A study of background lung lesions in a colony of specific pathogen free rats from weaning to 6-months of age

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This thesis is presented for the degree of Research Masters with Training (Health Sciences) of Murdoch University, 2015
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.

Louise Sullivan
Abstract

Background lung lesions in the laboratory rat have long been a frustrating and confounding issue for researchers. Reports of distinctive but poorly characterised lung lesions occurring in rats overseas prompted this study, which aimed to assess the baseline occurrence and progression of background lung lesions in rats maintained under specific pathogen free (SPF) conditions at a commercial rodent production facility in Australia.

The study design incorporated 200 rats (100 Sprague Dawley and 100 athymic nude) that were maintained under SPF conditions (either isolator or barrier housing) before being euthanased at 3, 6, 12, 18 or 24 weeks of age. Two additional groups of 10 rats were housed within external facilities and euthanased at 12 weeks of age. During postmortem examination, gross lesions were recorded and lung samples were collected for microbiological culture and histopathology.

A variety of microscopic and macroscopic lung lesions were identified within the study population. Where appropriate, classification and/or grading schemes were utilised or developed to further characterise these lesions. The most prevalent lesions were lymphohistiocytic interstitial pneumonia and perivascular inflammatory cell cuffing and prevalence of these lesions tended to increase with rat age, regardless of rat strain or housing type. There were no consistent trends in lesion prevalence across rat strain and housing type.

The predominant combination of lesions was distinctive and compatible with
descriptions of the disease known as “rat respiratory virus” and later, infectious interstitial pneumonia. Previously a poorly characterised entity of unknown aetiology, during the course of this study, *Pneumocystis carinii* was reported by others (Livingston, Besch-Williford et al. 2011, Henderson, Dole et al. 2012) to be the cause of this disease. Subsequently in the current study, histochemical staining demonstrated fungal organisms in a large proportion of affected athymic nude rats and *P. carinii* DNA was also detected in some rats via PCR assay. While there were minor differences in lesion progression and imperfect agreement between histopathologic lesions and PCR results, the results of this study are consistent with the recent literature outlining a causative role for *P. carinii* in this distinctive pneumonia in the rat.
Acknowledgements

This research project would not have been possible if it were not for the contributions of the many people that provided invaluable support, technical assistance and encouragement.

First and foremost, I would like to express my gratitude to my principal supervisor Associate Professor Mandy O’Hara whose expertise, understanding, guidance and patience has been integral within all aspects of this project. Mandy has been a valued mentor for many years, not only in research and veterinary pathology, but also at times, in life, and I cannot thank her enough for all she has contributed.

I am also very appreciative of the efforts of my co-supervisors, Dr Deborah Hopwood and Associate Professor Phil Nicholls in assisting me with this work. It was Deb who initially came up with the idea and rationale for this project, and she also assisted greatly with the development of the study design and obtained animal ethics approval for this project. In addition, Deb was also responsible for the logistics of sourcing and maintaining animals within the Animal Resources Centre for the duration of this project, as well as liaising with and transporting animals to external facilities. Lastly, I am also very grateful to Deb for her advice and comments in regards to the writing and content of this thesis. I would like to thank Phil Nicholls for advice regarding study design, thesis structure and content, and lesion interpretation. His time and expertise were greatly appreciated.

Mr Michael Slaven and Mr Gerard Spoelstra provided excellent advice and technical
expertise in histotechnology. Mr Gary Allen provided similar advice and expertise in providing microbiology services. Professor Giselle Hosgood and Dr Jo Moore provided advice and technical expertise regarding statistical analysis.

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The contributions of Bob Stevenson from Cerberus Sciences, Adelaide, are gratefully acknowledged. In particular, I want to acknowledge Bob and Cerberus Sciences for generously providing and performing the *Pneumocystis carinii* PCR assay that was an important part of this study. I also wish to thank him for his technical advice and acknowledge Cerberus Sciences as the provider of serology testing performed as part of this study.

I would also like to thank the staff of the Animal Resources Centre and Murdoch University Animal House who were involved in the care and transport of the rats. I would similarly like to thank the staff from the interstate research facility. Although this facility requested anonymity for the purposes of this study, the input efforts and communication of the involved staff was greatly appreciated and is therefore acknowledged here.
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# Table of Contents

Declaration .................................................................................................................................................. ii
Abstract .......................................................................................................................................................... iii
Acknowledgements ........................................................................................................................................ vi
Table of Contents ......................................................................................................................................... viii
List of Abbreviations ..................................................................................................................................... x
Introduction .................................................................................................................................................... xi

## Part One: Survey of Lung Lesions in a Colony of Specific Pathogen Free Rats

1. **Introduction** ........................................................................................................................................... 2
   1.1 The laboratory rat: historical aspects ................................................................................................. 2
   1.2 Impacts of disease on animal research ............................................................................................... 4
   1.3 Specific pathogen free technology ...................................................................................................... 6
   1.4 Housing systems for microbial exclusion .......................................................................................... 8
   1.5 Terminology of animal microbial status ............................................................................................. 12
   1.6 Principles of microbiologic monitoring ............................................................................................. 13
   1.7 Post-indigenous disease ................................................................................................................... 16
   1.8 Conditions associated with inflammatory lung lesions in rats ....................................................... 18
   1.9 Miscellaneous lesions ....................................................................................................................... 37
   1.10 Summary .......................................................................................................................................... 39
   1.11 Aims .................................................................................................................................................. 40
   1.12 Hypotheses ....................................................................................................................................... 41

2. **Materials and Methods** ....................................................................................................................... 42
   2.1 ARC-housed rats ............................................................................................................................... 42
   2.2 External facility-housed rats ............................................................................................................. 48

3. **Results** .................................................................................................................................................. 50
   3.1 Animal monitoring ............................................................................................................................. 50
   3.2 Gross pathology .................................................................................................................................. 50
   3.3 Histopathological findings ............................................................................................................... 57
   3.4 Ancillary diagnostics ......................................................................................................................... 99
   3.5 Summary and conclusions ............................................................................................................... 101
Part Two: Evaluation of Infectious Interstitial Pneumonia within the ARC Colony and in Rats Sourced from the ARC Colony ......................................................... 104

1. Introduction ........................................................................................................ 105
   1.1 Guidelines for the histopathological diagnosis of rat respiratory virus ...... 105
   1.2 Search for an aetiologic agent ................................................................. 108
   1.3 Aims ........................................................................................................... 109

2. Materials and Methods ................................................................................... 111
   2.1 Infectious interstitial pneumonia classification ........................................ 111
   2.2 Infectious interstitial pneumonia severity grading ..................................... 113
   2.3 Histochemical staining .............................................................................. 114
   2.4 Pneumocystis carinii PCR ........................................................................ 114

3. Results .............................................................................................................. 116
   3.1 Infectious interstitial pneumonia classification ........................................ 116
   3.2 Infectious interstitial pneumonia severity grading ..................................... 124
   3.3 Histochemical staining .............................................................................. 128
   3.4 Pneumocystis carinii PCR assay .............................................................. 131

Part Three: Discussion ....................................................................................... 134
   3.1 Introduction ................................................................................................... 135
   3.2 Infectious interstitial pneumonia (IIP) characterisation ............................. 138
   3.3 Detection of Pneumocystis carinii ............................................................... 148
   3.4 Effects of age, strain and housing on prevalence and severity of IIP ....... 156
   3.5 Other histopathological findings ............................................................... 165
   3.6 Infectious interstitial pneumonia classification ........................................ 178

Summary, Conclusions & Areas for Further Research ....................................... 183

Appendix A: ......................................................................................................... 189

Appendix B: ......................................................................................................... 191

Appendix C: ......................................................................................................... 194

Appendix D: ......................................................................................................... 195

References .......................................................................................................... 197
List of Abbreviations

ARC: Animal Resources Centre (SPF laboratory animal production facility in Perth, Western Australia)
B: barrier (refers to housing type)
BALT: bronchus-associated lymphoid tissue
CAR bacillus: cilia-associated respiratory bacillus
CBH - Chester Beatty Hooded (inbred rat strain)
ELISA: Enzyme-linked immunosorbent assay
H&E: haematoxylin and eosin (histochemical stain)
HEPA: high-efficiency particulate arrestance (type of air filter)
IFA: immunofluorescence assay
IIP: infectious interstitial pneumonia (historically referred to as rat respiratory virus (RRV) and more recently, pneumocystis pneumonia)
Is: Isolator (refers to housing type)
Nude: CBH-rnu/Arc (athymic nude) rat
PAAS: periodic acid ammoniacal silver (modified silver histochemical stain)
PCR - polymerase chain reaction
PRC: Parker’s rat coronavirus
PVM: pneumonia virus of mice
RADIL: Research Animal Diagnostic Laboratory (RADIL), University of Missouri
RRV: rat respiratory virus (historical term used to refer to the disease more recently referred to as infectious interstitial pneumonia (IIP) and pneumocystis pneumonia.
SD: Sprague Dawley rat
SDAV: sialodacryoadenitis virus
SPF: specified pathogen free
Introduction

When laboratory animals are used as an experimental model of disease, it is critical that the pathological changes that occur as a result of experimental manipulation are able to be distinguished from those changes that occur as a result of other influences, such as strain, age and environmental conditions. In the laboratory rat, diseases of the respiratory tract, particularly those caused by infectious agents, have historically been of great significance due to their ability to confound experimental results (National Research Council 1991). Whilst the introduction of caesarean derivation and gnotobiotic methodology has greatly decreased the incidence and thus the impact of infectious diseases, these diseases still occur in conventionally housed animals and even in specified pathogen free (SPF) colonies. The strict microbial exclusion required for maintenance of SPF status requires a tremendous degree of commitment, effort and monitoring, and animals can become inadvertently infected following breakdown of any aspect of barrier protocol (Weisbroth 1996, Shek 2008). Lastly, SPF colonies may also become infected with newly emerging and/or undiscovered pathogens that are not tested for as part of routine health surveillance monitoring (Weisbroth 1996).

The Animal Resources Centre (ARC) is a large laboratory animal production facility located in Perth, Western Australia. This facility produces and supplies SPF rats and mice to biomedical researchers in Australia and a number of neighbouring countries. A number of measures are utilised aimed at preventing inadvertent microbial contamination and thereby maintaining the SPF status of animals, including operating as a closed colony, with rederivation required prior to entry of new stock as well as
strict and rigorous environmental management, monitoring and microbial testing.

The interpretation of unexpected histopathological lesions in rat lungs has been a recurring query fielded by veterinary staff at the Animal Resources Centre over many years (D. Hopwood, personal communication). During 2008, veterinary staff at the ARC received several customer queries regarding the finding of unexpected inflammatory lung lesions (specifically multifocal interstitial pneumonia and perivascular inflammatory cell cuffing) of unknown origin in rats purchased from the facility. The described lesions seemed distinct from those described in previous sporadic customer queries, which had typically involved reports of peribronchiolar inflammatory cell infiltrates rather than perivascular cuffing. Most cases involved Sprague Dawley rats that had been born and reared at the ARC before being routinely transported to external research facilities, where they were held for varying periods of time, as required by experimental protocols. In the cases where attempts had been made to determine the aetiology, routine microbial culture and serological testing for known rodent respiratory pathogens were reportedly negative. Customers were interested in knowing whether any similar lesions had been identified within individual animals at the ARC. This question was the primary motivation for this study, as the answer was considered essential for determining whether the problem was related to pre-existing disease in the rats originating at the ARC or if the observed lesions were related to experimental variables/procedures, microbial contamination, or alternatively environmental changes to which the rats were exposed upon transfer to facilities with lower levels of microbial control and/or different husbandry procedures.
Part One: Survey of Lung Lesions in a Colony of Specific Pathogen Free Rats
1. Introduction

1.1 The laboratory rat: historical aspects

For over 100 years, the laboratory rat has been a commonly used animal model in all areas of biomedical research, being second only to the mouse as the most frequently utilised mammal in scientific investigation (Kohn and Clifford 2002). The majority of laboratory rat strains available today are either albino or have been derived from an albino background (Koolhaas 1999). Historical records indicate that albino rats, originally bred for use in rat-baiting or/or show purposes, were first brought into laboratories in the early nineteenth century (Hedrich 2000, Lindsey and Baker 2006). Whilst there is some evidence to suggest that rats were used in nutritional experiments as early as 1828 (Hedrich 2000); the work most commonly recognised as the first utilisation of the rat in biomedical research, which documented the effects of adrenalectomy in the albino rat, was not published until 1856 (Philippeaux 1856). Shortly after, the first recorded breeding experiments using rats (both albino and wild) were performed in Germany by Crampe (Lindsey and Baker 2006), although the use of the rat as a model remained only sporadic in both Europe and the United States until about 1890 (Kohn and Clifford 2002). Much of the credit for the early development of the rat for research, particularly in the United States, is attributed to the neurologist H.H. Donaldson, who by 1906 was heavily involved in the production and standardisation of early laboratory rat stocks at the Wistar Institute in Philadelphia (Kohn and Clifford 2002, Lindsey and Baker 2006). By the 1930s, Rattus norvegicus was well established as a common experimental animal in many areas of research (Porter 2000).
Today, numerous strains of laboratory rats, both inbred and outbred, are commercially available for research purposes. In recent times, many strains have been developed utilising a variety of genomic technologies, often for specific purposes such as the creation of animal models of human disease, by utilising mutant, gene knockout and even transgenic technology (Hedrich 2000). Although in recent times use of the laboratory mouse has increased in frequency over the rat as an experimental model in many studies due to greater ease of application of genetic molecular techniques (targeted mutations), the rat retains certain advantages over its smaller counterpart due to its larger size, permitting easier access for repeated samplings and as a model for surgical procedures (Hedrich 2000, Hedrich 2006). Similarly, although the number of commonly inbred rat strains is dwarfed by those that exist for the mouse, many of these strains represent important models for research into a variety of disease conditions (Kohn and Clifford 2002). The importance of the rat in biomedical research is clearly summarised in the following statement:

“Among the great discoveries of the 20th century, the rat as an experimental model has been invaluable in advancing knowledge across many areas of biomedical research, including transplantation, immunology, cancer research, biochemistry, physiology, genetics, neuroscience and ageing” (Gill 1985, Gill 1989).

