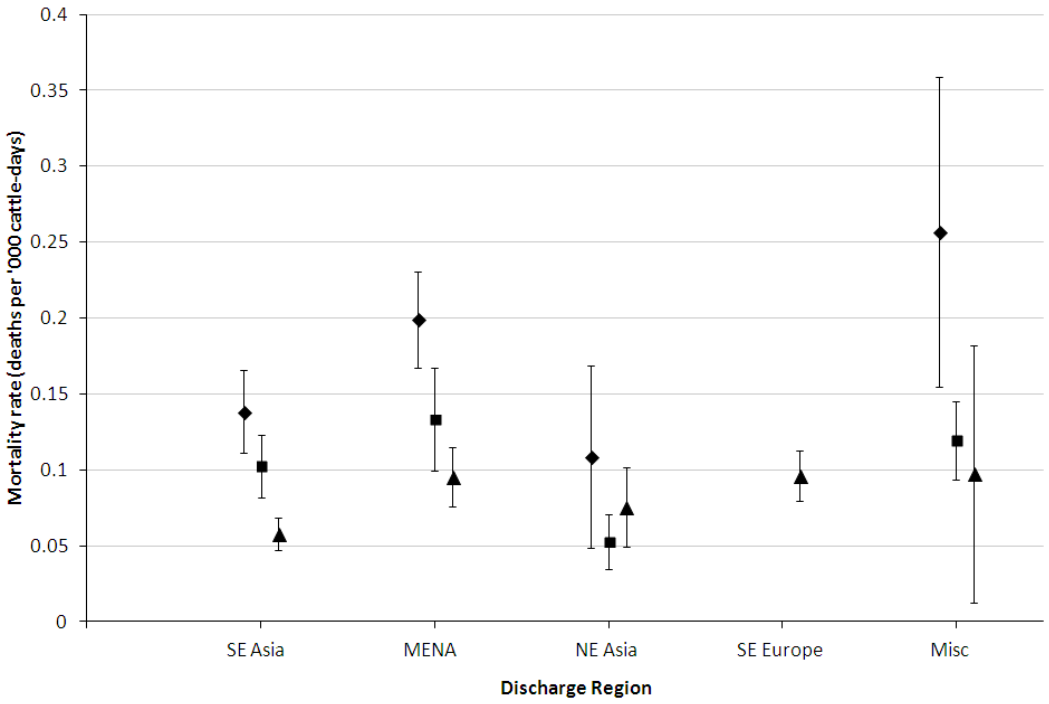


**Figure 6.5.** Voyage mortality rate by destination region and year. Diamonds, 1995-2002; squares, 2003-2009; triangles, 2010-2012. Bars represent 95% confidence intervals.

### 6.4.2 Daily mortality data

Data were obtained from 20 voyages. Summary statistics from the 20 voyages are presented in Table 6.2. Output from univariable screening tests, with adjustment for clustering at the voyage level, are presented in Table 6.3.

A graph of daily mortality rate estimates over time, produced as predictions following a Royston-Parmar survival model, can be found in Figure 6.6. The line is curved, indicating that the daily mortality is dynamic, changing from day to day throughout the voyage. Peak daily mortality rate occurs at between approximately three and four weeks after departure. There is also a reduction in the slope of the curve between day 10 and 15. Daily mortality rates for voyages to the Red Sea and Persian Gulf (MENA) are consistently lower than voyages to the Aegean Sea, Black Sea and Sea of Maramara (Turkey, Russian Federation), although this difference was not significant ($P$ = 0.07, Table 6.3).

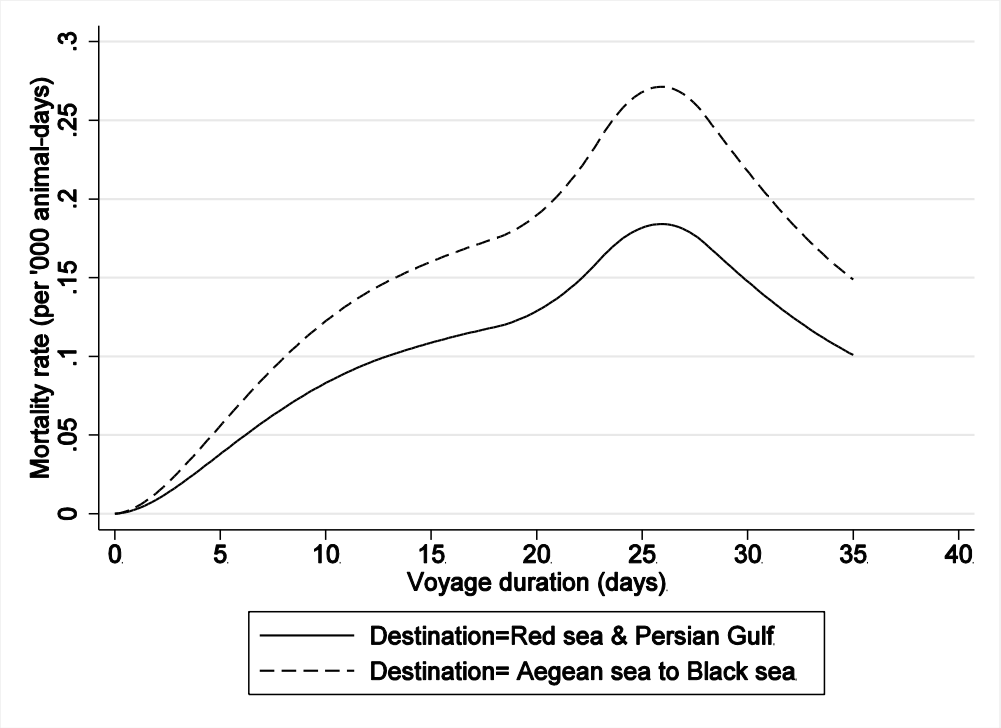


**Figure 6.6.** Daily mortality rate estimates over time for voyages to the Red Sea and Persian Gulf and Aegean Sea to Black Sea produced as predictions following a Royston-Parmar model.

**Table 6.2.** Voyage data for voyages used for analysis of daily mortality data. Voyage number 19 is not included since its destination (China) was not MENA or SE Europe. *Number of deaths per '000 cattle days, † Voyages for which animal movement data were available.

| Voyage ID | Load month (n) | Total cattle loaded (n) | Total deaths (n) | Voyage mortality percentage (%) | Total voyage length (days) | Voyage mortality rate* | First discharge (days) | Destination | Split discharge | Split load |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 3 | 9,430 | 16 | 0.170 | 21 | 0.081 | 17 | Red Sea | Yes | No |
| 2 | 5 | 9,213 | 13 | 0.141 | 24 | 0.059 | 22 | Mediterranean Sea | No | No |
| 3 | 8 | 5,090 | 4 | 0.079 | 19 | 0.041 | 18 | Red Sea | No | No |
| 4 | 10 | 10,428 | 11 | 0.105 | 18 | 0.059 | 15 | Red Sea | No | No |
| 5† | 1 | 19,990 | 148 | 0.740 | 24 | 0.308 | 18 | Aegean Sea | Yes | No |
| 6 | 1 | 3,994 | 21 | 0.526 | 30 | 0.175 | 27 | Black Sea | No | No |
| 7 | 1 | 16,255 | 25 | 0.154 | 35 | 0.044 | 22 | Sea of Marmara | Yes | No |
| 8† | 3 | 17,484 | 52 | 0.297 | 34 | 0.087 | 22 | Red Sea | Yes | Yes |
| 9 | 3 | 10,237 | 60 | 0.586 | 42 | 0.140 | 27 | Sea of Marmara | Yes | No |
| 10 | 5 | 12,763 | 54 | 0.423 | 34 | 0.124 | 30 | Black Sea | No | Yes |
| 11 | 6 | 9,000 | 106 | 1.178 | 37 | 0.318 | 36 | Sea of Marmara | No | Yes |
| 12† | 7 | 9,239 | 27 | 0.292 | 23 | 0.127 | 22 | Red Sea | No | Yes |
| 13 | 7 | 1,350 | 6 | 0.444 | 20 | 0.222 | 18 | Persian Gulf | No | Yes |
| 14 | 8 | 4,274 | 15 | 0.351 | 31 | 0.113 | 29 | Sea of Marmara | Yes | Yes |
| 15 | 9 | 12,256 | 61 | 0.498 | 30 | 0.166 | 27 | Sea of Marmara | No | Yes |
| 16 | 1 | 9,811 | 17 | 0.173 | 18 | 0.096 | 17 | Red Sea | No | No |
| 17 | 4 | 7,811 | 39 | 0.499 | 32 | 0.156 | 30 | Sea of Marmara | No | Yes |
| 18 | 4 | 9,068 | 33 | 0.364 | 34 | 0.107 | 33 | Sea of Marmara | No | Yes |
| 20 | 5 | 11,538 | 37 | 0.321 | 36 | 0.089 | 30 | Sea of Marmara | Yes | Yes |
| 21 | 6 | 8,209 | 21 | 0.256 | 26 | 0.098 | 23 | Red Sea | No | Yes |
| **Total** | | 197,440 | 766 | 0.388 | | | | | | |