Today, the laboratory rat remains a standard animal model in a wide range of research, including studies on behaviour, nutrition, endocrinology and neurology (Kohn and Clifford 2002). Certain strains are also utilised as models of human diseases, such as diabetes mellitus and hypertension (Gill 1985), and are also commonly used in toxicological, teratological and carcinogenesis testing conducted by a large range of
pharmaceutical, academic and governmental institutions (Kohn and Clifford 2002).

1.2 Impacts of disease on animal research

As with all scientific investigations, animal experiments require strict adherence to the principles of scientific merit in order to ensure the resulting data is both reliable and reproducible (National Research Council 1991). Biological systems, however, are exceedingly complex and subject to fluctuations in biological responses secondary to both endogenous factors (i.e. genetics and age) and numerous exogenous factors encompassing a variety of physical, chemical and microbial factors. These factors have the potential to influence and thus interfere with experimental outcomes, usually by producing confounding lesions and/or altering physiological or behavioural responses (National Research Council 1991, Nicklas, Homberger et al. 1999, Allmann-Iselin 2000, Lipman and Perkins 2002, Faith and Hessler 2006). Thus, when laboratory animals are used in research experiments, it is critical that any changes in biological responses occurring as a result of the experiment be distinguished from those changes that occur due to the influence of non-experimental variables. In particular, a variety of infectious agents (microbial factors) are able to severely influence and confound the results of animal experiments through a diverse range of local or systemic effects (National Research Council 1991, Jacoby and Lindsey 1997, Baker 1998, Nicklas, Homberger et al. 1999, Lipman and Perkins 2002).

Infectious agents are capable of influencing laboratory animal populations in many different ways (Lipman and Perkins 2002) and the effects of infectious agents on animal research have been the subject of several detailed reviews (Baker, Lindsey et al.
1979, Bhatt, Jacoby et al. 1986, National Research Council 1991, Baker 1998, Nicklas, Homberger et al. 1999, Lipman and Perkins 2002). Whilst some microbes are highly pathogenic (e.g. virulent strains of rat coronavirus) and capable of inducing clinical disease with a range of morbidity and mortality, the majority of natural pathogens encountered in laboratory rat populations induce only subclinical disease in immunocompetent animals, without observable morbidity or mortality (National Research Council 1991, Nicklas, Homberger et al. 1999, Otto and Franklin 2006). It is important, however, to note that many of these agents are capable of interfering with research even in the absence of observable clinical manifestations of disease; either through altering the background physiology of experimental animals or by causing alterations in the response to a variety of experimental variables (National Research Council 1991, Otto and Franklin 2006). Two of the specific biological processes that may be altered by the presence of infectious agents include immune system function and the induction and progression of neoplasia (Otto and Franklin 2006). This is particularly relevant, as the fields of immunogenetics, transplantation and cancer biology are considered the disciplines that most heavily rely on the use of rats as an experimental model (Gill 1989, Otto and Franklin 2006). Similarly, pathogens inciting either liver or kidney dysfunction can significantly alter the metabolism and excretion of experimentally administered chemical compounds, thereby altering experimental outcomes in a variety of different research disciplines (Lipman and Perkins 2002).

Of particular concern and considerable frustration are infectious agents that become activated through stress caused by an experimental treatment (i.e. exposure to toxins, immunosuppressive agents or the induction of tumours), allowing manifestation of a subclinical disease in the treated group that is not seen in control groups and
subsequent misinterpretation of the results (Festing and Peters 1999). Additionally, many of these normally minimally pathogenic agents are capable of inducing serious disease, sometimes with significant morbidity and mortality in individuals with inherited or acquired immunodeficiency (Otto and Franklin 2006, Weisbroth, Kohn et al. 2006). Lastly, in addition to influencing the results of whole animal experiments, infectious agents may also adversely impact in vitro studies utilising isolated organs, cell cultures or other biological materials such as sera, viruses and parasites (Nicklas, Homberger et al. 1999).

1.3 Specific pathogen free technology

The historical battle to control infectious disease in laboratory rodents has been reviewed in extensive detail by Weisbroth (1996), who describes how domesticated rodents brought with them a variety of infectious agents, which had co-evolved alongside their hosts. With time, many of these pathogens and their associated disease conditions became increasingly uncommon or disappeared completely from captive laboratory populations due to improvements in hygiene, nutrition and indoor maintenance. Other infectious agents however, including several primary bacterial, viral and mycoplasmal pathogens, flourished in the confined environment provided by high-density indoor housing. Therefore, despite the disappearance of some diseases, disease problems continued to be encountered in captive rodent populations, with the general health status and standards of care for these animals remaining poor into the 1950s (Weisbroth 1996).
In the period following the end of World War II, expansions in biomedical research led to increased demand for laboratory rodents (Weisbroth 1996, Faith and Hessler 2006). This, in turn, led to a rapid increase in the sophistication of colony management techniques, primarily driven by efforts to standardise research animals to allow confirmation and direct comparison of research findings between different laboratories (Waggie, Kagiyama et al. 1994, Faith and Hessler 2006). To this end, there was increasing pressure to reduce the incidence of infectious diseases that were continuing to impose serious restrictions onto studies utilising rodent models (Baker, Lindsey et al. 1979, Faith and Hessler 2006). Whilst the improvements in colony management (husbandry and sanitation) developed between the late nineteenth century and the early 1950s had led to a reduction in the quantitative incidence of rodent pathogens, the qualitative nature of the pathogens harboured by rodent colonies had not been affected (Weisbroth, Kohn et al. 2006) and the occurrence of infectious rodent diseases continued to result in the cancellation of studies. Given that the standard veterinary approaches (vaccination and chemotherapeutics) were ineffective at ameliorating the impact of these infectious diseases, attention turned to looking for alternative methods of disease control (Weisbroth 1996).

Given that the majority of natural pathogens in rodents cause subclinical disease, it was recognised that the prevention of infection, rather than just prevention of clinical disease, was essential if the adverse affects of these agents on research were to be avoided (Kunstyr and Nicklas 2000). As such, in the late 1950s it was perceived and shown that the principles of gnotobiotic methodology, previously developed for studying the realm of germ-free biology, could be applied to large scale production of laboratory rodents for the elimination of horizontally transmitted pathogens from rodent
stocks (Cumming and Elias 1957, Foster 1958, Trexler and Barry 1958, National Research Council 1991, Weisbroth 1996). This process entails the surgical removal of the full term, gravid uterus from a dam originating from a contaminated colony, followed by transfer of the uterus through disinfectant solution into a sterile isolator. The pups are then removed and subsequently are foster-reared on axenic (i.e. germ-free) dams. After being inoculated with the microbial flora required for normal gastrointestinal function, these rederived animals (termed gnotobiotes) are typically maintained in isolators to function as nucleus breeders, to generate offspring destined for large barrier production colonies. The offspring of these animals, in turn, are made available for sale or use as research animals of known health status (Weisbroth 1996, Shek 2008). This methodology proved an effective means of eliminating horizontally transmitted diseases from rodent stocks and as such, soon became the standard method of commercial rodent production (Weisbroth 1996).

1.4 **Housing systems for microbial exclusion**

Despite the significant advances in health status provided by the introduction of gnotobiotic methodology in rodent production, many research facilities were unable to maintain the health status of new arrivals, which frequently become unwell or seropositive shortly thereafter (Shek 2008). Even in contemporary rodent colonies, some agents of concern may remain endemic and even if absent, there is the continual risk that one or more of these agents may be introduced (Otto and Franklin 2006). Therefore, beyond the production of specific pathogen free rodents, the continued maintenance of rodents free from these specified pathogens requires a strict adherence
to transport and maintenance programs designed for continual bioexclusion (National Research Council 1991). A key component of this is the use of rodent housing systems that are designed to prevent microbial contamination of the enclosed animals.

Generally, systems for housing rodents can be categorised based on the degree of biosecurity and microbial exclusion. At one end of the spectrum are facilities that utilise few or no specific measures to prevent microbial contamination. Such facilities and the animals enclosed therein are often referred to as conventional facilities and animals, respectively. In conventional facilities, animals tend to be housed in open cages and rooms with unrestricted access (National Research Council 1991). In contrast, other rodent housing systems utilise a variably strict combination of methods aimed at excluding pathogens and other unwanted microorganisms from the contained animal population. Such methods revolve around the concept of a ‘barrier’, which refers to any combination of physical arrangements, operating procedures and routines that have been instituted within an animal facility with the intent of minimising the potential for inadvertent microbial contamination (National Research Council 1991, Clough 1999). The concept of a barrier can be applied at a variety of different levels including the entire facility, room, rack, and/or cage level (National Research Council 1991). Given that there are many possible sources and routes of contamination with unwanted microbial organisms, it is necessary to simultaneously intervene in multiple areas to minimise the potential for inadvertent microbial contamination of a laboratory animal facility (Clough 1999).

The exact combination of approaches utilised is dependent on the specific research requirements and the objectives of investigators and/or institutions using the
enclosed animal population, however barrier programs typically consist of a number of essential elements. These include sourcing high quality animals demonstrably free of unwanted organisms (i.e. specific pathogen free animals), appropriate housing and facility design, thorough management and maintenance of the physical environment and thorough microbial monitoring of the enclosed animals to ensure barrier effectiveness (National Research Council 1991, Clough 1999). Some specific examples of barriers commonly used within laboratory rodent facilities are outlined in Table 1.1.1 (adapted from Clough 1999).

The simplest and most common form of rodent housing to utilise bioexclusion methodology are known as barrier facilities or barrier rooms (White, Anderson et al. 1998). The principles of a barrier in regards to rodent housing have been previously described, and in this type of housing, these concepts are applied to an entire facility or part(s) of a facility (e.g. barrier room). Depending on the number and type of bioexclusion and biosecurity measures utilised, barrier facilities can be further subclassified as being minimal, moderate, high or maximum-security barrier facility (National Research Council 1991).

An example of a maximum-security barrier is a methodology known as “isolator maintenance”, which provides the highest standard of biosecurity and microbial exclusion. An “isolator” refers to a sterilisable life-support chamber, typically constructed using either stainless steel or plastic and utilising high efficiency particulate air (HEPA) filtered laminar air flow technology, in which gnotobiotic animals can be continuously maintained free from unwanted microbial contamination (National Research Council 1991). Whilst the use of traditional flexible film and semi-rigid
isolators provide the most effective method of excluding microbial contaminants from an animal colony, limitations include a relatively large space requirement per animal, high cost and limited access for investigators to their study animals (Shek 2008).

<table>
<thead>
<tr>
<th>Facility design:</th>
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<tbody>
<tr>
<td>Physical separation of the building away from contaminated animal populations (wild and infected captive populations)</td>
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<tr>
<td>Physical separation of buildings within the animal facility</td>
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<th>Building design:</th>
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<tr>
<td>Encouraging one-way “flow” of animals between “clean” and “dirty” operations</td>
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<tr>
<td>Provision of spare room(s) to allow periodic room sterilisation</td>
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<tr>
<td>Provision of rodent and insect barriers at all building entrances and exits</td>
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<tr>
<td>Use of adequate ventilation supply and exhaust air filtered to the required level and the facility, room +/- cage level (at least 12-15 air changes per hour in each room)</td>
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<tr>
<td>Use of air-lock systems and air pressure differentials to minimise airborne cross-contamination</td>
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<th>Personnel management:</th>
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<tr>
<td>Restricted entry minimising the number of people allowed entry to the facility</td>
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<tr>
<td>Strict adherence to operating protocols including personnel decontamination (i.e. use of footbaths, change of footwear and outer clothing, followed by showering and complete change into protective, high-coverage clothing, wearing of facemasks and gloves)</td>
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<tr>
<td>Routine screening of staff for undesirable animal pathogens and limiting staff ownership of pets (particularly species maintained within the facility)</td>
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<th>Facility management:</th>
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<tr>
<td>Use of disinfectants for surface cleaning</td>
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<tr>
<td>Sourcing of animals from trusted sources of high quality (i.e. specific pathogen free) animals</td>
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<tr>
<td>Routine quarantine for all incoming animals</td>
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<tr>
<td>Provision and use of sterilising equipment for all cages, cage equipment, bedding, food, water, personnel clothing etc.</td>
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<tr>
<td>Thorough maintenance of equipment to ensure optimal function (i.e. autoclaves and air handling systems)</td>
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<tr>
<td>Implementation of effective health surveillance programs</td>
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<tr>
<td>Use of rederivation (by caesarian or embryo transfer) to eradicate pathogens</td>
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*Table 1.1.1 Barrier techniques commonly employed in laboratory animal facilities: adapted from Clough (1999)*
1.5 Terminology of animal microbial status

The subsequent widespread application of SPF technology and housing systems for microbial exclusion in the commercial production of laboratory rodents has resulted in the development of a “bewildering” array of terms used for the designation of the microbial status of animals, which frequently convey imprecise meanings leading to confusion regarding animal quality (National Research Council 1991). Typically, animals can be categorised in two ways based on a) the microbial exclusion methods utilised in colony maintenance and b) the microbiological status of individual animals.

As a result of the development and subsequent widespread usage of dedicated barrier housing systems for laboratory rodents, it is possible to produce and maintain animals with a known, or defined, pathogen status. Firstly, animals derived by hysterectomy as previously described, that are continually maintained in an isolator utilising germfree methods, should be demonstrably free from all microorganisms including bacteria, viruses, protozoa, fungi and other parasitic or saprophytic forms of life (except for endogenous viruses such as leukaemia viruses that occur in all mice). Such isolator-maintained animals are termed “germfree” (or “axenic”) animals and differ from animals defined as being “gnotobiotic” only in that the latter have been intentionally inoculated with one or more known non-pathogenic microorganisms (i.e. the “Schlaedler cocktail” consisting of several non-pathogenic bacteria required for normal gastrointestinal function) (National Research Council 1991).

The next major category of designated microbial status for animals includes “pathogen free” animals. In the strictest sense when referring to laboratory animal
quality, a “pathogen” refers to an infectious agent that is either capable of causing overt disease and/or of altering the biological responses of an animal during an experiment. There is, however, no universal agreement as to which agents have pathogen status, consequently such terminology is confusing. As such, a designation of “pathogen free” means little if the list of the pathogens for which the animal(s) are supposedly free of is not specified. As such, the term “specific pathogen free” (SPF) was coined to refer to animals free of a specified list of pathogens. These animals must be strictly barrier-maintained to order to ensure ongoing maintenance of their SPF status. In addition the designation of any given subpopulation of animals into the SPF category must be supported by a variety of current, appropriate test results from the group of animals in question to demonstrate the absence of the specified pathogens from within that group. Lastly, the term “conventional animal” refers to animals with uncontrolled and unknown microbial burden in which little or no routine monitoring of microbial status occurs. Such animals are typically housed under conditions of no containment, typically in open top cages and under husbandry conditions lacking measures to prevent pathogen contamination (National Research Council 1991).

1.6 Principles of microbiologic monitoring

Whilst the combination of rederivation and microbial exclusion programs has greatly decreased the range of diversity and the incidence of infectious diseases over the past 60 years or so, the maintenance of gnotobiotic and Specific Pathogen Free (SPF) rodent colonies requires a tremendous degree of commitment, effort and monitoring. There exists a continual risk of infectious agents being introduced into contemporary
colonies and additionally, a number of infectious agents of concern remain present at varying levels of prevalence within many institutional colonies (Pritchett-Corning, Cosentino et al. 2009). Animals can become inadvertently infected following breakdown of any aspect of barrier protocol (Weisbroth 1996, Shek 2008). For example, in many research settings, groups of rodents harbouring subclinical infections with potentially pathogenic microorganisms may be reared and/or maintained in the same facilities as animals of SPF status, often with little regard to the microbiologic status of the former. Practices like these have the potential to lead to the inadvertent introduction of infection to a previously SPF colony, resulting in disease outbreaks amongst immunologically naïve animals (Waggie, Kagiyama et al. 1994).

As barriers can, and do, fail, routine monitoring of the microbiological status of SPF maintained rodent colonies is essential in order to verify the continued absence of specified microorganisms (Shek 2008). Colony health monitoring (used synonymously with the term microbiologic monitoring) refers to scheduled and repetitive testing of an animal population to document the presence or absence of infectious agents within a colony, typically with the aim of ensuring that the previously specified microbiologic status of the colony is being maintained (Waggie, Kagiyama et al. 1994, Otto and Franklin 2006). As the microbiologic status of the enclosed animals is directly influenced by colony management techniques, health monitoring programs provide a retrospective indication as to the adequacy of colony management practices (Waggie, Kagiyama et al. 1994). Whilst the benefits of comprehensive colony health monitoring in protecting the validity and reproducibility of experimental data are clear (Otto and Franklin 2006), there are numerous opinions regarding the optimal design and required extent of individual programs (National Research Council 1991).
Almost 100 potential pathogens have been reported for rats and mice (Waggie, Kagiyama et al. 1994) and for reasons of practicality it is typically impossible to test for all known infectious agents or even to test for all agents with the theoretical potential to interfere with a specific study. As such, microbial testing is, by necessity, selective, and inclusion of an agent in a test battery is based on the significance of a pathogen and its likelihood of interfering with research (National Research Council 1991). As dissimilar studies will be variably affected by different pathogens, testing should wherever possible be matched to meet the requirements of specific research programs. For example the presence of certain microbes in animals used in surgical research may have no effect on the outcome of the study, whilst the presence of the same organism in an immunological study could have a devastating effect on the results and therefore validity of the entire study (Waggie, Kagiyama et al. 1994).

The potential pathogens of rats can be divided into five categories to assist in selection of pathogens for monitoring (Waggie, Kagiyama et al. 1994), as outlined in Table 1.1.2. Agents within categories A and B have the potential to cause problems regardless of the experimental objectives and include zoonotic pathogens and pathogens capable of causing disease fatal to animals, respectively. As such agents in these categories should be included within all monitoring programs. Inclusion of agents with categories C-E is typically dependent on the specific aims of the experiment as discussed in the previous paragraph. Category C includes pathogens not fatal to animals but which retain the capacity to cause disease and affect physiological functions of the test species. The remaining categories encompass opportunistic pathogens (category D) and microbes that indicate the general microbiologic status of an animal (or colony) (category E) (Waggie, Kagiyama et al. 1994).
### Table 1.1.2: Categories of microbial pathogens of rats: adapted from (Waggie, Kagiwama et al. 1994).

<table>
<thead>
<tr>
<th>Category</th>
<th>Microbes</th>
</tr>
</thead>
</table>
| A        | Hantavirus  
          | Lymphocytic choriomeningitis virus  
          | Salmonella spp.  
          | Dermatophytes  
          | Streptobacillus moniliformis |
| B        | Sendai virus |
| C        | Sialodacryoadenitis virus  
          | Pneumonia virus of mice  
          | Mycoplasma pulmonis  
          | Kilham rat virus  
          | Cilia-associated respiratory bacillus (CAR)  
          | Bacillus piliformis  
          | Corynbacterium kutscheri  
          | Pasteurella pneumotropica  
          | Bordetella bronchiseptica  
          | Streptococcus pneumoniae  
          | Spironucleus muris |
| D        | Pneumocystis carinii  
          | Pseudomonas aeruginosa  
          | Staphylococcus aureus |
| E        | Syphacia spp. (pinworms) |

1.7 Post-indigenous disease

In addition to the constant threat of introduction of known infectious agents due to the breakdown of microbial exclusion protocols, specific pathogen free colonies may also become infected with newly emerging and/or undiscovered pathogens not tested for in routine health surveillance monitoring. These diseases, referred to as post-indigenous diseases, comprise a group of diseases that have not been defined through history and experience as being indigenous to the particular rodent host (Weisbroth 1996). Rather,
this group of diseases incorporates a number of new conditions that are being recognised diagnostically over time. The question of whether these agents are recent arrivals, have been present for some time but have remained unrecognised, or a combination of both of these factors, often remains uncertain (Weisbroth 1996). The main contributor to the recognition of these previously unrecognised agents has been the introduction and use of more sensitive serologic assays as well as development of molecular genetic techniques, especially the polymerase chain reaction (PCR) methodology. The unrecognised and often clinically silent nature of these agents means that they may remain undetected in SPF colonies despite intense microbiologic monitoring. As a result, these agents can be inadvertently transferred between both colonies and institutions prior to their recognition. Strictly maintained isolator-maintained colonies rederived prior to the discovery of such agents have been described as being uniformly free of these agents, as have the majority of barrier maintained colonies where stock is sourced directly from such isolators (Shek 2008). Examples of such emerging or recently recognised agents in rats and mice include *Helicobacter* spp. infections, atypical parvovirus infections and murine norovirus (Weisbroth 1996, Shek 2008). The importance of eradication and maintenance of colonies free from such pathogens is somewhat dependent on the characteristics of specific agents as well as on the immune status of the animals within a colony. It is, however, important to note that as discussed previously, even non-pathogenic agents may also be capable of interfering with research (Shek 2008). In the case of recently discovered diseases, a specific example of potential research interference is the ability of some atypical parvovirus strains to modulate immune function by infecting proliferating lymphocytes and tumour cells (Gigliotti, Harmsen et al. 1993, Shek 2008).
1.8 Conditions associated with inflammatory lung lesions in rats

Given the circumstances prompting this study and the importance of eliminating known agents as a potential cause, it is pertinent to review the various types and major causes of inflammatory lesions that have been documented in the lungs of rats used for biomedical research. As for other body systems, lung structure and function in rats may be influenced by a variety of different factors, including environmental factors, diet and a variety of infectious agents (Castleman 1992). Infectious diseases of the respiratory tract in rats are of particular importance and are considered among the most frequently encountered health problems in this species (National Research Council 1991). In addition, lung disease in rats has historically been a major confounding factor in the use of this species for research. The importance of this organ system in this regard is reflected in the vast amount of literature available pertaining to infections of the respiratory tract in rats and is summarised by Lindsey and others, who wrote “most laboratory animals have one or more organ systems so frequently diseased as to seriously restrict the usefulness of that species for research purposes. In the rat, this distinction clearly belongs to the respiratory system” (Lindsey, Baker et al. 1971). The severe impact of murine respiratory mycoplasmosis (caused by Mycoplasma pulmonis infection) as a confounding factor in experiments utilising rodents is also credited as being the main motivating factor in prompting the search for novel methods of disease control, which led to the subsequent development of specific pathogen free technology (Weisbroth 1996).

A variety of infectious agents, including bacterial, viral and fungal agents have the ability to induce necrosis and/or inflammation in the lungs of rats (Boorman and
Eustis 1990). At least 14 specific respiratory pathogens of rats have been identified and, while these agents vary widely in pathogenicity, the disease course for all agents is frequently subclinical with overt clinical disease being uncommon. When clinical manifestations of disease do occur, this is usually due to dual or multiple infections whereby synergistic interactions between pathogens have more than an additive effect in producing signs of disease. It is, however, important to note that as previously discussed, many infectious agents of rodents, including those of the respiratory tract, are capable of causing research interference in the absence of clinical disease (National Research Council 1991, Baker 1998). For example, necrosis of airway epithelial cells or changes in the mucus layer can lead to a marked decrease in clearance of particulate matter from the lung, which can have a marked effect on the outcome of inhalational toxicology studies. Similarly, infectious agents can induce an increased rate of cell turnover, which can alter the response to an administered carcinogen. Lastly, the presence of pathogen-induced morphological changes in the lung can mask the presence of subtle experimental-related changes in the lung (Boorman and Eustis 1990).

1.8.1 Pneumonia

I. Bronchopneumonia

Murine respiratory mycoplasmosis

The cause of murine respiratory mycoplasmosis, *Mycoplasma pulmonis*, is without a doubt one of the most important infectious agents of rats and mice. A member of the order Mycoplasmatales, *M. pulmonis* is a small, fastidious bacterium that lacks a cell wall (Ganaway 1994). Transmission may be horizontal, via aerosol (Hill 1972, National Research Council 1991, Zenner and Regnault 2000) and possibly sexually
(Gaillard and Clifford 2000), or alternately may be vertical following uterine infection (Domachowske, Bonville et al. 2002). Husbandry and the presence of other infectious agents will also influence disease transmission and prevalence. In the absence of other respiratory pathogens and under optimal husbandry conditions (including frequent bedding changes and low intracage ammonia levels) horizontal transmission is considered unlikely (Boorman and Eustis 1990). Conversely, in situations with co-occurring infections with other respiratory pathogens and high intracage ammonia levels, horizontal spread may occur rapidly between both cagemates and adjacent cages (Baker 2003). In poorly managed conventional colonies the rate of infection may approach 100%, however, the advent of caesarean derivation and strict microbial exclusion procedures proved a highly effective means of eliminating *M. pulmonis* infection from rodent stocks (Weisbroth 1996, Baker 2003). As such, *M. pulmonis* infections in laboratory rat colonies today are infrequent and are typically limited to a small proportion of conventional rodent facilities. That said, the continued presence of this pathogen in wild rodents and in a subset of conventionally housed rodents means that frequent and thorough testing (predominately via ELISA testing supported by PCR in some cases) is required to ensure ongoing freedom from infection (Weisbroth, Kohn et al. 2006).

Following entry, principally via the respiratory tract, *M. pulmonis* readily colonises the epithelial surfaces of the respiratory tract, middle ear and female genital tract (Baker 2003). Outcome of infection with *M. pulmonis* is dependent on a variety of factors including intrinsic (i.e. strain related) factors as well as extrinsic factors such as husbandry conditions and the presence/absence of infection with other respiratory pathogens (National Research Council 1991, Gaillard and Clifford 2000, Baker 2003).
In general, the prevalence and severity of disease expression tends to increase both with increasing age of the rat and in the presence of other stressors whether they be environmental, experimental or due to the co-occurrence of other respiratory pathogens (Weisbroth, Kohn et al. 2006). Under conditions of optimal husbandry and in the absence of other respiratory pathogens, morbidity and mortality with *M. pulmonis* infection is extremely low and infection is typically sub-clinical (Baker 2003). Indeed under ideal conditions for the host, it has been suggested that *M. pulmonis* is probably a commensal (National Research Council 1991). Conversely, less favourable circumstances for the host, such as co-infection with other respiratory pathogens (Ward, Hamlin et al. 1983, Liang, Schoeb et al. 1995) and/or under poor husbandry conditions such as high intra-cage ammonia levels (Ganaway, Allen et al. 1973, Ganaway 1994) lead to increasing morbidity and mortality, beginning at 6-8 weeks of age and increasing in severity with age (Baker 2003). The observable increase in severity occurring with age has been attributed to factors such as declining immune function, chronic disease progression and increased lung involvement in older rats (Broderson, Lindsey et al. 1976, Gaillard and Clifford 2000). Other extrinsic factors reported to influence disease outcome in *M. pulmonis* infection in rats include exposure to toxic chemicals (such as hexamethylphosphamide) (Cassell, Lindsey et al. 1973), nutritional deficiencies (vitamins A and E) (Cumming and Elias 1957), and inoculation dose and virulence of the *M. pulmonis* strain (Lindsey, Baker et al. 1971, Brix, Nyska et al. 2005). Additionally, differences in strain susceptibility have also been noted, with LEW rats having been shown to develop more extensive and severe disease post-inoculation compared to F344 rats (Davis and Cassell 1982, Bhatt, Jacoby et al. 1986).