**Table 6.3.** Output from univariable screening tests, run as separate Cox proportional hazard models with adjustment for clustering at the voyage level. HR, hazard ratio; se, standard error; z, z statistic; p, p-value; CI, confidence interval.

| Variable | Level | HR | se | z | p | 95% CI | |
|---|---|---|---|---|---|---|---|
| | | | | | | Upper | Lower |
| Destination | Red Sea, Persian Gulf , Mediterranean Sea | ref | | | | | |
| | Aegean & Black Seas | 1.532 | 0.363 | 1.8 | 0.072 | 0.963 | 2.44 |
| Destination | Red Sea | ref | | | <0.001 | | |
| | Aegean Sea | 3.204 | 0.240 | 15.52 | <0.001 | 2.766 | 3.712 |
| | Mediterranean Sea | 0.609 | 0.046 | -6.62 | <0.001 | 0.526 | 0.705 |
| | Persian Gulf | 2.806 | 0.247 | 11.71 | <0.001 | 2.361 | 3.335 |
| | Russia | 1.181 | 0.158 | 1.25 | 0.21 | 0.909 | 1.535 |
| | Sea of Marmara | 1.197 | 0.264 | 0.82 | 0.41 | 0.777 | 1.844 |
| Season of loading | Summer | ref | | | 0.87 | | |
| | Autumn | 0.729 | 0.377 | -0.61 | 0.54 | 0.265 | 2.006 |
| | Winter | 0.870 | 0.489 | -0.25 | 0.80 | 0.289 | 2.619 |
| | Spring | 0.767 | 0.414 | -0.49 | 0.62 | 0.267 | 2.207 |
| Discharge period (time-varying) | Before start of first discharge | ref | | | | | |
| | After start of first discharge | 0.822 | 0.324 | -0.5 | 0.62 | 0.379 | 1.782 |
| Split load | No | ref | | | | | |
| | Yes | 0.996 | 0.373 | -0.01 | 0.99 | 0.477 | 2.08 |
| Split discharge | No | ref | | | | | |
| | Yes | 0.901 | 0.331 | -0.28 | 0.78 | 0.439 | 1.85 |

### 6.4.3 Cattle movement data

Whole-of-shipment RFID identifier lists were available for 3 study voyages (voyage number 5, 8 and 12) (Table 6.2). Movement data were available for all animals on these lists exported from Western Australia, allowing analysis of 28,152 of the 38,068 (74%) cattle movement histories. Over the lifetime record, the number of property to property movements ranged from 1 to 10, covering from 6 to 3790 kilometres and originating from 105 shires. In the 90 days prior to export, animals recorded between 1 and 8 moves, and travelled between 5 and 2375 kilometres, and starting from 1 of 98 shires. The range of the movements is shown in Figure 6.7.

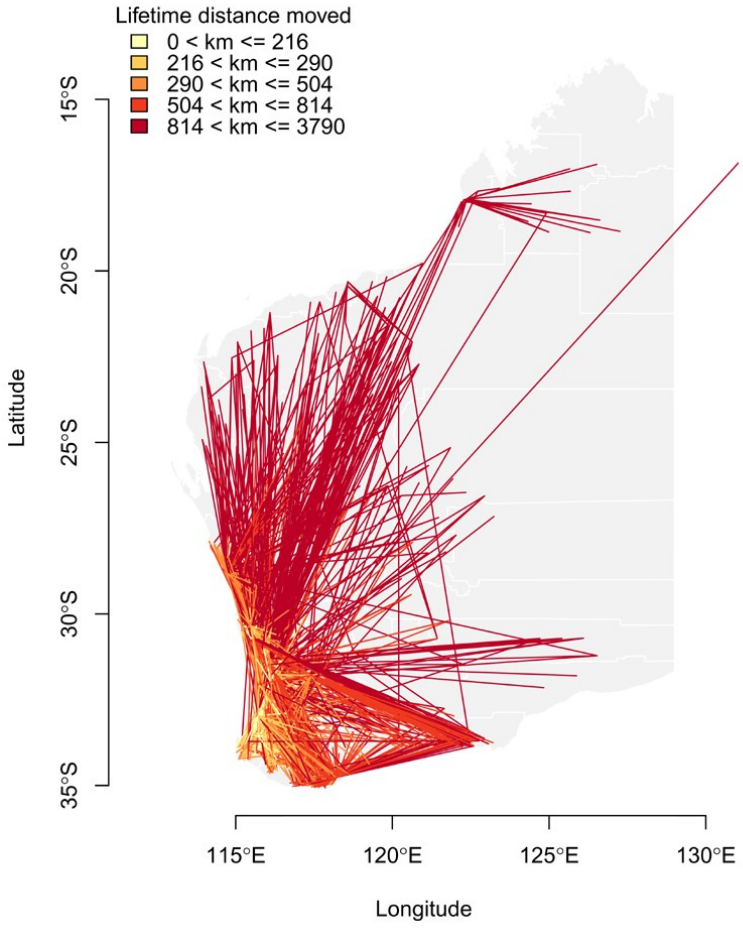


**Figure 6.7.** Lifetime movement pathways of all cattle exported on 3 voyages as reported to the National Livestock Identification System.

## 6.5 Discussion

In this paper we have described trends in and risk factors for mortality based on retrospective analysis of records from 6,447 voyages between 1995 and 2012, analysis of daily mortality data from 21 voyages between 2010 and 2012, and stock movement records for cattle from three voyages between 2010 and 2012.

The number of cattle exported varies year on year in response to market forces including consumer demand, emerging diseases and the value of the Australian dollar (Agra CEAS Consulting, 2008). Although a detailed discussion of the local and global economic trends affecting Australia's live export industry is beyond the scope of this report, a number of general comments can be made.

Fluctuations in exports to SE Asian countries reflect periods of economic downturn and recovery in the SE Asian economies. For example, there was a dramatic decrease in the number of cattle exported to SE Asia in 1997 following the financial crisis in that region (Agra CEAS Consulting, 2008). During the same period live cattle exports to the MENA increased as cattle were redirected from SE Asian markets (Johnston, 2000) and trade restrictions were imposed on imports from European and South American countries in response to outbreaks of bovine spongiform encephalopathy and foot-and-mouth disease in these regions (Agra CEAS Consulting, 2008).

The NE Asian market is dominated by the export of dairy breeding stock to China. This market has grown since the early 2000s in response to Chinese government policy for increased dairy consumption, and increased demand for cattle to rebuild the national herd following the China tainted milk scandal in 2008 (Parry, 2008).

Voyages to the Russian Federation began in 2006 and those to Turkey in 2012, largely to supply beef and dairy breeder cattle to support increased red meat and dairy consumption as incomes rise with economic growth (Australian Trade Commission, 2014; MLA, 2014).

In the SMDB deaths during the loading, voyage and discharge phases of a voyage are reported separately. In the study reported herein the number of voyage deaths and discharge deaths were significantly correlated, which suggests that deaths during discharge may be the final expression of an outcome that has been heavily influenced by voyage events. Therefore, the total number of cattle that died during each voyage was calculated by summing the number of cattle that died during the loading, voyage and discharge phases of each voyage.