Clinical signs are commonly absent even in cases where significant pulmonary
lesions exist (Weisbroth, Kohn et al. 2006). When present, clinical signs in infected rats are non-specific and may include “snuffling”, weight loss, inactivity, dyspnoea, polypnoea, hunched posture and/or torticollis (Lindsey, Baker et al. 1971, National Research Council 1991, Gaillard and Clifford 2000, Weisbroth, Kohn et al. 2006). As with clinical outcome, gross and microscopic expression of disease is highly variable, being influenced by a variety of intrinsic and extrinsic factors as discussed above. On the mildest end of the spectrum, some rats may not have gross or microscopic lesions. Rats of all ages may have evidence of rhinitis, otitis media, otitis interna, laryngitis and/or tracheitis that may vary from acute to chronic in nature depending on the timing of the disease course. Epithelial changes such as pseudoglandular hyperplasia and squamous metaplasia may also be observed (Lindsey, Baker et al. 1971, Bhatt, Jacoby et al. 1986, National Research Council 1991, Gaillard and Clifford 2000). Additionally, older rats, from about four weeks of age often have additional lesions of the lower respiratory tract associated with extension of infection via the trachea and bronchioles (Gaillard and Clifford 2000, Baker 2003). As with the upper respiratory tract, lesions may be acute or chronic in nature. Grossly, early lesions typically present as pinpoint grey foci progressing to variably sized, mucous or pus-filled foci as bronchiectasis develops (Percy and Barthold 2007). Focal areas of pulmonary consolidation may be apparent accompanied by a highly viscous exudate being present in the airways (Baker 1998). In severe cases the presence of this exudate may lead to patchy pulmonary emphysema and additionally multifocal areas of abscessation may also be seen. Lung lesions may be unilateral or bilateral, are usually cranioventral in distribution and are frequently asymmetric (Percy and Barthold 2007). Microscopically, a range of lesions may be observed including hyperplasia of bronchus-associated lymphoid tissue, hyperplasia/metaplasia of mucosal epithelium, suppurative bronchitis/bronchiolitis,
suppurative to non-suppurative alveolitis, pulmonary abscessation, bronchiectasis/bronchiolectasis and peribronchial, peribronchiolar and/or perivascular lymphocyte and plasma cell cuffing (Lindsey, Baker et al. 1971, Gaillard and Clifford 2000, Elmore 2006, Percy and Barthold 2007). The latter feature is typically prominent in all stages of the disease process (Percy and Barthold 2007).

Cilia-associated respiratory bacillus

A Gram negative, filamentous bacillus of uncertain classification (Baker 2003), known as cilia-associated respiratory (CAR) bacillus, colonizes ciliated airway epithelium and was first described in association with naturally occurring respiratory disease in rats in 1980 (van Zwieten, Solleveld et al. 1980). Analyses of rat isolate CAR bacillus 16S ribosomal RNA sequences indicate that the bacillus is most closely related to members of the Flavobacterium and Flexibacter groups (Schoeb, Dybvig et al. 1993, Kawano, Nenoi et al. 2000).

Clinical signs and the nature/severity of lesions attributed to CAR bacillus infection are variable and are influenced by a number of factors including bacterial isolate (Schoeb, Davidson et al. 1997), host age and the presence of co-pathogens (Weisbroth, Kohn et al. 2006). Whilst CAR bacillus infection is considered a common co-pathogen (Baker 2003), monospecific experimental CAR bacillus infection has been shown to cause clinical signs and lesions extremely similar to those seen with murine respiratory mycoplasmosis (Ganaway, Spencer et al. 1985, Matsushita and Joshima 1989). Like many other infectious agents of the rodent respiratory tract, infections with CAR bacillus typically start as an upper respiratory tract infection, with chronic inflammation seen in the nasal cavity, middle ears and trachea. Subsequently,
mucopurulent bronchitis and bronchiolitis occurs, with marked associated peribronchiolar inflammatory cell infiltration and multifocal necrosis of airway epithelium, which may also be accompanied by bronchiectasis and abscess formation. (Gaillard and Clifford 2000, Weisbroth, Kohn et al. 2006). Bronchus-associated lymphoid tissue hyperplasia may also occur and silver stains demonstrate large numbers of filamentous bacilli on the surface of the respiratory epithelium throughout the respiratory tract. In cases with secondary bacterial infection, additional lesions of suppurative bronchopneumonia may also be noted (Gaillard and Clifford 2000).

**Other bacteria**

A variety of other bacterial agents have been shown have tropism for the respiratory tract and have an ability to cause clinical disease in rats. Infection with these agents is generally subclinical in immunocompetent animals, with clinical disease typically only occurring following immunosuppression or other insults such as experimental stress, dietary deficiencies or concurrent infections (National Research Council 1991, Nicklas, Homberger et al. 1999, Weisbroth, Kohn et al. 2006). These organisms generally cause a suppurative bronchopneumonia and/or abscessation, often with varying degrees of necrosis and/or pleuritis. Specific bacteria implicated include *Corynebacterium kutscheri, Streptococcus pneumonia*, and *Pasteurella pneumotropica* (National Research Council 1991, Waggie, Kagiyama et al. 1994, Gaillard and Clifford 2000, Weisbroth, Kohn et al. 2006).

**II. Bronchointerstitial pneumonia**

Bronchointerstitial pneumonia in the rat has been defined as pneumonia having an acute exudative phase with pronounced bronchiolar orientation that is followed by a predominately interstitial orientation in the latter stages. (Dungworth, Ernst et al. 1992).
Major causes of this pattern of pneumonia in this species include Sendai virus and other murine respiratory viruses including pneumonia virus of mice (PVM), sialodacryoadenitis virus (SDA) and rat coronavirus (RCV). As previously outlined, these infectious diseases are most commonly encountered in conventional housing facilities that may be endemically infected, however surveillance for these diseases is crucial for specific pathogen free colonies which may become inadvertently infected following breakdown of barrier protocols (Dungworth, Ernst et al. 1992, McInnes, Rasmussen et al. 2011).

**Sendai virus**

An ssRNA virus of the family *Paramyxoviridae*, genus *Paramyxovirus*, Sendai virus is extremely contagious and is considered one of the most important pathogens of laboratory mice and rats (Baker 2003). As with many rodent infectious agents, natural infection in rats is typically asymptomatic (Castleman 1992, Baker 2003, Percy and Barthold 2007), although prior serological surveys suggest that it has been relatively widespread amongst rat colonies in the past (Percy and Barthold 2007, McInnes, Rasmussen et al. 2011). Differences in viral strain and host factors such as age, strain susceptibility and host immune status lead to variability in lesion severity (Castleman 1992, Baker 2003) and co-infection with other respiratory pathogens such as *M. pulmonis* will further increase lesion variability and severity (Baker 2003). In the initial stages, infection leads to transient necrosis, hypertrophy and hyperplasia of nasal and lower respiratory tract epithelium with associated infiltration of neutrophils, lymphocytes, macrophages and plasma cells into airway epithelium, walls and lumina (Baker 2003, Percy and Barthold 2007). Once the virus reaches the lungs, multifocal interstitial pneumonia is seen with hyperplastic changes and interstitial infiltration of
similar inflammatory cells, which is most severe at the level of the terminal bronchioles but also involves the alveolar septa (Castleman 1992, Baker 2003). As virus replication ceases approximately one week post infection, lesions typically resolve quickly (Baker 2003) and in the later and resolving stages of infection, typically include lymphoplasmacytic perivascular and peribronchiolar inflammatory cell cuffing. Non-suppurative inflammatory cell infiltrates may be found within the alveolar septa for several weeks post infection, sometimes in conjunction with interstitial fibrosis (Percy and Barthold 2007). Residual lesions attributed to early life infection with Sendai virus have also been described in aging Sprague Dawley rats, with peribronchiolar and perivascular infiltrates of macrophages and lymphocytes and small bronchiolar and multifocal bronchiolar mural fibrosis having been documented in rats at least 18 months after neonatal infection (Castleman 1992).

**Pneumonia virus of mice**

Another ssRNA virus of the *Paramyxoviridae* family, this virus is classified within the genus *pneumovirus* and multiple strains of varying pathogenicity exist (Baker 2003). Although the prevalence in contemporary rodent colonies is generally considered to be extremely low (Pritchett-Corning, Cosentino et al. 2009), high seroprevalence has been documented in some rodent colonies (Zenner and Regnault 2000, Percy and Barthold 2007), with up to 100% prevalence in weanling rats from colonies with enzootic infection (Brownstein 1996). Pneumonia virus of mice (PVM) is known to be capable of altering gene expression leading to production of a number of pro-inflammatory factors, including cytokines and adhesion molecules, in mice (Domachowske, Bonville et al. 2002, Baker 2003). Whilst some authors describe a lack of pathological lesions in naturally infected immunocompetent rats (Baker 2003, Jacoby
and Gaertner 2006); others have described both gross and histopathological lesions in naturally infected rats, as well as histopathological lesions in experimentally infected rats (Brownstein 1996). Where documented, gross lesions in adult, naturally infected rats are reportedly rare, although focal or multifocal, less than 2 mm grey to plum-coloured pulmonary foci have been described (Brownstein 1996). Histopathological lesions reported in rats following recent serological conversion for PVM include bronchus-associated lymphoid tissue (BALT) hyperplasia and multifocal perivascular and other interstitial mononuclear inflammatory cell infiltrates (Castleman 1992, Brownstein 1996). Other features included a mixed inflammatory cell infiltrate within alveolar lumina and prominent bronchiolar epithelium (Castleman 1992). These lesions of PVM may persist for up to several weeks in the rat (Percy and Barthold 2007). Lastly, in experimentally infected F344 rats, non-suppurative vasculitis and acute interstitial pneumonia have also been reported (Vogtsberger, Stromberg et al. 1982).

**Rat coronaviruses**

Two major naturally occurring coronaviruses have been described in the rat, Parker’s rat coronavirus (PRC) and sialodacryoadenitis virus (SDAV). Given the context in which the two prototype strains were identified, the nomenclature for this group of viruses is somewhat confusing (Jacoby and Gaertner 2006). In 1970 a coronavirus isolate was found in the lungs of clinically healthy rats and became known as Parker’s rat coronavirus (Parker, Cross et al. 1970). A second coronavirus was later identified that caused sialodacryoadenitis, and was descriptively named sialodacryoadenitis virus (Bhatt, Percy et al. 1972). Subsequently, however, PRC was later shown experimentally to produce identical lesions to those seen in SDAV inoculated rats (Percy and Williams 1990). Additionally, as coronaviruses are single-
stranded RNA viruses, the potential for errors during viral replication can lead to divergent strains emerging during natural infections, and indeed additional rat coronavirus isolates have been described subsequent to PRC and SDAV (Jacoby and Gaertner 2006). As such, sialodacryoadenitis virus is now considered a morphological designation that includes all coronavirus isolates that cause sialodacryoadenitis. Similarly it has been suggested that these viruses all be considered members of a single biological group, referred to as rat coronaviruses (RCV), as it is likely that this group contains multiple strains that vary in virulence and are constantly changing (Percy and Barthold 2007).

As for many infectious respiratory pathogens in rats, RCV used to be relatively common, however, it is now rarely seen in well-managed facilities (Percy and Barthold 2007). In addition to differences with infecting viral strain, disease severity may be altered by non-viral factors such as rat strain (Liang, Schoeb et al. 1995) and synergistic infection with other respiratory pathogens such as M. pulmonis (Baker 2003). Clinical signs in enzootic infections are usually limited to suckling rats, which may show signs of conjunctivitis. Infection in adults and weanlings is usually unapparent, however periparturient females occasionally develop conjunctivitis and exophthalmos. Clinical signs in epizootic infections are typically more severe, affecting rats of any age and including sneezing, conjunctivitis, corneal and nasal discharge, cervical oedema and corneal ulceration (Baker 2003). Whilst tissue tropism can vary with viral strain, SDAV shows tropism for serous or mixed tubuloalveolar glands (National Research Council 1991) and infection typically leads to necrosis and associated secondary mixed mononuclear and polymorphonuclear inflammatory cell infiltration within the parotid, submandibular and orbital salivary glands and the lacrimal glands (including the
Harderian gland) (Baker 2003, Percy and Barthold 2007). Some strains also cause lesions in the respiratory tract, including multifocal rhinitis, tracheitis, bronchitis/bronchiolitis and alveolitis (Baker 2003). These lesions are characterised by leukocyte infiltration and hyperplasia of respiratory epithelium with attenuation and loss of ciliated cells (Percy and Barthold 2007).

III. Interstitial pneumonia

Interstitial inflammatory cell infiltrates, consisting predominately of perivascular and peribronchial/peribronchiolar lymphocytes, have been described within the lungs of ageing SPF rats, especially as cuffs surrounding small pulmonary veins. The number and size of these infiltrates is considered a strict function of colony husbandry and hygiene, with this lesion not being documented in lungs from clean control colonies. When present, these interstitial infiltrates indicate probable prior infection with one of the murine respiratory viruses, previously described under the category of bronchointerstitial pneumonia (Dungworth, Ernst et al. 1992). Additionally rats maintained in stable SPF conditions have been documented as having relatively inconspicuous BALT, with no appreciable increase with age. Therefore conspicuous BALT, especially with germinal centre development, is similarly considered evidence of pulmonary infection with immunostimulatory bacteria or viruses (Ward, Hamlin et al. 1983)

“Rat respiratory virus”

In the late 1990’s distinctive and apparently novel pulmonary inflammatory lesions were identified and described in the lungs of laboratory rats from multiple institutions, first in the USA (Elwell, Mahler et al. 1997, Gilbert, Black et al. 1997,
Riley, Purdy et al. 1997) and subsequently in Europe (Slaoui, Dreef et al. 1998) and Asia (Clifford and Albers 2003). The first report, published in 1997 in the Journal of Toxicologic Pathology, described “a spectrum of inflammatory lesions of unknown aetiology” within the lungs of F344 rats used in prechronic toxicity studies at a number of different laboratories across the United States. In rats with extensive inflammatory lesions, “pale or tan foci” were seen macroscopically in the lungs. Histopathological examination revealed perivascular inflammatory cell cuffing throughout the lungs, accompanied by alveolar infiltrates of macrophages, neutrophils and lymphocytes, variably increased amounts of BALT and type II pneumocyte hyperplasia. Extensive serological testing, bacterial culture and protozoal identification testing failed to identify a causative agent. Electron microscopy failed to demonstrate the presence of viral-particles, although structures interpreted as bacterial bacilli were observed in the majority of rats with lung lesions (Elwell, Mahler et al. 1997).

Similar lung lesions were subsequently reported in Sprague Dawley rats (Farrar and LaRegina 1997, Gilbert, Black et al. 1997) and the next year, in Wistar rats used for toxicology studies in the Netherlands (Slaoui, Dreef et al. 1998). As in the Elwell et al report, variably extensive attempts undertaken to establish a cause for these lesions were unsuccessful in identifying a causative agent, however the authors’ frequently noted that lesion incidence and severity tended to decrease over time during the course of a study, leading to speculation of an acquired immunity (Slaoui, Dreef et al. 1998). Clinical signs of respiratory disease were not reported in association with the respiratory lesions in any of the above studies.

At a similar time as the above studies were reported, research was being
Conducted into similar idiopathic lung lesions noted in laboratory rats at the Research Animal Diagnostic & Investigative Laboratory (RADIL) at the University of Missouri (Riley, Purdy et al. 1997, Riley, Simmons et al. 1999). In order to better understand lesion pathogenesis and progression, groups of Sprague Dawley rats, from historically affected colonies, were examined at various ages (2/3, 6, 8, 10, 12 and 18 weeks) for evidence of pulmonary disease and blood and tissues were also collected for extensive ancillary diagnostic testing. As in the other reports, gross lesions were seen in some rats (multifocal grey-white raised lesions that were distributed randomly across all lung lobes) between the ages of 8 and 18 weeks. Histopathological lesions were first noted in the 6 week-old rats and consisted of mild multifocal perivascular infiltrates of lymphocytes. Lesion severity increased in 8 week-old rats, with multiple perivascular infiltrates of lymphocytes and neutrophils, and peaked in the 10-12 week groups with moderate perivascular lesions that were frequently found in conjunction with foci of interstitial pneumonia and occasional type II pneumocyte hyperplasia. These lesions were occasionally of sufficient severity to cause localised pulmonary consolidation (Riley, Simmons et al. 1999).

Extensive ancillary diagnostic testing was performed in an attempt to identify an aetiologic agent, including microbiological culture, histochemical staining and PCR testing (including a generic bacterial PCR with primers targeting a conserved region of the 16S rRNA gene in all known eubacterial species). Other tests performed included evaluation of tissues under polarised light to evaluate for foreign material, fungal and Pneumocystis carinii screening (including silver stains and PCR) and serological testing for known respiratory and non-respiratory viral agents of rats. Lastly, multiple mammalian cell lines were inoculated with tissue homogenates from the lungs of
affected rats, which subsequently produced cytopathic effects in 2/15 cell lines. This effect was not seen with use of tissue homogenates from unaffected rats. Additionally, affected rat sera reacted with the “infected” mammalian cell cultures in immunofluorescence assays, a finding not repeatable when using sera from colonies with no history of lung lesions. As such, it was concluded that these results suggested the presence of antibodies to a cytopathic virus in affected rats that was not present in unaffected individuals (Riley, Purdy et al. 1997, Riley, Simmons et al. 1999).

The researchers went on to report the isolation and culture of a virus from affected lung tissue, designated “rat respiratory virus” (RRV). Subsequent attempts to characterise and identify the virus were severely hampered by difficulties that arose during efforts to propagate the virus in vitro, with viral titres in cell culture reportedly being very low (Riley, Purdy et al. 1997, Riley, Simmons et al. 1999). Despite these limitations however, preliminary characterisation studies, utilising immunofluorescence assays, western blot analysis and electron microscopy, led to a tentative classification of the virus within the Hantavirus genus (Simmons and Riley 2002, Percy and Barthold 2007). An immunofluorescence assay was subsequently developed as a method of detecting infected rats and preliminary results showed good correlation between histologic findings from both affected and unaffected rats (Riley et al 1997; 1999).

Despite these early findings, other early attempts at virus isolation from infected lungs, using a variety of different cell lines (Rhesus monkey kidney, Hep-2, human neonatal kidney cell and human embryonic lung fibroblast), failed to demonstrate the presence of haemagglutinating, cytopathic or syncytial forming viruses (Farrar & LaRegina 1997). Additionally, the results of later studies indicated that the positive IFA
reactions reported by Riley et al. were likely false-positive results resulting from non-specific binding to “sticky” nucleocapsid proteins of Hantaviruses. It has also been reported that PCR assays using hantavirus-specific primer sets have failed to demonstrate any relationship with other members of the *Hantavirus* genus (Percy and Barthold 2007). As such, at the time of commencement of the study culminating in this thesis, a definitive cause of the distinctive lung lesions attributed to “rat respiratory virus” had not been identified. Additional information and discussion regarding subsequent updates to the knowledge regarding this disease are provided later in Parts two and three of this thesis. As with most contemporary infectious agents of laboratory rodents, the impact of RRV lies with its ability to interfere with research, with the associated morphological changes within the lung being reported as a confounding factor in inhalational and immunotoxicity studies (Gilbert, Black et al. 1997, Gore, Gower et al. 2004).

**Polyoma virus**

A polyoma virus affecting rats was first documented in athymic nude rats exhibiting a syndrome of wasting, pneumonia and parotid gland sialoadenitis. Lesions included the presence of prominent intranuclear inclusion bodies in the bronchiolar and alveolar epithelial cells, as well as within salivary glands. Lung lesions reported in this report were highly variable and included hyperplastic bronchitis and bronchiolitis, bronchiolitis obliterans and bronchiectasis, suppurative bronchopneumonia and abscessation, as well as focal interstitial pneumonia (Ward, Lock et al. 1984). Additionally, *Staphylococcus* sp. and *Streptococcus* sp. were isolated from a few of the affected rats. Anecdotal descriptions by other authors describing diagnostic material have reported polyoma viral inclusions in athymic nude rats exhibiting weight loss and
interstitial pneumonia (Percy and Barthold 2007). Given these observations, it seems likely that the suppurative inflammation and bronchopneumonia noted in the original report were the result of superimposed bacterial infections within the colony, and that uncomplicated polyoma virus infections induce interstitial pneumonia without concurrent suppurative inflammation.

**Pneumocystis carinii**

*Pneumocystis* pneumonia was first described in guinea pigs by Chagas in 1909, but it was mistaken for a form of *Trypansoma cruzi* and it was not until 1912 that it was recognised as a new organism, named *Pneumocystis carinii*, this time causing pneumonia in rats (Weisbroth, Kohn et al. 2006). Prior to the era of radiation and anti-inflammatory research in the 1950s and 1960s (Davis and Cassell 1982), however, there were few additional reports of this organism causing disease, and it is now recognised that the increased incidence of this disease noted during those decades was due to the introduction of research protocols causing immunosuppression (Weisbroth 2006). The taxonomic classification of *Pneumocystis* was a matter of uncertainty and considerable debate for years. Historically, the majority of investigators considered *P. carinii* to be a protozoan, however relatively recently; molecular assays including sequence analysis of 16S rRNA and subsequent cloning and sequencing of other *P. carinii* genes, have firmly demonstrated that *P. carinii* is in fact a genus of unusual single-celled fungi within the phylum ascomycetes (Ward, Yoon et al. 2001, McInnes, Rasmussen et al. 2011).

Natural infection with *Pneumocystis carinii* has been described in humans and numerous other mammalian species (Waggie, Kagiyama et al. 1994). Infection is
transmitted via the airbourne route (Hughes 1982), and experimental studies in rats have confirmed that *P. carinii* infection is acquired very shortly after birth (Icenhour, Rebholz et al. 2002). A variety of antigenic and genetic differences have been identified among isolates from different host species and from within the same host species (Halliwell 1997). Additionally, these isolates demonstrate significant host specificity and have been shown to not be universally transmissible between mammalian hosts (Jacoby and Lindsey 1997). At present, two main species are recognised in laboratory rats. The designation of *P. carinii* is retained for the most common species, while the less common of the two was named *P. wakefieldi* (formerly *P. carinii f. sp. ratti*) (Cushion, Keely et al. 2004, Weisbroth 2006). Additional species have also been identified in wild rats, and co-infection with more than one species has also been documented (Hook 1991).

Clinical signs of *Pneumocystis* infection are not seen in immunocompetent animals and therefore is considered an opportunistic pathogen that requires immunodeficiency in order to cause clinical disease, either through inherited immunodeficiency (e.g. immunodeficient strains of mice and rats) or through naturally acquired or experimentally induced immunosuppression (e.g. prolonged corticosteroid administration (Konish and Higashiguchi 1996, Baker 1998, Weisbroth 2006). Despite the subclinical nature of the infection in immunocompetent hosts, shedding of the organism still occurs (Menotti, Emmanuel et al. 2013) and the importance of immunocompetent hosts as maintenance reservoir for the propagation of infection to immunocompromised hosts is now well recognised (Weisbroth 2006, Morris, Wei et al. 2008).
The subclinical nature of *Pneumocystis* infection in immunocompetent hosts means that that infection is often overlooked (Waggie, Kagiyama et al. 1994). In contrast, disease in immunocompromised animals leads to chronic progressive pneumonia with high morbidity and mortality (Nicklas, Homberger et al. 1999). Clinical signs of infection in immunocompromised hosts include chronic wasting, rough hair coat, dyspnoea, cyanosis and death (National Research Council 1991, Weisbroth 2006). Gross lesions are not apparent in occult infections (Gaillard and Clifford 2000), but in clinical infections affected lungs are enlarged, heavier than normal with a rubbery consistency (National Research Council 1991, Baker 1998), and sometimes have multiple grey-brown foci of consolidation (Gaillard and Clifford 2000). Microscopically, the classic lesion is alveolar thickening and alveolar filling with amorphous, eosinophilic foamy to honeycombed material, that consists of a mixture of organisms, serum protein, pulmonary surfactant, and dead host cells (Baker 1998). The lesion usually affects contiguous alveoli with minimal concurrent inflammation (Boorman and Eustis 1990), although infiltrates of lymphocytes have also been described (Waggie, Kagiyama et al. 1994). The septal thickening seen is due to type II pneumocyte proliferation and interstitial fibrosis (Nicklas, Homberger et al. 1999). Application of special stains (e.g. GMS) demonstrates multiple discoid cysts (asci) within alveoli (Weisbroth, Kohn et al. 2006).

Infection with *Pneumocystis carinii* in laboratory rats can have significant research implications. Infection not only leads to a high incidence of disease and death in all congenitally immunodeficient or experimentally immunosuppressed animals, but infection has also been documented to alter a variety of physiological and immunological responses (Nicklas, Homberger et al. 1999). These alterations have been
comprehensively reviewed by Nicklas and Homberger et al, but include alterations in lung compliance (Brun-Pascaud, Pocidal et al. 1985) and surfactant production (Kernbaum, Masliah et al. 1983), the release of numerous inflammatory mediators including tumour necrosis factor-alpha (Hoffman, Standing et al. 1993) and both the activation and inhibition of cellular innate immune responses (Vargas, Ponce et al. 2013).

1.9 Miscellaneous lesions

1.9.1 Alveolar histiocytosis

While some texts use the term “alveolar histiocytosis” to refer to accumulations of foamy (lipid-laden) macrophages within alveoli (Boorman and Eustis 1990), the term has also been used to refer to a variety of different lesions, ranging from localised accumulations of alveolar macrophages to mixed inflammatory lesions where large foamy macrophages or even fibrosis predominate (Dungworth, Ernst et al. 1992). These lesions are often found in conjunction with cholesterol crystals, type II pneumocyte hyperplasia, and patchy infiltrates of inflammatory cells, including neutrophils (Dungworth, Ernst et al. 1992, Sells, Brix et al. 2007). Haemosiderin (Renne, Brix et al. 2009), lipofuscin and carbon have also been observed within a proportion of these alveolar macrophages (McInnes 2012), and increased phospholipid material within the alveoli has also been described (Boorman and Eustis 1990). Foci of alveolar histiocytosis occur predominately in the peripheral regions of the lung, most commonly in subpleural regions followed by alveoli surrounding large blood vessels and airways (Dungworth, Ernst et al. 1992). Foci of alveolar histiocytosis have been
described within control animals and the prevalence tends to increase with age, with one study documenting a prevalence of 80.3% in control rats at the end of a 2-year bioassay (Brix, Nyska et al. 2005). Minor lesions of alveolar histiocytosis are too small to be detected macroscopically, however more extensive lesions appear as multiple grey-white, plaque like foci that can be seen from the pleural surface (Dungworth, Ernst et al. 1992). These more extensive lesions have been seen following infection with respiratory pathogens (Dungworth, Ernst et al. 1992), but have also been induced by a variety of different experimental mechanisms, including chronic exposure to airborne irritants/toxicants and parenchymal necrosis (Renne, Brix et al. 2009). Accumulation of lipid-laden macrophages has also been induced secondary to dietary lipid imbalance (Dungworth, Ernst et al. 1992) or altered endogenous lipid metabolism by a number of cationic amphiphilic drugs (alveolar phospholipidosis) (Halliwell 1997) and by hypophysectomy (Konish and Higashiguchi 1996).