Traditionally voyage mortality is reported as the percentage of cattle loaded that died. However, the used of this metric has the potential to bias any comparison of voyages since two voyages can have exactly the same risk of mortality each day but if one voyage has a longer duration then more cattle will die. Therefore, in the study reported here voyage mortality was expressed as deaths per 1000 cattle days to adjust for the duration of each voyage and produce a measure that could be compared between voyages of different lengths. Although the voyage mortality rate provides a more accurate reflection of voyage mortality risk than a simple mortality percentage, this metric cannot be directly compared to reportable mortality thresholds that are based on percentages of cattle loaded (0.5% for voyages ≤10 days and 1% for voyages >10 days). In addition as shown in Figure 6.6, mortality rate does not remain constant during the voyage, instead it shows a pattern of rising mortality rates to a peak between 25-30 days and then declines.

Average annual mortality rates were relatively high during the period 1995-2002, reduced between 2003 and 2009, and reduced further in 2010-2012. These trends are likely to have

been influenced by factors including market demand, and changes to regulation, risk management and quality assurance systems in the livestock export supply chain.

During the 1980s and 1990s the Australian live cattle export industry experienced a period of rapid growth which led to the emergence of a number of problems including increased mortalities, difficulty sourcing appropriate animals, and increasing concern for animal welfare (Stinson, 2008). In addition, government and industry recognised that the legislative system of the time was inadequate and so developed the Australian Live Export Standards (ALES), which were released in 1997 (Stinson, 2008). The release of ALES appears to have had little immediate effect on mortality rates.

Concerns about the lack of scientific information about the health and welfare of live export cattle led to industry funded research to investigate causes of death and risk factors for mortality in cattle during sea transport from Australia (Meat & Livestock Australia project SBMR.001) (Norris and Creeper, 2000). Heat stroke was the most common cause of death, accounting for 50/92 (54.3%) of deaths for which a diagnosis could be made. This finding was in agreement with an earlier study on causes of death in slaughter cattle exported to Japan in the late 1980s (Hedlefs, 1988). Based on results from the Norris et al. (2003) study it is reasonable to assume that the majority of voyage deaths during the period 1995 until at least 2003 were due to heat stress. Therefore trends in overall mortality during this period most probably reflect trends in deaths due to heat stress.

Recommendations arising from project SBMR.001 aimed to reduce deaths due to heat stress and included preferential selection of *Bos indicus* cattle and minimising exports from southern ports during the southern hemisphere winter and northern ports during summer (Norris and Creeper, 2000). The incorporation of these recommendations into industry standards (ALES) and their implementation through the Live Export Accreditation Program (LEAP) beginning in 1998 (Stinson, 2008) likely made a significant contribution to the

stabilisation and then decline in average yearly mortality rates after 1998. The drop in mortality rate for 1999 has been attributed to an increase in the proportion of *Bos indicus* cattle being exported to the MENA following the collapse of the trade to SE Asia associated with the Asian currency crisis (Norris et al., 2003).

High mortality events on individual voyages in the early 2000s (Stinson, 2008) resulted in substantial changes to the selection and management of animals during export. The Livestock Export Heat Stress Risk Assessment Model (HotStuff) was implemented in 2003 to help exporters to estimate and minimise the risk of heat stress mortalities occurring during a voyage (Maunsell Australia, 2003). The HotStuff model takes account of weather at destination and en route, animal acclimatisation, coat and condition, and the ventilation characteristics of the ships, and outputs optimal stocking densities based on a particular set of starting conditions (Maunsell Australia, 2003).

The Australian Standards for the Export of Livestock (ASEL) came into effect in 2004 and include standards for on-farm preparation of livestock, land transport, management of cattle in pre-export quarantine facilities, vessel preparation and loading, and management of livestock during voyages, including reporting of mortalities to the Australian government (DAFF, 2011). Since 2004 there have been improvements to the design and construction of livestock export vessels including automatic ventilation systems, automatic feed and water dispensers, flexible pen design to accommodate sheep and cattle, to meet ASEL requirements.

In addition to changes to industry Standards and regulation, between 1995 and 2012 there has been a change in the type of cattle sent for live export, mainly in response to import trade tariffs, consumer education, and government initiatives in importing countries. Liveweight restrictions in many markets have resulted in a proportional decrease in exports of older, heavier, fatter “slaughter” cattle and their replacement with younger, lighter

"feeder" cattle which cope better with on-board conditions during voyages (Moore et al., 2014).

Consumer education programs, particularly in MENA and SE Asian markets, have increased consumer acceptance of *Bos indicus* and *Bos indicus*-infused cattle entering these markets. Voyage mortality risk for *Bos indicus* cattle are lower than for *Bos taurus* (Norris et al., 2003; Stinson, 2008). This has been attributed to physiological adaptations, including reduced heat production (Swett et al., 1961; Vercoe, 1970; Worstell and Brody, 1953) and increased ability to dissipate heat (Blackshaw and Blackshaw, 1994; Finch, 1986), that allow *Bos indicus* cattle to cope better with hot and humid conditions during voyages than *Bos taurus* cattle. Therefore increased consumer acceptance of *Bos indicus* animals resulting in an increased *Bos indicus* content of shipments to MENA and SE Asian markets may have contributed to the observed reduction in mortality observed during this period.

Between 2010 and 2012 the dairy cattle market increased from 8.8% to 12.5% of cattle exported, primarily to meet the demands of the growing Chinese market. Dairy cattle are worth nearly three-times as much as feeder and slaughter cattle ($2294.96 versus $793.70 per head in 2012 (LiveCorp, 2012)) which provides a strong economic incentive for exporters to minimise mortality in this class of cattle. For example, during voyages dairy cattle are often stocked at a density lower than that stipulated in ASEL (Standard 4.4 (DAFF, 2011)), housed in more comfortable areas of the vessel, and provided with deeper bedding. This is reflected in the fact that mortality rates for voyages to NE Asia, of which the majority are China dairy cattle, are consistently lower than those to all other regions (Figures 6.4 and 6.5).

All of these factors are likely to have contributed to the industry achieving relatively low and stable mortality rates from 2003 to 2012. However, sporadic mortality incidents –

voyages with a mortality greater than 0.5% (voyage length ≤ 10 days) or 1% (>10 days) – continue to occur, indicating that there is still room for improvement.

Differences in mortality rate for load and discharge regions may be related to differences in the type of cattle loaded out of northern versus southern regions of Australiana, and in the type of cattle preferred by different export market regions. However, explanations for these differences are complex and inter-related and require further work to elucidate.

The majority of cattle exported from northern ports are tropically-adapted *Bos indicus* feeder and slaughter animals which are fattened and/or processed in SE Asian markets (Farmer, 2011). Producers in northern areas of Australia prefer tropically adapted *Bos indicus* cattle to *Bos taurus* cattle due to the former's superior performance in tropical climates, hardiness and tick resistance (DAFWA, 2009). Similarly, SE Asian importers, particularly feedlot operators, prefer *Bos indicus* cattle because they perform well in hot and humid environments (Farmer, 2011). Voyages to SE Asia are relatively short and *Bos indicus* cattle have been observed to have lower deaths rates during voyages than *Bos taurus* cattle (Norris et al., 2003; Stinson, 2008) so mortality rates for voyages from northern ports and voyages to SE Asia are relatively low.

In contrast, the majority of cattle exported from southern ports are *Bos taurus* feeder and slaughter animals destined for MENA. The temperate, mostly tick-free climate of southern Australia allows the production of *Bos taurus* cattle which grow more rapidly, mature earlier, and produce superior quality meat compared to *Bos indicus* animals. Voyages to NE Asia, MENA and SE Europe are relatively long and *Bos taurus* cattle carry a higher mortality risk that *Bos indicus* cattle (Norris et al., 2003; Stinson, 2008). Thus it is not surprising that mortality rates for voyages loaded out of southern ports and voyages to MENA are relatively high.

Most cattle exported to NE Asia and SE Europe are breeder cattle which, anecdotally, have lower mortality rates than other classes of cattle for the reasons discussed above. This helps to explain why mortality rates for voyages to NE Asia and SE Europe are lower than both SE Asia and MENA, which primarily accept feeder and slaughter animals. The fact that voyages to NE Asia and SE Europe together account for only ¼ of the voyages and cattle from southern regions (with the remaining ¾ of voyages and cattle destined for the MENA) suggests that the relatively high mortality rates for southern ports is due mostly to voyages to MENA.