Alveolar histiocytosis can also be a feature of alveolar lipoproteinosis, a condition where there is progressive accumulation of acellular material, composed predominately of phospholipids derived from pulmonary surfactant within alveoli (Dungworth, Ernst et al. 1992, Renne, Brix et al. 2009). This condition is readily induced by repeated experimental exposure to cytotoxic materials such as silica, however macrophages are relatively scarce within fully developed lesions (Renne, Brix et al. 2009).
1.10 Summary

As outlined in the preceding review, both endogenous (i.e. genetics and ageing) and exogenous (physical, chemical and microbial) factors have the potential to produce confounding lesions in animals used in biomedical research. Background lung lesions, particularly those caused by infectious agents, have long been a frustrating and often confounding issue for biomedical researchers. While the use of SPF technology in commercial laboratory animal production facilities has helped reduce the incidence and impact of these infectious agents, there remains a continual risk of accidental introduction of infectious agents. Additionally, animals may become infected by newly emerging pathogens not tested for as part of colony health monitoring programs.

Following the identification of unexpected lung lesions by researchers using rats sourced from the Animal Resources Centre (ARC), a large laboratory rodent production facility located in Perth, Western Australia, questions arose as to whether the problem was related to pre-existing disease in the rats originating at the ARC or rather if the observed lesions were related to experimental variables/procedures, microbial contamination, or environmental changes to which the rats were exposed upon transfer to facilities with different levels of microbial control and/or husbandry procedures. These questions, combined with reports of distinctive but poorly characterised lung lesions in laboratory rats overseas, known at the time as “rat respiratory virus”, prompted this study.
1.11 Aims

This primary aim of this study was to survey the ARC rat population for evidence of background lung lesions. To achieve this, a combination of gross post-mortem examination, histopathology and microbiological culture were used to evaluate the lungs of two common rat strains (Sprague Dawley and athymic nude) reared at the ARC. Samples were also collected and stored so that molecular techniques could be performed if indicated. In the event that lung lesions were identified, additional aims were to assess for differences in lesion prevalence and severity between rats reared and maintained in different SPF housing conditions (isolator and barrier) as well as between rats of different age groups. To further investigate any potential effects of housing on lesion prevalence and/or severity, a second, smaller experiment was conducted to evaluate two additional groups of rats, which were sourced from the ARC, but housed at external research institutions under routine experimental conditions, for evidence of gross and microscopic lung disease. In the event that pulmonary lesions were identified in the initial survey, additional testing would be performed in order to try to ascertain the cause of the observed lesion(s).
1.12 Hypotheses

As outlined above, this study aimed to investigate the presence and, if relevant, the prevalence and severity of lung lesions in rats of different strains that were sourced from the Animal Resources Centre in Perth, Western Australia and held in a variety of housing conditions. The following hypotheses were tested in this project:

1. There are no identifiable gross or microscopic lung lesions within the ARC rat colony or within ARC-sourced rats housed within external facilities.

2. Age has no effect on the occurrence of gross and microscopic lung lesions within the ARC rat colony.

3. Rat strain has no effect on the occurrence of gross and microscopic lung lesions within the ARC rat colony.

4. Housing conditions have no effect on the occurrence of gross and microscopic lung lesions within the ARC rat colony.
2. Materials and Methods

2.1 ARC-housed rats

2.1.1 Study design

The main experiment utilised 200 rats (100 Sprague Dawley (SD) and 100 athymic nude (CBH-mu/Arc) rats that were reared and maintained under SPF conditions at the Animal Resources Centre (ARC), located in Perth, Western Australia, for the entire study period. Fifty rats of each strain were maintained in each of the barrier and isolator facilities. At weaning (between 3-4 weeks of age), 10 rats of each strain were removed from each location for euthanasia and necropsy examination. This procedure was then repeated at four subsequent time points, namely 3, 9, 15 and 21 weeks post weaning (approximately 6, 12, 18 and 24 weeks of age, respectively). This experimental design is summarised in Table 1.2.1.

<table>
<thead>
<tr>
<th>Age of Rat</th>
<th>Number of rats</th>
<th>Strains</th>
<th>Housing</th>
</tr>
</thead>
<tbody>
<tr>
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<td>10</td>
<td>SD, N</td>
<td>Is, B</td>
</tr>
<tr>
<td>3 weeks post weaning (~6 weeks old)</td>
<td>10</td>
<td>SD, N</td>
<td>Is, B</td>
</tr>
<tr>
<td>9 weeks post weaning (~12 weeks old)</td>
<td>10</td>
<td>SD, N</td>
<td>Is, B,</td>
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<tr>
<td>15 weeks post weaning (~18 weeks old)</td>
<td>10</td>
<td>SD, N</td>
<td>Is, B</td>
</tr>
<tr>
<td>21 weeks post weaning (~24 weeks old)</td>
<td>10</td>
<td>SD, N</td>
<td>Is, B</td>
</tr>
</tbody>
</table>

Table 1.2.1: Experimental outline. Key: SD = Sprague Dawley; N = Athymic nude; Is = Isolator; B = Barrier.

The study included equal numbers of male and female SD rats (50 each), with
each experimental SD group containing 5 males and 5 females. For the athymic nude rats, small litter sizes led to the necessary inclusion of 75 female rats and 25 male rats. As a result, most athymic nude groups contained more females than males.

2.1.2 Animal care and housing

Male and female Sprague-Dawley (Crj:CD(SD)IGS) and athymic nude (homozygous CBH-rnu/Arc) rats were obtained from the Animal Resources Centre (ARC) (Perth, Western Australia). Rats in this part of the study were maintained exclusively within either the barrier or isolator facility at the ARC for the entire study period. Approval for this study was obtained from the Animal Ethics Committees of Murdoch University and the Animal Resources Centre.

Animals in the isolator facility were housed within large flexible film isolators that receive HEPA filtered air. Within the barrier facility, which contains multiple distinct production rooms separated physically and by air locks containing HEPA filter air scrubbers, animals were housed in open top cages. In addition, as per standard ARC operating procedures, multiple protocols aiming to prevent microbiological contamination were followed throughout the entirety of the study period, including personnel showering, sterilised clothing including hoods and masks and personnel movement controls. All animal care and use procedures complied with the Australian Code for the Care and Use of Animals for Scientific Purposes (Menotti, Emmanuel et al. 2013) and all operations were ISO9001:2008 compliant (Choukri, Aliouat el et al. 2011).

Athymic nude rats are normally housed exclusively within the isolator facility
and Sprague Dawley rats within the barrier facility at the ARC. In order to produce exclusively isolator reared and maintained Sprague Dawley rats, gravid dams were removed from their original location into the isolator facility during late-stage pregnancy (at least 16 embryonic days) and pups were born within a week of the transfer. In contrast, athymic nude rats were transferred to the barrier facility prior to being bred, and as such were maintained in the barrier facility for the entire gestation period. For all rats other than the groups euthanased and examined at weaning (~3 weeks), following weaning, the pups were separated into male and female groups, which comprised 2-4 cages for each experimental group of 10. The rats then remained in these groups and cages in their allocated facility for the remainder of the experimental period.

All rats were kept on Purachip Coarse Aspen Bedding (Able Scientific, Canning Vale, Western Australia) and had *ad libitum* access to autoclaved feed (Rat and Mouse diet; Speciality Feeds, Glen Forrest, Western Australia) and acidified water (pH 2.2 – 2.4). Animals were maintained on a light cycle of 15 hours on and 9 hours off. All cage materials such as racks, cages, bottles and bedding were autoclaved, while non-autoclavable materials were fumigated with formaldehyde (Choukri, Aliouat el et al. 2011).

### 2.1.3 ARC colony health monitoring

Routine ARC colony health monitoring reports were obtained for the barrier facility and the applicable part of the isolator facility in which animals were housed for
the duration of the study period. Serological testing and Helicobacter spp. testing was performed at Cerberus Sciences, Adelaide and the Murine Virus Monitoring Service of the Institute of Medical and Veterinary Science, Adelaide. Bacteriological cultures were performed at the Animal Health Laboratory, Western Australian Department of Agriculture and Food. External parasitological screening was conducted at the Murdoch University Veterinary Clinical Pathology Laboratory. Specific details of the quality control monitoring procedures, including the infectious agents tested for, test information and testing intervals can be found in Appendix A.

Given the unusual circumstance of housing older, immunocompetent rats in an isolator facility within a commercial rodent production facility, rats from the 12, 18 and 24 week-old isolator-housed groups were maintained in a separate part of the isolator facility to the sentinel rats used for routine colony health monitoring. As such, routine health monitoring data from sentinel rats was not available for this area during the time period of the study. Therefore, paired sera samples from four randomly selected rats from each of these age groups, were submitted for serological testing for rat coronavirus, pneumonia virus of mice, *Mycoplasma pulmonis*, Sendai virus, reovirus type 3, hantavirus, cilia-associated respiratory bacillus and rat parvoviruses (Kilham rat virus, rat parvovirus, Toolan’s H1) (Cerberus Sciences, Adelaide).
2.1.4 Sample collection and processing

I. Euthanasia and blood collection

At the time points outlined in the experimental design, each group of rats was euthanased by intraperitoneal pentobarbitone injection (Virbac (Australia) Pty. Ltd, Milperra, New South Wales) at a dosage of approximately 200 mg/kg. This method of euthanasia was chosen in an effort to avoid the artefacts of alveolar haemorrhage and congestion that are known to be associated with euthanasia performed using CO₂ asphyxiation (Fawell, Thomson et al. 1972). Immediately post euthanasia, blood was collected via cardiac puncture. Following clotting at room temperature, the serum was routinely separated within one hour and stored frozen at -20°C.

II. Necropsy examination

A systematic necropsy examination was performed on each rat (detailed procedure outlined in Appendix B). Any gross lesions evident were described and photographed. The distal half to one-third of each the right cranial and right middle lung lobes were collected aseptically for microbiological testing and collection into approximately 10 times the tissue volume of RNAlater® RNA Stabilization Reagent (Qiagen, Chadstone Centre, Victoria, Australia), respectively. RNAlater® samples were stored at 2-3°C for 24 hours and subsequently frozen at -20°C. Following removal from the thoracic cavity, the lungs were filled with 10% neutral buffered formalin via intratracheal infusion and then immersed in formalin for a minimum of 24 hours. Routine samples from other organs were collected and formalin-fixed for archival purposes.

III. Microbiological culture

The distal portion of the right cranial lung lobe, collected aseptically during
necropsy examination, was submitted for aerobic and anaerobic bacteriological culture (Murdoch University Veterinary Hospital microbiology laboratory, Murdoch, Western Australia). Culture plates were incubated for seven days at 35 °C. Sheep blood agar and MacConkey agar plates were used for aerobic culture while additional sheep blood agar plates were utilised for anaerobic culture. Oxoid gas packs and jars were used for the anaerobic plates. Additionally, aseptically collected lung samples from the 6 and 18 week-old athymic nude rat barrier-housed group were also submitted for routine fungal culture using Sabaroud’s agar plates plus Sabaroud’s with gentamicin and colistin incubated at room temperature for 4 weeks.

IV. Tissue selection and Processing

Following fixation, lungs with obvious gross lesions were photographed. Samples of each lung lobe were selected for processing from each rat, including a single sagittal section from each of the left and right caudal lobes, a transverse section from the accessory lobe, and transverse sections of the remaining right middle and cranial lobes, at their widest point. The lung tissue was then processed routinely, being dehydrated in increasing strengths of alcohol then cleared in xylene and impregnated with paraffin wax (Tissue-Tek VIP 2000) before embedding in paraffin (Leica EG1150H embedding station). Histological sections were then cut at 5 µm (Leica RM2135) and stained with haematoxylin and eosin. For a small number of lungs, additional slides from these paraffin blocks were later obtained and stained with a modified Llewlyn’s Sirius red for eosinophils (detailed procedure outlines in Appendix C) or a Perls’ Prussian blue for iron (Dole, Wunderlich et al. 2011).

2.1.5 Histopathological Examination of Lungs

All haematoxylin and eosin stained slides were examined for morphologic
lesions via light microscopy. Slides were examined in random order for the presence of lung lesions with the observer being blinded to rat strain, age, and housing condition. Based on this examination, a comprehensive results table was generated to record any changes seen. Where practical, the actual number of lesions was noted or more commonly, the relative frequency (rare, occasional or common) of individual lesions was subjectively assessed.

2.2 External facility-housed rats

The second and smaller part of this study aimed to simulate the experimental conditions under which rats, sourced from the ARC, are held in external facilities during the course of biomedical experiments. To do this, two groups of 10 SD rats (five male and five female) were transferred from the ARC at six weeks of age to one of two external conventional animal facilities, each part of a separate external research institution. The first facility was the Animal House at Murdoch University, which is located less than one km from the ARC production facility in Perth, Western Australia. The second facility, which requested anonymity for the purposes of this study, was a research institute and ARC customer located in one of the eastern states of Australia. Again, approval for this study was obtained from this institution’s Animal Ethics Committee.

The rats from these external groups were removed from the ARC barrier facility at six weeks of age and transported, following ARC standard operating protocols, to one of the two external facilities. Each group was then housed under routine husbandry conditions for the respective facility for an additional six weeks. At 12 weeks of age, the
rats were euthanased and submitted for necropsy examination, sample collection and histopathological examination as outlined previously in the first part of this study. Exceptions to this protocol were that microbial culture was not performed in rats from the interstate facility group and that lung samples from the Murdoch University animal house group were submitted for routine fungal culture in addition to routine aerobic and anaerobic culture as outlined above. Additionally, while the Murdoch University Animal House group were autopsied by the author under the same conditions as the 200 ARC-housed rats; given logistical limitations, interstate facility group necropsies were performed by researchers at that facility, following the protocol outlined in Appendix B. The formalin-fixed lungs, along with routine samples from other organs, were then couriered to Murdoch University for tissue sampling and processing as previously described.
3. Results

3.1 Animal monitoring

No clinical signs of illness or injury, including respiratory signs such as snuffling or sneezing, were reported during routine monitoring of the rats during the duration of the study.

3.2 Gross pathology

3.2.1 Fresh (unfixed) lung tissue

Gross lesions were identified in the lungs of 14/200 ARC-housed rats at the time of necropsy. Lesions were multifocal to coalescing, roughly circular (1-3 mm diameter), pale tan to grey, flat to slightly raised and were randomly scattered across the surface of one or more lung lobes (Figure 1.3.1 A-D). In a few rats, occasional dark red, non-raised foci were observed (Figure 1.3.1 B). All affected rats were athymic nude and all but one (a 24 week-old rat), was housed in the barrier facility. Gross lesions were first noted in 12 week-old rats (n = 2), reaching maximum prevalence (n = 6) in the 18 and 24 week-old groups.

In the externally housed SD rats, similar gross lesions were seen at necropsy in 3/10 rats from the Murdoch University animal house group. No gross lesions were reported at necropsy of the interstate facility housed rats.
Figure 1.3.1 (A-D). A. Normal gross appearance of lungs at necropsy. B-D. The spectrum of gross lung lesions at seen at necropsy. Note the multifocal to coalescing, randomly scattered, roughly circular foci (arrows). These foci vary from pale tan (B & D), grey (D) to dark red (C) and larger foci are often slightly raised (encircled).
No significant lesions were noted in any other organ system. There was a low prevalence of acute haemorrhage and serosal reddening in the thoracic and/or peritoneal cavities. These lesions were considered to be the result of cardiac puncture to facilitate blood collection and intraperitoneal pentobarbitone injection, respectively. Mild skin lesions, including scaling, small crusts, papules and pustules, were noted in four 3 week old athymic nude rats, and three Sprague Dawley rats (2 x 18 week and 1 x 12 week old) had hydrosalpinx. These lesions were not investigated further.

3.1.2 Fixed lung tissue

The process of formalin infusion of the lungs, followed by immersion fixation for at least 24 hours, frequently revealed macroscopic pulmonary lesions that had not been evident at the time of necropsy. In rats with gross lesions at necropsy, in addition to revealing additional lesions that were not previously apparent, formalin fixation also enhanced previously noted lesions.

Gross lung lesions of variable severity were noted in 65/200 of the ARC-housed rats (Figure 1.3.2 A-F), and included rats of both strains and housing types within multiple age groups (data summarised later in Figure 1.3.4). In mildly affected lungs, these lesions were characterised by low numbers of randomly distributed, ≤1 mm pinpoint circular flat white foci over the surface of one or more lobes (Figure 1.3.2 A & D). With increasing severity, these foci became more numerous (Figure 1.3.2 E & F), often coalescing and/or becoming slightly raised (Figure 1.3.2 C). In some rats, lesions coalesced to form poorly demarcated, patchy white-pale tan areas (Figure 1.3.2 F). In the most severely affected rats, these regions of pallor were more extensive, raised and firm, giving the lungs a slightly nodular appearance (Figure 1.3.2 B).
Figure 1.3.2 (A-F): A: Normal (unaffected) lungs post formalin fixation. B-F: Spectrum of gross lung lesions post formalin-fixation. Note the variably sized multifocal, white to pale tan, circular foci (arrows), which sometimes coalesce (encircled regions). Lesions varied from few (D) to numerous and disseminated (C & F).
The trend of enhanced lesion visualisation post formalin-fixation was also seen in rats housed at the Murdoch University animal house. In this group, 3/10 rats had macroscopic lesions at necropsy (Figure 1.3.3 A) and following fixation (Figure 1.3.3 B). In two additional rats, lung lesions were only evident post-fixation, bringing the overall prevalence of gross lesions in this group to 50% (5/10). Interestingly, although no lung lesions were noted at necropsy in the interstate facility group, examination of formalin-fixed lungs following receipt at Murdoch University revealed gross lesions in 6/10 rats. Additionally, some lung lobes in a proportion rats from this group were suboptimally infused with formalin (Figure 1.3.3 C).

The prevalence of gross lung lesions (in both fixed and unfixed specimens) from the ARC and externally housed groups is summarised in Figure 1.3.4. This graph shows the same or an increased prevalence in gross lesions in 12 week-old Sprague Dawley rats housed either behind the barrier or in the animal house or interstate facility compared with 12 week-old athymic nude rats. Over time, there was a continual increase in the number of athymic nude rats with gross lung lesions regardless of housing type. However, by 24 weeks there was an overall reduction in gross lesions in barrier housed Sprague Dawley rats compared to 12 weeks. In contrast, there was an overall increase in gross lesions in isolator housed Sprague Dawley rats at 24 weeks compared to 18 and 12 weeks.
Figure 1.3 A&B: Macroscopic lung lesions in a Sprague Dawley rat housed at the Murdoch University animal house. Appearance at necropsy (A), note the poorly demarcated, dark red to pale tan regions (circled) and smaller circular foci of dark red discolouration (arrowhead). Lungs from the same rat post formalin-fixation (B). The red-tan foci are now pale tan. C: Macroscopic lung lesions in a Sprague Dawley rat housed at the interstate facility rat. Note the disseminated white-pale tan pinpoint foci throughout all lung lobes and the suboptimal inflation of the right lung lobes.
Figure 1.3.4: Prevalence of gross (unfixed and fixed lung tissue) lesions in all rats, grouped according to age (weeks), strain and housing type. SD: Sprague Dawley; RNU: athymic nude; B: barrier-housed (ARC); Is: isolator-housed (ARC); AH: Murdoch University animal house-housed; IF: interstate facility-housed.
3.3 **Histopathological findings**

As outlined in chapter 2.1.5, H&E stained lung sections from each rat were examined under light microscopy. Several different morphological changes were identified on histological examination of the study population. Inflammation, specifically lymphohistiocytic interstitial pneumonia and dense perivascular inflammatory cell cuffing, was common. Other lesions noted include other patterns of inflammation (other interstitial, alveolar and miscellaneous), bronchus-associated lymphoid tissue (BALT) hyperplasia, non-cellular deposits, and several lesions that were classified within a miscellaneous category.

3.3.1 **Interstitial inflammation**

1. **Lymphohistiocytic interstitial pneumonia**

   Lymphohistiocytic interstitial pneumonia was common in the study population, being noted in 76/200 ARC-housed and 15/20 external facility-housed rats, including rats of both strains and housing types within multiple age groups. This lesion was morphologically indistinguishable between athymic nude and Sprague Dawley rats, and was defined as alveolar septal thickening by an inflammatory infiltrate that was composed predominately of lymphocytes and macrophages (Figure 1.3.5 A-D). Frequently, there was concurrent type II pneumocyte hyperplasia (Figure 1.3.5 B) and over three-quarters of affected rats also had variable numbers of neutrophils, macrophages and cellular debris within adjacent alveoli (Figure 1.3.5 C & D). In the most severely affected regions, the inflammatory infiltrate completely filled alveoli, obscuring or obliterating alveolar septa and leading to variably extensive consolidation of the lung.
Figure 1.3.5 H&E. A-D: Interstitial lymphohistiocytic pneumonia. Note variably severe thickening of the alveolar septa (asterisks) and scattered inflammatory cells in the alveoli (arrows). A & B bar = 50 μm, C bar = 20 μm and D bar = 10 μm.
Effects of age, strain and housing on the prevalence of lymphohistiocytic pneumonia

The number and extent of the lymphohistiocytic pneumonia foci was variable between affected rats, and in many cases there was also marked variability between individual lung lobes within the same individual. Differences in the prevalence and severity of this lesion were also evident between groups of rats of differing age, strain and housing type. These data are summarised in Figure 1.3.6.

In athymic nude rats, the prevalence of lymphohistiocytic pneumonia increased with age, starting at a prevalence of 25% at 6 weeks, increasing slightly at 12 weeks to a prevalence of 30% and then increasing dramatically at 18 and 24 weeks with 100% prevalence in both isolator and barrier-housed rats. In their Sprague Dawley counterparts, however, lymphohistiocytic interstitial pneumonia was not seen until 12 weeks, at which time the overall prevalence of this lesion was slightly higher (45%) than in the athymic nude rats of the same age (30%). In contrast to the trend seen in athymic nude rats, the prevalence of this lesion in Sprague Dawley rats then decreased at 18 weeks (30%) and was highly variable at 24 weeks across the different housing types, decreasing further to 10% in the barrier housed group, but increasing to 90% in the isolator housed animals (Figure 1.3.6).

Differences were also noted when comparing the prevalence of lymphohistiocytic interstitial pneumonia in the 12 week-old ARC-housed Sprague Dawley rats (barrier and isolator) to that in Sprague Dawley rats housed in external conventional facilities (Murdoch University animal house and the interstate facility). The prevalence of this lesion in both externally housed groups (60% Murdoch
University animal house and 90% interstate facility) was higher than in their ARC-housed counterparts (50% barrier and 40% isolator) (Figure 1.3.6).

Lymphohistiocytic interstitial pneumonia severity scoring was performed for each rat, based on the number of foci of lymphohistiocytic interstitial pneumonia within the lung sections examined for each rat (1 focus = score 1, 2 foci = score 2 and 3 or more foci = score 3). These results are presented graphically, also in Figure 1.3.6, which also categorises the severity of the lymphohistiocytic pneumonia according to rat age, strain and housing. Overall, lymphohistiocytic pneumonia severity scores increased with age across all groups, except in the barrier housed SD rats, in which the severity of the lymphohistiocytic pneumonia decreased after 12 weeks of age.
Figure 1.3.6: Prevalence and severity scoring of lymphohistiocytic interstitial pneumonia in the lungs of Sprague Dawley (SD) and athymic nude (RNU) rats categorised by age, strain and housing. LHIP = lymphohistiocytic interstitial pneumonia. Score 1 = 1 focus of interstitial pneumonia within the sections of lung examined; score 2 = 2 distinct foci of interstitial pneumonia within the sections of lung examined; and score 3 = 3 or more distinct foci of interstitial pneumonia within the sections of lung examined. SD: Sprague Dawley; RNU: athymic nude; B: barrier-housed (ARC); Is: isolator-housed (ARC); AH: Murdoch University animal house-housed; IF: interstate facility-housed.
II. Dense perivascular inflammatory cell cuffing

Dense perivascular inflammatory cell cuffing was common, being identified in 41% (81/200) of ARC-housed rats and 95% (19/20) of the 12 week-old external facility-housed rats, again affecting rats of both strains and housing conditions, and in the ARC-housed groups, rats of varying age. Again, this lesion was morphologically indistinguishable between athymic nude and Sprague Dawley rats and the lungs of affected rats contained one or more dense perivascular inflammatory cell cuffs, composed predominately of lymphocytes and macrophages. In some rats, a variable proportion of these cuffs also contained variable numbers of plasma cells and/or granulocytes. It was often difficult to distinguish neutrophils from eosinophils on routine H&E examination (Figures 1.3.8 A & B), however examination of Sirius red stained sections revealed positive red granular cytoplasmic staining in many of these granulocytes, compatible with eosinophils (Figure 1.3.9).
Figure 1.3.7: Four examples of perivascular inflammatory cell cuffing (arrows) accompanied by lymphohistiocytic interstitial pneumonia (asterisks) in the lungs of athymic nude 24 week-old isolator-housed rats. H&E. A: bar = 100 μm. B: bar = 50 μm. C: bar = 50 μm. D: Image B, higher magnification. bar = 20 μm.
Figure 1.3.8A: Perivascular inflammatory infiltrate (encircled) composed almost entirely of granulocytes. BV = blood vessel lumen. H&E, bar = 20 μm.

Figure 1.3.8B: Higher magnification of Figure 1.3.8A. Note the granulocytes (arrows) within a perivascular inflammatory cell cuff. H&E, bar = 10 μm.
Effects of age, strain and housing on the prevalence of perivascular inflammatory cell cuffing

As was seen with lesions of lymphohistiocytic interstitial pneumonia, the number and extent of dense perivascular inflammatory cell cuffing were also highly variable between rats and in many cases, there was also marked variability between individual lung lobes from the same individual. Similarly, differences in lesion prevalence and severity were also evident between groups of rats of differing age, strain and housing type. These data are summarised in Figure 1.3.10.

Overall the prevalence of the dense perivascular cuffs in the different strains at the different timepoints and housing showed a similar pattern to the prevalence of lymphohistiocytic pneumonia in the different strains at the different timepoints and housing conditions. The main differences included that perivascular cuffing was observed earlier (6 weeks in a small proportion of isolator and barrier-housed Sprague
Dawley rats and isolator-housed athymic nude rats). Additionally, in the athymic nude rats and isolator housed Sprague Dawley rats, there was an increase in the prevalence of perivascular cuffing over time for the duration of the study, whereas in barrier-housed Sprague Dawley rats, the prevalence of this lesion peaked at 18 weeks (Figure 1.3.10).

Differences in prevalence were also noted when comparing 12 week-old ARC-housed SD rats (barrier and isolator) to SD rats housed in external facilities (Murdoch University animal house and the interstate facility). The prevalence of this lesion in both externally housed groups (90% Murdoch University animal house and 100% interstate facility) was higher than in their ARC-housed counterparts (60% barrier and 20% isolator) (Figure 1.3.10).