Seasonal variation in mortality in feedlot cattle has been reported in North America, with peak mortality occurring in the autumn-winter in most studies (Jensen et al., 1976; Loneragan et al., 2001; Ribble et al., 1995a) and in spring-summer in one study (Babcock et al., 2013). However, Ribble et al. (1995a; 1995b) reported that patterns of mortality in feedlot cattle in the U.S. and Canada appeared to reflect incoming numbers of cattle and time on feed and not underlying effects of time of year or changes in weather. Similarly, a recent study of patterns of respiratory disease in Australian feedlot cattle indicated that mortality risk appeared to be related to incoming numbers and time post feedlot entry (induction) and not related to season (Perkins, 2013).

The observed rise in mortality rate in live export cattle between December and March may be related to seasonal variation in the type of cattle sent for export. Northern areas of Australia experience summer monsoon rains so the number of cattle exported from these areas is highest during the winter dry season (May to September). In contrast, the number of cattle exported from southern areas is highest from November to March largely due to seasonal restrictions on exports of southern cattle between May to October as stipulated by ASEL (Standard 1.5A (DAFF, 2011)). Therefore southern *Bos taurus* cattle comprise the majority of animals exported from Australia during the summer months. As discussed

above, *Bos taurus* cattle appear to carry a higher voyage mortality risk than *Bos indicus* cattle (Norris et al., 2003; Stinson, 2008). Furthermore, the high demand for southern cattle during the non-restricted period (November to April) may result in more aggregating and mixing of cattle and therefore greater pathogen exposure. Taken together these factors may account for the higher mortality rate observed between December and March.

The epidemiology of mortalities during voyages, at least with regards to the timing of the peak mortality period, appear to be similar to those in land-based feedlots in both Australia and North America. During voyages the daily mortality rate peaked at 3-4 weeks post-sailing. The most common cause of death during these voyages was BRD, which accounted for approximately 50% of deaths overall and 60% of deaths for which a diagnosis could be made (Moore et al., 2014). In land-based feedlots in North America and Canada peak mortality due to BRD is observed at approximately 2-4 weeks post arrival (Kelly and Janzen, 1986; Ribble et al., 1995a; Wilson et al., 1985). A recent survey of the Australian feedlot industry found that peak mortality occurred slightly later, at between 4-6 weeks after feedlot entry (Perkins, 2013). The timing of peak mortality risk is hypothesised to be related to the timing of the main period of exposure of cattle to potential pathogens prior to induction, for example during mixing in saleyards or at aggregation depots (Ribble et al., 1995a; Ribble et al., 1995b). The later occurrence of peak mortality in Australian compared to North American cattle may be because in Australia most feedlot cattle are transported directly from pasture to feedlot without going through saleyards or interim aggregation depots (Perkins, 2013). It may be that there is a relatively consistent time window of 4-6 weeks from initial pathogen exposure to peak mortality risk with varied time from induction to peak mortality being explained by variation in when exposure occurs (before induction or at induction).

The daily mortality rate curve shows a slight flattening (slowing of the progressive rise in daily mortality rate) before it rises more steeply to peak between days 25-30. These patterns may reflect changes in the intensity of factors influencing mortality risk over time, or a transition where the presence or absence of some risk factors may change over time. The previous paragraph discussed infectious disease epidemiology with animal mixing and timing of exposure to infectious agents contributing to possible delayed patterns for expression of disease. There are additional time-based factors on export voyages associated with progressive changes in the pen environments over time (e.g. accumulation of waste products) and changes in the local climate and sea conditions that may be associated in part with the geographic position of the vessel as it crosses the Indian Ocean. For example, hot, humid conditions with little wind in the Intertropical Convergence Zone followed by relatively favourable conditions between the Intertropical Convergence Zone and Gulf of Aden.

The apparently higher mortality rate in cattle destined for the Aegean and Black Sea ports (Turkey, Russian Federation) compared to the Red Sea and Persian Gulf (MENA) is probably due to a combination of the type of cattle on these voyages and seasonal climatic conditions. Six out of the 9 voyages to MENA carried cattle with a high *Bos indicus* content while voyages to Turkey and the Russian Federation comprised *Bos taurus* feeder and breeder cattle. In addition, 3 out of 11 voyages to Turkey and the Russian Federation sailed from Australia in January, i.e. from the southern hemisphere summer to the northern hemisphere winter, and were thus going from one climatic extreme to another. Exposure to temperature extremes without sufficient time for physiologic adaptation may have increased the mortality risk for these voyages, particularly around discharge.

There was interest in deriving variables from NLIS data including origin and travel details and using these to investigate possible associations with voyage mortality outcomes.

Unfortunately there were problems with model convergence that were likely to be due to sparseness of data and low overall mortality rates.

The development of methods for spatial analyses coupled with NLIS data allowed the description of patterns of animal movement prior to export. The cost to obtain this data (recording the NLIS tag details and date of death) is negligible, and protocols for analysing the data have now been developed. Extension and application of these methods to more complete datasets present an exciting avenue through which to improve our understanding of patterns of animal movement prior to export and the potential impact of spatial and climatic factors on mortality risk during voyages.

Retrospective analysis of voyage mortality data indicate that annual mortality rates and yearly and monthly fluctuations in mortality have reduced significantly since 2000. This has been achieved through changes to cattle selection and management prior to and during voyages which have been driven by market demand and risk management and quality assurance systems in the livestock export supply chain. However, despite these changes sporadic mortality incidents continue to occur.

The HotStuff model has recently been validated (Meat & Livestock Australia project LIV.0276) to ensure that it is still relevant to the ships and animal husbandry practices in use today, over 10 years since the model was first released. Results of this validation will be available soon.

Since at least 2006 respiratory disease appears to have taken the place of heat stress as the most important cause of death in live export cattle, particularly for cattle on long-haul voyages (DAFF, 2014; Moore et al., 2014). The epidemiology of BRD in live export cattle appears to be similar to feedlot cattle (Moore et al., *in press*) so mortality reduction practices used in land-based feedlots in Australia and overseas, including yard weaning,

backgrounding, vaccination and metaphylactic antibiotic treatment (Edwards, 2010), could be used to reduce the risk of mortality due to BRD during voyages.

Further research on management strategies to manage the impact of BRD and heat stress during voyages including determining optimal stocking densities, deck washing schedules and ventilation requirements, is likely to have a positive effect on the health and welfare of live export cattle in the future.

## 6.6 Author's contributions

SJM performed statistical analysis of retrospective data and drafted the Chapter. BM extracted relevant data from the NLIS database and performed the spatial analysis. GN provided the SMBD data and daily mortality data. NP advised on statistical analysis of retrospective data and led the analysis of daily mortality data. All authors provided editorial comment on the Chapter.

## 6.7 Acknowledgements

Thank you to Bob Vassallo and John Creeper for helping with livestock movement data.

# Chapter 7: General Discussion

## 7.1 Introduction

This study was initiated partly as a result of high mortalities on a number of shipments of cattle in 2006 which were attributed to outbreaks of respiratory disease (DAFF, 2014). That respiratory disease was the primary cause of death on these voyages was in contrast to previous mortality investigations which had concluded that heat stress was the most important cause of death in live export cattle (DAFF, 2014; Hedlefs, 1988; Norris et al., 2003). These findings highlighted a need to: 1) provide an update on mortality risks and causes of death in Australian live export cattle, and 2) to identify the viral and bacterial organisms important in the development of respiratory disease (BRD) during voyages.

## 7.2 Research Methods and Limitations

The research objectives were achieved through investigations in three broad study areas:

Firstly, collection and examination of necropsy samples from animals that died during 20 research voyages provided information on causes of death during voyages and allowed viral and bacterial organisms present in the lungs and nasal secretions at death to be identified. In previous studies of causes of death in live export cattle sample and data collection was performed by designated research staff which, due to time and cost restrictions, limited the number of voyages that could be sampled ($n$ = 10 and $n$ = 4 respectively) (Hedlefs, 1988; Norris et al., 2003). The study reported here used a novel approach to sample and data collection, which was performed by the Australian Quarantine and Inspection Service (AQIS, now the Department of Agriculture Fisheries and Forestry Biosecurity) accredited veterinarian (AA Vet) that accompanied each voyage,

instead of designated research staff. This sampling strategy allowed a greater number of voyages to be sampled than would have been possible with a single operator and thus represented the most efficient use of available resources. However, an increase in the number of operators performing necropsies also introduced the possibility of an increased risk of sampling bias and mis-diagnosis. To manage these risks standardised necropsy protocols and data recording forms were developed and the AA Vets collecting the samples received training on identifying gross necropsy changes typical of common disease conditions. The standardized protocols for the examination and reporting of voyage deaths and training programs for AA Vets developed during the course of this project provide a powerful tool for ongoing monitoring and investigation of mortality in cattle (and other species) sent for live export.

In addition to using a novel approach to sample and data collection during voyages, this project is the first time that quantitative PCR assays have been applied to samples collected from live export animals, of any species, that have died during voyages. The results from this testing provided valuable information on the viral and bacterial organisms that are present in live export cattle that die during voyages, as well as indicating which organisms are or are not associated with respiratory disease. These data can be used to inform on management strategies to maximise animal health and welfare on live export voyages.

Secondly, collection of nasal swab and blood samples from animals in the pre-export quarantine facilities allowed organisms present in the nasal secretions prior to loading to be identified, and the seroprevalence of antibodies to viruses of interest to be determined. This is the first time that nasal swabs have been collected from live export cattle in the pre-export assembly depot and the first time that serological samples from live export cattle have been analysed in a research capacity. Pre-export serological testing of live export cattle to meet the health requirements of the importing country has been performed since

as early as 1985 (Littlejohns and Horner, 1990) and since the late 1960s there have been a number of serosurveys for antibodies to BVDV, BoHV, BPIV-3 and BRSV in Australian cattle (Dunn et al., 1995; Durham and Paine, 1997; French, 1962; Littlejohns and Horner, 1990; St George, 1983; St George et al., 1967; Taylor et al., 2006; Zyambo et al., 1973). A range of samples, including nasal swabs, collected from Australian cattle with clinical signs of respiratory disease have been tested for the presence of viruses and bacteria known to be associated with BRD (Hick et al., 2012; Mahony and Horwood, 2006).

Matched nasal swabs and serological samples (i.e. nasal swab and blood samples collected simultaneously from the same animal) have been used to investigate the relationship between nasal shedding, serostatus, and BRD in feedlot cattle in the U.S. (Cho et al., 2001; Frank and Smith, 1983; Fulton et al., 2005; Fulton et al., 2002a; Fulton et al., 2002b; Fulton et al., 2011; Hanzlicek et al., 2011; Hasoksuz et al., 2002a; Lathrop et al., 2000b; Lin et al., 2000; Morter and Amstutz, 1983; Plummer et al., 2004; Storz et al., 2000b; Thomas et al., 2006), Italy (Decaro et al., 2008b), and Canada (Thomson et al., 1975; Van Donkersgoed et al., 1994; Yates et al., 1983) although many of these studies have tested for only 1 or 2 organisms.

A limitation of this phase of the project was the number of samples and range of animals (with regards to load port, class, type and sex) that could be sampled, and this was determined by available finances, time/labour, and industry tolerance. Cohorts of cattle were selected to provide a representative subset of the final composition of the shipment from as many assembly depots and properties as possible, and to collect enough samples to be able to perform statistical comparisons between explanatory variables, while minimising the impact of study sampling on processing of cattle. In total, nasal swab samples were collected from 1484/55,902 (2.6%) and matched serum samples from 334 (0.6%) of cattle across 4 study voyages. This means that, particularly in the case of

serological results, the prevalences reported for sampled animals may not necessarily represent the prevalence of these organisms across the entire shipment, or in the general population. Also, due to the relatively small number of voyage deaths it was not possible to investigate associations between prevalence of organisms in the pre-export assembly depot and mortality risk during voyages. Nevertheless, analysis of nasal prevalence and seroprevalence data, combined with available animal and epidemiological data, enabled the identification of pre-export risk factors that may influence morbidity and mortality during voyages.

Thirdly, examination of voyage data collected by industry for voyages between 1995 and 2012, and daily mortality data from research voyages, provided detailed, up-to-date data on mortality risks during voyages. In addition, spatial analysis of animal movement data allowed patterns of animal movement prior to export to be described for a subset of research voyages.

Voyage data were extracted from the Shipboard Mortality Database (SMDB), which is funded by Meat & Livestock Australia and administered by the Department of Agriculture and Food, Western Australia. Annual summary statistics for the SMBD are made available to industry and the public each year and allow the industry, government, and other stakeholders to monitor mortality trends in the Australian livestock export industry (Norris and Norman, 2013). In this PhD project novel statistical analysis methods were applied to the SMDB data (negative binomial modelling with an offset for animal days at risk and incorporating ship as a cluster variable) to allow meaningful comparisons to be made between voyages of different lengths. However, data detailing the causes of death, type of cattle (Bos *taurus* versus *Bos indicus*), and class of cattle (feeder, slaughter, breeder) are not routinely reported so these explanatory variables could not be included in risk factor analysis of SMDB data.

This is the first time that daily mortality data for live export cattle have been analysed. This analysis allowed patterns of mortality during voyages to be described and peak periods of mortality to be identified. Data from 20 research voyages to the Middle East and North Africa (MENA) and the Russian Federation were analysed; analysis of data from a larger number of voyages to a greater range of destination regions would improve the power of the analysis.

This is the first time that spatial analysis has been used to describe pre-export movement patterns for live export cattle. Raw data for Western Australian cattle loaded onto 3 research voyages were extracted from the National Livestock Identification System (NLIS) which is a nation-wide system for identification and traceability of livestock (MLA, 2012b). Due to the small number of voyages for which data were available and low overall mortality rates it was not possible to investigate associations between voyage mortality risk and cattle movement. However, the application of methods developed during this PhD project to more complete datasets could be used to investigate the influence of spatial and climatic factors on voyage mortality risk.

## 7.3 Mortality risks and causes of death in live export cattle

Annual voyage mortality rates increased sharply between 1995 and 1998 but have since declined markedly and appear to have stabilised at a relatively low level. The significant reduction in mortality rates observed since 1998 is likely due to a combination of factors including increased industry awareness of causes of death during voyages, changes to cattle selection and management prior to and during voyages (primarily to reduce the incidence of deaths due to heat stroke), improved vessel design and a gradual shift in the type of cattle being exported (guided by consumer education programs in importing countries).

The voyage mortality rate for cattle exported from ports in southern Australia remains higher than those exported from northern regions, and that for cattle exported to the MENA is higher than those exported to other destination regions. Interestingly, the mortality rate for cattle exported from southern regions to SE Asia is higher than for those to the MENA, although the reason for this is unclear. Given these findings, further refinement of cattle selection and management programs to take into account differences in mortality risk for *Bos taurus* versus *Bos indicus* cattle, may have a positive effect on voyage mortality rates.

The daily mortality rate changes from day to day during the voyage and this is likely to be influenced by a number of factors including prevailing environmental conditions. This information has important implications for management of cattle during voyages. For example, optimising temperature and humidity control through adjustments to stocking densities, deck washing schedules, and ventilation may help to reduce deaths during risk periods.

Daily mortality risk on long-haul voyages peaks at 3-4 weeks post-departure. The timing of the peak mortality period appears to be similar to that observed in land-based feedlots in both Australia (Perkins, 2013) and North America (Kelly and Janzen, 1986; Ribble et al., 1995a; Wilson et al., 1985) which suggests that mortality control measures used in feedlot cattle (reviewed in (Edwards, 2010)) may also be effective in live export cattle.

The cause of death in 215 cattle that died on 20 long-haul live export voyages between 2010 and 2012 was investigated using gross, histological, and/or molecular techniques. Of the animals for which a cause of death could be determined (*n* = 180) the most commonly diagnosed cause of death was respiratory disease (107, 59.4%), followed by lameness (*n* = 22, 12.2%), ketosis (*n* = 12, 6.7%), septicaemia (*n* = 11, 6.1%), and enteric disease (*n* = 10, 5.6%).

### 7.3.1 Deaths due to heat stroke

Heat stroke has previously been reported as the most common cause of death in studies by Hedlefs (1988) and Norris et al. (2003), accounting for 50/92 (54.3%) and 58/180 (32.2%) of deaths for which a diagnosis could be made respectively. In contrast, heat stroke was identified as the primary cause of death in only 2 animals in the present study (<1% of deaths) although hyperthermia secondary to other disease processes, for example severe bronchopneumonia and septicaemia, was recorded in an additional 9 animals. This marked reduction in deaths due to heat stroke is likely to be due to changes that have occurred in the industry since the late 1990s as a result of increased industry awareness of risk factors for heat stress, and modifications to the selection and management of cattle prior to and during export. In addition, the livestock export vessel fleet has been upgraded with a move away from refurbished freight vessels and car carriers to the production of purpose-built livestock carriers.

In this author's opinion, the incorporation of recommendations arising from the Norris et al. study (Norris and Creeper, 2000; Norris et al., 2003) – 1) that cattle for the MENA be sourced from northern rather than southern areas during the southern hemisphere winter, 2) that tropically-adapted *Bos indicus* and *Bos indicus*-infused cattle be sourced in preference to *Bos taurus* breeds when possible, and 3) that the management of cattle during voyages needed to be reviewed particularly with regards to ventilation, stocking densities, and feed and water management – into industry standards (Australian Live Export Standards (ALES), 1997 (Stinson, 2008)) and, later, into mandatory standards for animal health and welfare (Australian Standards for the Export of Livestock (ASEL), 2004 (DAFF, 2011)) has probably made the single most significant contribution to reducing mortalities due to heat stress since 1998. This is supported by results of analysis of retrospective voyage data that show that average yearly mortality rates began to decline after 1998, the year that ALES was implemented through the Live Export Accreditation

Program (LEAP) (Stinson, 2008), and stabilised at a relatively low level in 2003-2004 which coincides with the introduction of the Heat Management Risk Assessment Model (HotStuff) and the ASEL.

However, despite the apparent success of ASEL and HotStuff, heat stress incidents continue to occur. For example, between January 2006 and August 2013 heat stroke was identified as being the primary cause of mortality and/or heat stress as making a significant contribution to mortality in 6/23 voyage consignments that were reported to the Australian government for breaching the reportable mortality threshold (0.5% for voyages <10 days duration, 1% for voyages ≥10 days) (DAFF, 2014). This indicates that more can be done to reduce the incidence of heat stress and heat stroke.

Inadequate ventilation and/or ventilation failure contributed to deaths due to heat stress on a number of voyages. The ASEL includes minimum requirements for ventilation, e.g. the minimum number of complete air changes in a specified period of time (described in the Marine Orders Part 43, Appendix 4, Section 3) and each ship must pass an annual inspection to be allowed to continue to carry livestock. However, assessment of ventilation capacity is carried out when the vessel is not carrying any livestock so may not provide an accurate reflection of air flow when the ship is fully loaded. Therefore ventilation capacity should be assessed when the ship is loaded, or adjustments made to unloaded values to take into account the effect that livestock have on air flow.

Over half of the mortality investigations concerning cattle between 2006 and 2013 recommended a 10% increase above ASEL requirements in the amount of space allocated per animal to reduce the risk of mortalities due to heat stress and other causes (DAFF, 2014). Maximum stocking densities on board vessels, as set out in the ASEL, are up to 7 times higher than those in land-based feedlots. These relatively high stocking densities may have a negative impact on an animal's ability to rest (since there is insufficient space for all

animals in a pen to lie down at the same time), escape intimidation by more dominant animals, consume enough feed and water, and dissipate heat (Ferguson and Lea, 2013). These impacts may be exacerbated if hospital pens are full or if a sick animal cannot be moved from its home pen. In these cases a hospital pen may be created within the home pen, resulting in a temporary increase in the stocking density of the home pen to above ASEL requirements. Research has been carried out on associations between stocking density and morbidity, mortality and liveweight (Ferguson and Lea, 2013; Morton, 2008). However, cattle voyages described in these studies ($n$ = 1 and $n$ = 19 respectively) were all short-haul voyages (average voyage length approximately 7 days) carrying *Bos indicus* and *Bos indicus*-infused cattle (Ferguson and Lea, 2013; Morton, 2008). Therefore there is a need for empirical investigation of the effect of stocking density on welfare and mortality risk on long-haul voyages, particularly those with a high *Bos taurus* content.

### 7.3.2 Deaths due to lameness

Trauma, including lameness and misadventure, accounted for 29/215 (13.5%) deaths and was the second most common cause of death for cases for which a diagnosis could be made. Deaths in the lameness category were generally the result of the animal being euthanized because its injuries were severely compromising its welfare. The incidence of deaths due to trauma (13.5%) was lower than that reported previously (Hedlefs, 1988; Norris et al., 2003); 22.8% and 24.6% respectively. This is likely to be due to a combination of improved vessel design to meet ASEL requirements, and a change in the type of cattle being exported, for example, shipments comprising a higher proportion of smaller, lighter feeder cattle and a higher proportion of hardy, tropically adapted *Bos indicus* and *Bos indicus*-infused animals.

However, deaths due to trauma under-represents the true impact that leg trauma and sequelae have on animal health and welfare. In this author's experience and through anecdotal evidence gathered from AA Vets, morbidity due to lameness can be significant. There are no working instructions of the best veterinary treatments for the management on leg injuries sustained on-board ship and no consistent decision making protocols for when to treat and when to euthanase. Injuries sustained in the pre-export period may not heal once the animal is on the boat due to ongoing maceration and bacterial contamination through constant contact with manure. Therefore careful attention should be paid to yard maintenance and animal handling prior to loading onto the vessel to reduce the risk of foot and leg injuries during this period. To meet ASEL requirements, LiveCorp offers a training program for live export stockmen with responsibilities for the care of livestock exported by sea. This program includes information and practical training in low stress cattle handling techniques. Education of stockmen, truck drivers, and stevedores involved with transport of animals from the farm or origin through to loading onto the vessel may help to reduce injuries in the pre-export period.

The final veterinary inspection of live export cattle occurs as they are unloaded from trucks at the wharf and loaded onto the vessel. Exporters want the cattle loaded as quickly as possible which means that the time taken for veterinary inspection may be very brief and difficult if the animals' legs are dirty. Allowing more time for veterinary inspection at the wharf may help to reduce the number of injured animals that are loaded onto the vessel and thus reduce morbidity and mortality due to lameness during the voyage.

During the voyage rough seas can lead to traumatic injury (DAFF, 2014) but most cases of lameness develop secondary to injuries and abrasive floor surfaces (Banney et al., 2009). Lame animals are reluctant to stand which increases the risk of their being trampled by pen-mates, and can lead to inappetence and dehydration. During the voyages decks are

hosed down periodically to remove manure and soiled bedding. While deck washing improves the cleanliness of the pens it may also lead to an increase in humidity, especially on closed decks (SJ Moore, *personal observation*), and provide a route for transfer of waste material and associated potential pathogens which can increase mortality risk.

Research has been carried out on cattle selection and bedding management to reduce the incidence of lameness (Banney et al., 2009) but this important cause of morbidity and mortality would benefit from further attention. For example, guidelines for optimal deck flooring materials and, where a bituminised aggregate flooring material is used, optimal aggregate size; assessment of the adequacy of ship bilge systems, particularly with regards to the amount of bedding that can be used; investigation of optimal deck washing schedules, which will depend on stocking density, type of cattle, voyage route, time of year etc. to manage lameness and heat stress.

### 7.3.3 Deaths due to respiratory disease

Respiratory disease was the most commonly diagnosed cause of death during voyages, accounting for 107/215 (49.8%) of deaths overall, and 107/181 (59.1%) of deaths for which a diagnosis could be made. This is higher than that previously reported for live export cattle (18.9% of deaths) (Norris et al., 2003) but broadly similar to that reported in beef feedlots in Canada (46–65%) (Church and Radostits, 1981; Edwards, 1996; Gagea et al., 2006b; Janzen, 2003; Martin et al., 1982) and the U.S. (55–75%) (Jensen et al., 1976; Loneragan et al., 2001). The incidence of respiratory disease was not reported by Hedlefs (1988). Pneumonia was also identified in 33% of animals for which respiratory disease was not considered the primary cause of death, indicating that respiratory disease plays a role on both morbidity and mortality during voyages.

The apparent increase in the proportion of animals dying from respiratory disease in our study compared to the Norris et al. (2003) study may be related to the reduction in deaths due to heat stroke and lameness. That is, as prevention and management of heat stress and lameness has become more effective, for example through increased industry awareness and changes to animal selection and management to meet the requirements of the ASEL and HotStuff, respiratory disease has become more prominent. This is supported by results from Australian government investigations of high mortality voyages which indicate that since the mid-2000s respiratory disease appears to have taken the place of heat stress as the most important cause of death in live export cattle, particularly for cattle on long-haul voyages (voyage duration ≥10 days) (DAFF, 2014).

Bovine respiratory disease is the most important and costly disease of beef feedlot cattle in Australia (Cusack et al., 2007; Dunn et al., 2000; Dunn et al., 1995) and North America (Griffin, 1997; Irsik et al., 2006; Smith, 2000). It is not surprising that respiratory disease is also a significant cause of death in live export cattle, given that cattle management processes and housing in the live export and feedlot industries are similar. For example, both industries involve transport of cattle from their farm of origin, co-mingling of cattle from different sources at feedlot/assembly depot entry, maintenance of cattle at stocking densities higher than those on their property of origin, and feeding of a complete ration. Indeed, a live export vessel is essentially a floating feedlot, albeit with higher stocking densities and fewer opportunities to isolate individual animals for treatment.

## 7.4 Organisms involved in bovine respiratory disease in live export cattle

The qPCR techniques developed during this study provided sensitive methods (diagnostic sensitivity of <10 gene copies for most organisms) for the detection of nucleic acids from viruses and bacteria of interest in swab and tissue samples: Bovine coronavirus (BCoV, *Betacoronavirus 1*), Bovine herpesvirus 1 (BoHV-1), Bovine viral diarrhoea virus (BVDV), Bovine respiratory syncytial virus (BRSV), Bovine parainfluenza virus 3 (BPIV-3), *Histophilus somni*, *Mycoplasma bovis*, *Mannheimia haemolytica* and *Pasteurella multocida*.

Two-thirds (130/195) of animals from which lung samples were collected had histological changes and/or positive qPCR results indicative of infectious lung disease: 93/130 (72%) had evidence of bacterial infection, 4 (3%) had viral infection, 29 (22%) had mixed bacterial and viral infections, and for 4 (3%) the causative organism could not be identified. For all 4 bacteria of interest, the presence of bacterial nucleic acid in lung samples was significantly correlated with histological evidence of pneumonia. In contrast, no significant correlation was detected between viral nucleic acid and pneumonia, although the presence of BCoV in nasal swab samples was associated with a higher odds of death due to respiratory disease.

These findings support our understanding of the different roles that viruses and bacteria play in the pathogenesis of BRD. That is, viruses are primary pathogens that damage the respiratory tract and may also inhibit the immune system, thus facilitating secondary invasion by bacteria that may go on to cause a fatal bacterial pneumonia (Panciera and Confer, 2010; Taylor et al., 2010), by which time the virus(es) may no longer be detectable.

### 7.4.1 Bovine coronavirus

This is the first time that BCoV has been demonstrated in live export cattle and the second time that BCoV has been reported in Australian cattle. The first report of BCoV in Australian

cattle occurred during the course of this PhD project, associated with an outbreak of respiratory disease in beef feedlot cattle in eastern Australia (Hick et al., 2012).

For cattle that died during voyages, the presence of BCoV in nasal swab samples collected from animals at necropsy was associated with significantly higher odds of a final diagnosis of respiratory disease, compared to animals without BCoV in their nasal secretions. In addition, the average prevalence of BCoV in the assembly depot (40%) was much higher than that for the next most commonly detected virus (BVDV, 3%). An association between nasal shedding of BCoV and clinical signs of respiratory disease has been reported in some studies (Hasoksuz et al., 2002a; Storz et al., 2000b; Storz et al., 1996; Thomas et al., 2006) but not others (Cho et al., 2001; Hasoksuz et al., 2005). In feedlot cattle, the presence of BCoV in nasal secretions has been linked to an increased risk of treatment for respiratory disease (Fulton et al., 2011; Lathrop et al., 2000a; Plummer et al., 2004), reduced weight gain (Thomas et al., 2006) and increased incidence of pulmonary lesions at slaughter (Lathrop et al., 2000a). These results suggest that BCoV is important in the pathogenesis of BRD in Australian live export cattle.

### 7.4.2 Bovine viral diarrhoea virus

Bovine viral diarrhoea virus was detected in 17/151 (11%) lung samples and 5/82 (6%) nasal swabs collected from animals that died during voyages, and was not significantly associated with either lung pathology or death due to respiratory disease. In the pre-export assembly depot the average nasal prevalence of BVDV was 3% and the seroprevalence was 58%.

To the authors' knowledge, this study is the first time that BVDV has been detected in individual animal nasal swabs from naturally exposed cattle with (Decaro et al., 2008a; Storz et al., 2000b) or without (Fulton et al., 2005; Fulton et al., 2002a) clinical signs of BRD.

Seven out of 20 (35%) BVDV positive animals that died during voyages were from consignments in which acutely or persistently infected (PI) animals had been identified by serum protein A antibody-capture ELISA (PACE) and removed from the consignment prior to loading. This suggests that a single test to remove persistently infected animals does not guarantee the cessation of virus circulation from the consignment. In the assembly depot, the cohort with the highest BVDV nasal prevalence (12%) was detected in a cohort of cattle that had been carried over from a previous voyage for reasons including a positive PACE result. The presence of animals which are shedding BVDV in the pre-export assembly depot, combined with the finding that approximately 40% of animals are susceptible to infection (seronegative, nasal swab negative), suggests that acute BVDV infection caused by exposure to PI animals may pose a risk for BRD morbidity and mortality during voyages.

### 7.4.3 Bovine herpesvirus 1 and Bovine respiratory syncytial virus

The significance of BoHV-1 and BRSV infections is difficult to determine. These viruses were isolated infrequently from animals that died during voyages (BoHV-1 from 6% of the 168 animals tested, BRSV from 5%) and neither virus was significantly associated with deaths due to respiratory disease. A study on Australian feedlot cattle reported that BoHV-1 isolated from post-mortem material was significantly associated with deaths from respiratory disease in (Dunn et al., 2000). The proportion of susceptible animals in the Dunn et al. study (87% seronegative) was 1.5 times higher than in our study (61%), which may explain the differences in the association between BoHV-1 and terminal respiratory disease in these two studies.

In the pre-export assembly depots the nasal prevalence of both viruses was low (BoHV-1 = 1%, BRSV = 1.2%). To this author's knowledge, this is the first time that BRSV has been detected in nasal swabs from beef cattle over the age of 4 months with (Decaro et al., 2008a; Hick et al., 2012; Storz et al., 2000b; Van der Poel et al., 1997) or without (Fulton et

al., 2005; Fulton et al., 2002a) clinical signs of respiratory disease. There was a significant increase in BRSV nasal prevalence between depot entry and re-resting 9 days later, which is likely to indicate transmission of BRSV to naïve animals during this period. Bovine respiratory syncytial virus infection has previously been associated with clinical but not fatal respiratory disease in Australian cattle (Dunn et al., 2000; Dunn et al., 1995) and seroconversion during the feeding period has been linked to an increased risk of treatment for BRD (Caldow et al., 1988; Thomas et al., 2006). The seroprevalence of BRSV was 46%, indicating that more than 50% of animals are susceptible to infection. Therefore, BRSV may be contributing to BRD morbidity but not mortality during voyages.

### 7.4.4 Bovine parainfluenzavirus 3

The seroprevalence for BPIV-3 (87%) was the highest out of the four viruses tested. This, combined with a low nasal prevalence (1.4%) and lack of evidence for an association between BPIV-3 and respiratory disease in Australian live export cattle or feedlot cattle in Australia (Dunn et al., 2000; Dunn et al., 1995; Van Vuuren, 1990) or North America (Fulton et al., 2002a), suggests that BPIV-3 plays a secondary role, if any, in the development of BRD in Australian live export cattle.

### 7.4.5 Bacteria

Bacteria of interest were detected in nasal and lung swabs from animals that died during voyages and in nasal swabs collected from cattle in the pre-export assembly depots. However, the significance of individual bacteria is difficult to determine given the polymicrobial nature of BRD, and the fact that all 4 bacteria can be found as commensal organisms in apparently healthy animals as well as causing respiratory disease as primary or, more commonly, secondary disease agents.

At least 1 of the 4 bacteria of interest were detected in lung samples from up to 60% of animals necropsied during study voyages, and all bacteria were significantly associated with

histological pneumonia. The presence of *M. bovis*, *M. haemolytica* and/or *P. multocida* in nasal swab samples was significantly associated with pneumonia and with respiratory disease as the primary cause of death. The relatively high prevalence of *P. multocida* compared to *M. haemolytica* in both voyage and pre-export samples supports previous reports of the increasing isolation of *P. multocida* in association with BRD (Rice et al., 2007; Welsh et al., 2004). The identification of histological lesions consistent with *H. somni* – associated myocarditis, thrombotic meningoencephalitis and/or pneumonia suggest that *H. somni* may play a role in some cases of sudden death during voyages. Therefore, the importance of *P. multocida* and *H. somni* should not be overlooked when considering management of voyage mortalities due to BRD and other causes.

In the pre-export assembly depot, at least 1 of the bacteria of interest was detected in up to 42% of animals, and 1 or more viruses and concurrent bacteria were detected in 38% of cattle. The prevalence of *M. bovis* and *M. haemolytica* increased significantly between depot entry and retesting approximately 1 week later. This increase is likely to be due to a combination of stress-induced proliferation of commensal bacteria and transmission of bacteria between animals.

## 7.5 Managing the impact of bovine respiratory disease in live export cattle

The live export cattle supply chain has many features in common with management of cattle in feedlots. This study has revealed that the pathogenesis and epidemiology of BRD in live export cattle is similar to that in feedlot cattle. Therefore cattle selection and husbandry strategies used to prevent and control mortality in feedlot cattle are likely to also be effective in minimising the incidence and costs associated with morbidity and mortality (due to BRD and other diseases) in live export cattle. Prevention and control

strategies aim to minimise pathogen exposure, stimulate herd immunity, and manage risk factors that potentiate the spread of disease (Edwards, 2010).

Mixing of cattle of unknown health status from different properties is an important risk factor for pathogen exposure (Mahony TJ, *unpublished data*) (Sanderson et al., 2008; Taylor et al., 2010). The majority of cattle sent for export, particularly those on long-haul voyages, are sourced directly from their property of origin so co-mingling at the assembly depot presents an important BRD risk. In an ideal world, cohorts of cattle could be tested at their property of origin, vaccinated or treated with antibiotics as required, and mixed strategically in the assembly depot to minimise exposure of susceptible animals to potential pathogens. Unfortunately, this type of on-farm preparation is not compatible with the existing live export cattle sourcing process.

Vaccination can be used to stimulate herd immunity. As described above, on-farm vaccination is unlikely to be considered feasible in the current live export supply chain. From a management perspective, a more cost effective and feasible alternative is the development of single shot, fast acting, efficacious vaccines that can be administered at arrival at the assembly depot. With regards to BCoV, intranasal vaccination may prove effective since intranasal vaccination of feedlot calves with a modified live BCoV vaccine at feedlot entry has been shown to reduce the risk of treatment for BRD (Plummer et al., 2004). Genomic sequencing of Australian BCoV isolates will aid in the development of effective vaccines. Antimicrobial sensitivity testing of bacterial isolates from Australian live export cattle may help to guide selection of metaphylactic and therapeutic antibiotics.

Exposure of cattle to physiological and environmental stressors can lead to inhibition of the immune response and increased susceptibility to BRD and other disease conditions (Panciera and Confer, 2010; Taylor et al., 2010). Yard weaning, where newly weaned calves are gradually introduced to close human contact and being handled through races and

yards, is associated with a reduced risk of BRD in feedlot cattle (Mahony TJ, *unpublished data*) (Condon, 2013; Edwards, 2010). During the voyage, informed management of stocking rates, ventilation, and bedding management (including bedding substrate and amount, and deck washing schedules) could be used to minimise the adverse effects of the often challenging environmental conditions on-board livestock vessels.

## 7.6 Management Outcomes and Recommendations

The following recommendations for management of live export cattle are suggested for consideration in addition to existing ASEL requirements.

### 7.6.1 Pre-export assembly period

- Apply methods for spatial analyses coupled with NLIS data to more complete datasets to improve our understanding of patterns of animal movement prior to export and the potential impact of spatial and climatic factors on mortality risk.
- Keep newly received animals separated from carryover animals
- Minimise co-mingling by minimising the number of PICs per pen, avoid re-sorting cohorts on the vessel
- Consider the use of pre-export vaccination for organisms that appear to be important in the development of BRD during voyages: BCoV, BVDV, *M. haemolytica*. Currently, only BVDV and *M. haemolytica* vaccines are commercially available in Australia so additional vaccines may need to be imported. Vaccine efficacy could be tested using data and sample collection systems used in this project.
- Vaccines should be used according to the manufacturers' instructions with the aim of loading fully protected cattle. Where 2 treatments are required for vaccine efficiency cattle may need to be vaccinated on-farm and in the assembly depot.

Alternatively, the use of single-shot vaccines, administered in the assembly depot, may be more appropriate.

- Investigate the seroprevalence of BCoV.
- Investigate genetic factors that may make Australian BCoV isolates more/less virulent than isolates detected elsewhere in the world, for example, through genomic sequencing of BCoV isolates collected during this study.
- Identify BVDV persistently infected animals as early as possible and manage these animals to minimise exposure of naïve animals to persistently infected animals.
- Perform antimicrobial sensitivity testing of bacterial isolates to inform on metaphylactic antibiotic selection.
- Maintain yards, races and trucks in good condition.
- Practice low stress livestock handling during transport and in the assembly depot to minimise stress and foot/leg injuries.

### 7.6.2 Voyage

- Use tailored load plans which take into account hot spots within different vessels. Recognised stockman experience when formulating load plans. Higher risk cattle should be housed in better areas of the ship where possible.
- Revisit stocking densities taking into account temporary increases in stocking density associated with creation of in-pen hospital pens due to lack of facilities for isolation of individual animals.
- Revisit pen flooring, bedding material and amount, and deck washing guidelines with a view to reducing morbidity and mortality due to trauma and lameness. Encourage reporting of leg injuries to support this research.
- Develop consistent work instructions for the management of traumatic leg injuries.

- Review the use of antibiotics during voyages particularly with regards to withholding periods, which may be different between Australia and the importing country.
- Develop systems for ongoing morbidity monitoring and mortality reporting during voyages to support mandatory reporting requirements (e.g. Daily and End of Voyage Reports) and monitoring of mortality trends.

## 7.7 Conclusion

During the course of this PhD project the Australian live export trade was reviewed again (Farmer, 2011) in response to public outcry, this time to treatment of animals at their destination (Australian Broadcasting Corporation, 2011). Prior to the Farmer review the exporters' responsibility for their stock ended when the animal walked off the end of the boat ramp at its destination. The Farmer review has led to the establishment of a new regulatory system for feeder and slaughter (but not breeder) cattle after arrival at destination countries to enable traceability and accountability from farm to slaughter (Exporter Supply Chain Assurance System (ESCAS) (DAFF, 2013b)).

Results of this study have shown that an increased awareness and consideration of animal health and welfare has achieved better conditions for live export cattle, for example, as reflected in reduced mortality rates following the introduction of the LEAP, and maintenance of relatively low mortality rates under the ASEL and HotStuff. There is hope that the ESCAS will similarly lead to improvements in welfare in destination countries. In the future, the live export industry's social license to operate would benefit greatly from the industry being seen to be proactive, rather than reactive, in its approach to the health and welfare of live export animals.

The results reported in this thesis represent a contemporary description of causes of death, risk factors for mortality, and viruses and bacteria associated with BRD in Australian live export cattle. The development and application of novel sampling strategies, statistical analysis techniques and diagnostic tests have helped to improve our understanding of the pathogenesis and epidemiology of BRD in live export cattle. This information provides a foundation to build on through further research and upon which informed management decisions can be based.

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