Severity scoring for the lesion of dense perivascular inflammatory cuffing was also performed for each affected rat, based on the number of perivascular inflammatory cell cuffs seen within the lung sections examined for each rat (single perivascular cuff = score 1, two perivascular cuffs = score 2, and three or more perivascular cuffs = score 3). 19/80 rats had only a single dense perivascular cuff (score 1) evident within the lung sections examined. Eight rats had two dense perivascular cuffs (score 2), whilst the remaining 53 rats had three or more foci (score 3). The most severely affected rats, which were included in the score 3 category, had innumerable distinct, dense and complete perivascular cuffs within the examined sections of lung. As with overall lesion prevalence, the overall severity scores for this lesion also increased with age for almost all rat groups, with the exception of the barrier-housed Sprague Dawley rats, in which the severity scores peaked at 12 weeks and then decreased. These data are also summarised in Figure 1.3.10.
Figure 1.3.10: Prevalence and severity scoring of dense perivascular inflammatory cell cuffs in the lungs of Sprague Dawley (SD) and athymic nude (RNU) rats categorised by age, strain and housing. Score 1 = single perivascular cuff; score 2 = two perivascular cuffs; score 3 = three or more perivascular cuffs. PV = perivascular. B: barrier-housed (ARC); Is: isolator-housed (ARC); AH: Murdoch University animal house-housed; IF: interstate facility-housed.
3.3.2 Alveolar inflammation

I. Alveolar histiocytosis

Alveolar histiocytosis, characterised by increased numbers of alveolar macrophages in one or more regions of the lung, was noted in 103/200 ARC-housed rats and 17/20 external facility-housed rats. Affected rats comprised both strains and housing types within most age groups, with the only categories not represented being the 3 week-old groups and the 12 week-old athymic nude isolator-housed rats.

The cellular morphology of the macrophages seen within the alveoli of rats in this study was variable, especially within foci of alveolar histiocytosis (Figure 1.3.11 A-D). The majority of macrophages were morphologically identical to those seen within the alveoli of normal rats, despite being increased in number. In some rats, however, other morphological variants were seen, affecting a variable proportion of alveolar macrophages. These included alveolar macrophages with abundant foamy cytoplasm that ranged from clear with wispy eosinophilic strands to foamy and yellow to light golden brown (Figure 1.3.11 A). In some rats, only the occasional alveolar macrophage had this morphology, while in other rats over 30% of alveolar macrophages were affected. Over two-thirds of rats with foamy alveolar macrophages had concurrent foci of lymphohistiocytic interstitial pneumonia and/or dense perivascular inflammatory cell cuffing.

In over two-thirds of rats with alveolar histiocytosis, some alveolar macrophages contained phagocytised granulocytes or karyorrhectic debris (cytophagia) (Figure 1.3.11 B). Rarely (in only two rats), erythrophagocytosis by alveolar macrophages was also
seen (Figure 1.3.11 C). Lastly, in approximately 15% of rats, comprising both ARC and the externally housed rats, golden brown to dark brown granular pigment was seen within the cytoplasm of alveolar macrophages (Figure 1.3.11 D). The majority of these rats had concurrent alveolar histiocytosis and with the exception of the 3 week-old rat groups, these pigmented macrophages were seen in rats of all ages, strain and housing types. A section of lung from the rat that subjectively contained the most pigment-laden macrophages was stained with Perls’ Prussian blue for the demonstration of iron. Some, but not all, of this pigment was Perls’ positive suggesting that another pigment (such as lipofuscin or ceroid) was also present in addition to haemosiderin, but this was not investigated further.
Figure 1.3.11 Morphologic spectrum of alveolar macrophages. A: Foamy alveolar macrophages (arrows). H&E, bar = 20 μm. B: Interstitial pneumonia (asterisks) with scattered intra-alveolar macrophages, lymphocytes (arrowhead) and granulocytes (short arrow). Note the multinucleated macrophage that has phagocytosed a granulocyte (long arrow). H&E, bar = 10 μm. C: Alveolar macrophages (short arrow) and granulocytes (long arrow). Note evidence of recent erythrophagia (circled). H&E, bar = 10 μm. D: Pigment-laden macrophages (arrows), H&E, bar = 20 μm.
Effects of age, strain and housing on the prevalence of alveolar histiocytosis

As with the other lesions previously described, the number and extent of alveolar histiocytosis was variable both between affected rats and within individual lung lobes from the same individual. Some differences in prevalence and severity trends were also noted between rats of different ages, strains and housing types. These data are summarised in Figure 1.3.12.

Overall, the prevalence of alveolar histiocytosis increased with age. Alveolar histiocytosis was not seen in any of the 3 week-old rats. In athymic nude rats, the prevalence of alveolar histiocytosis was similar in 6 and 12 week old rats (50% and 40%, respectively). Within these age groups, however, the prevalence varied markedly with housing, with 80% of affected 6 week rats and 100% of affected 12 week rats being housed within the barrier. Then, as was the trend with the lesions of lymphohistiocytic pneumonia and the dense perivascular inflammatory cell cuffing, the prevalence increased dramatically, reaching 100% at both 18 and 24 weeks in both the isolator and barrier-housed groups. In contrast, in the Sprague Dawley rats, the prevalence of this lesion was relatively low at 6 weeks (15%), before increasing to 70% at 12 and 18 weeks, and then decreasing very slightly to 65% at 24 weeks. There was no consistent trend with housing within the Sprague Dawley groups, with the prevalence being slightly higher in barrier-housed rats for the 6 week group, equal prevalence between barrier and isolator-housed rats in the 18 week group and higher in isolator-housed rats in the 12 and 24 week groups (Figure 1.3.12).

The overall prevalence of alveolar histiocytosis was slightly higher in 12 week old Sprague Dawley rats housed within external facilities (Murdoch University Animal
House and the interstate facility) than in the 12 week old Sprague Dawley rats housed at the ARC (85% versus 70%, respectively). However, the prevalence was variable between groups, and the prevalence within the ARC isolator-housed group and the Murdoch University Animal House group was equal (80% for each) (Figure 1.3.12).

The number of foci within affected lungs was variable, varying from a single focus to being multifocal and extensive. Again, severity scoring was performed for each rat. For this lesion, severity scoring was based on the number and relative frequency of foci of alveolar histiocytosis. Rats with only a single focus were assessed as score 1. For lungs in which the lesion was multifocal (two or more foci of alveolar histiocytosis), the relative frequency of the lesion was subjectively assessed as being rare (score 2), occasional (score 3) or common (score 4). Within the ARC-housed groups, the higher alveolar histiocytosis severity scores (scores 3 & 4) were more frequently seen in older rats, and more athymic nude rats had an alveolar histiocytosis severity score of 4 than did Sprague Dawley rats. Within the 12 week-old rat groups, rats from the external facilities tended to have higher alveolar severity scores than did the isolator- and barrier-housed rats from the ARC. This data is summarised in Figure 1.3.12.

Concurrent lymphohistiocytic interstitial pneumonia was common in rats with alveolar histiocytosis (72/103 affected ARC rats), as was dense perivascular inflammatory cell cuffing (66/103 affected ARC rats). Over 50% of rats with alveolar histiocytosis also had lesion(s) of interstitial pneumonia and dense perivascular inflammatory cell cuffing. In these rats, the foci of alveolar histiocytosis were most common within alveoli located adjacent to regions of interstitial pneumonia and/or perivascular inflammation.
Figure 1.3.12: Prevalence of alveolar histiocytosis by age, strain and housing. 1 = single focus of AH; score 2 = two or more foci of AH (rare); score 3 = two or more foci of AH (occasional); score 4 = two or more foci of AH (common). SD: Sprague Dawley; RNU: athymic nude; B: barrier-housed (ARC); Is: isolator-housed (ARC); AH: Murdoch University animal house-housed; IF: interstate facility-housed; AH: alveolar histiocytosis.
II. Alveolar neutrophils

63/200 ARC-housed rats and 9/20 external-facility housed rats had variable numbers of neutrophils present within a variable proportion of alveoli (Figure 1.3.13 A-D and Figure 1.3.14). Although definitive distinction of neutrophils from eosinophils was often difficult, the nuclei were typically multilobulated and this, combined with the lack of staining with Sirius Red (Figure 1.3.13 C) in the small sample of lungs stained, was most compatible with neutrophils. In 59/63 ARC-housed rats and in 9/20 external facility rats, the alveolar neutrophils were seen in association with dense perivascular inflammatory cell cuffing and/or a concurrent lymphohistiocytic interstitial pneumonia as previously described (Figure 1.3.13 A-D). Attempts were made to quantify the number of neutrophils present, although subjectively, the number of neutrophils increased along with increasing severity of lymphohistiocytic interstitial pneumonia and dense perivascular inflammatory cell cuffing.
Figure 1.3.13: A: Numerous alveolar granulocytes and macrophages (asterisks) within an area of interstitial pneumonia, H & E, bar = 20 μm. B: Higher power view of granulocytes (long arrows) and macrophages (arrowheads) within alveoli, H & E, bar = 10 μm. C: Granulocytes within alveoli (arrows) not staining for Sirius red (neutrophils), Sirius Red, bar = 10 μm. D: Intra-alveolar foamy macrophages (within circled region) with admixed pyknotic and karyorrhectic nuclear debris (arrowheads) within a region of interstitial pneumonia. Note type II pneumocyte hypertrophy and hyperplasia (asterisk), H & E, bar = 100 μm.
Figure 1.3.14: Mild interstitial pneumonia (asterisks) with alveolar macrophages and granulocytes (long arrows). Some macrophages contain cytoplasmic debris (short arrows). H&E, bar = 10 μm.

Effects of age, strain and housing on the prevalence of alveolar neutrophils

As with other lesions already described, again, the prevalence of alveolar neutrophils was variable, both between affected rats and within different regions of lung within individual rats. This lesion was identified in athymic nude rats in the ARC-barrier at 6 weeks of age, but was not noted in isolator-housed ARC rats or in Sprague Dawley rats until 12 weeks of age. In athymic nude rats, lesion prevalence increased with age, irrespective of housing and was extremely prevalent in the older athymic nude rat groups, reaching 100% prevalence in both 24 week-old groups, as did the lesions of lymphohistiocytic interstitial pneumonia, dense perivascular inflammatory cell cuffing, and alveolar histiocytosis. In contrast, prevalence of alveolar neutrophils in Sprague Dawley rats in the barrier paralleled those of the athymic nude rats, increasing steadily with age, while with Sprague Dawley rats in the barrier, the lesion appeared for the first and last time in the 12 week-old groups, disappearing completely at 18 and 24 weeks. Lesion prevalence within the two ARC-housed groups and the two external facility-housed groups was similar. These findings are summarised in Figure 13.1.15.
Figure 1.3.15: Prevalence of alveolar neutrophils by age, strain and housing. SD: Sprague Dawley; RNU: athymic nude; B: barrier-housed (ARC); Is: isolator-housed (ARC); AH: Murdoch University animal house-housed; IF: interstate facility-housed.
3.3.3 Miscellaneous Interstitial Inflammatory Cell Infiltrates

In some rats, a variety of inflammatory cell infiltrates were noted within the pulmonary interstitium in regions that did not fit into any of the aforementioned categories.

I. Peribronchial and peribronchiolar inflammation

Inflammatory cell infiltrates composed of lymphocytes and occasional macrophages were present within the submucosa of bronchi and/or bronchioles of 31/200 ARC-housed rats and in 2/20 external-facility housed rats. Almost all of the affected rats were athymic nude rats (28/31) and 28/33 affected rats were 18 or 24 weeks-of-age. The diffuse and sometimes sparse nature of this infiltrate was not typical for BALT in either athymic nude (Hanes 2006) or immunocompetent rats (Elmore 2006), and was therefore categorised separately to BALT hyperplasia, although concurrent expansion of the bronchus-associated lymphoid tissue (BALT) was noted to a variable degree in 16/33 affected rats (including 15/16 athymic nude rats) (BALT hyperplasia). Variably sized airways, varying from large to small, were affected (Figures 1.3.16 -1.3.18), however the number of airways affected within each lung as well as the thickness/density of the inflammatory cell infiltrate varied between rats. In many affected athymic nude rats, this predominately lymphocytic infiltrate expanded the submucosa and rarely, low numbers of intra-epithelial lymphocytes were present within the overlying airway epithelium (Figures 1.3.17 and 1.3.18). In one rat, there was a single airway that contained a small amount of intraluminal inflammatory cell exudate composed of predominately neutrophils and macrophages (Figures 1.3.19 A & B). In the five affected Sprague Dawley rats, the submucosal infiltrates were rare and minimal (low density and without expanding the submucosa).
Figure 1.3.16: Bronchial submucosal lymphoid infiltrate (asterisks) in an 18 week old athymic nude rat, forming a variably thick cuff around a large airway. H & E, bar = 100 μm.

Figure 1.3.17: Higher magnification of a bronchial submucosal lymphoid infiltrate (asterisks) in an athymic nude rat forming a peribronchial cuff around a medium sized airway. H & E, bar = 50 μm.
Figure 1.3.18: Peribronchiolar lymphoid infiltrate (asterisks) surrounding a small diameter airway. Low numbers of lymphocytes can be seen transmigrating across the airway epithelium (arrow). H & E, bar = 20 µm.

Figure 1.3.19 A: Peribronchiolar lymphoid cuffing. Note the diffuse submucosal lymphoid infiltrate (asterisks), low numbers of lymphocytes transmigrating across the respiratory epithelium (arrows) and an intraluminal inflammatory cell exudate (encircled). H & E, bar = 50 µm.
Figure 1.3.19 B: Higher magnification image of Figure 5.31 A - intraluminal granulocytes (neutrophils; short arrows) and macrophages (long arrows). H & E, bar = 20 μm.

II. Subpleural nodular inflammatory cell aggregates

Nodular aggregates of inflammatory cells immediately subjacent to the pleura were identified in 115/200 ARC-housed rats and in 11/20 external facility-housed rats. These foci were composed predominately of lymphocytes with lower numbers of macrophages and very rarely, low numbers of eosinophils or plasma cells were also present. The lesions included within this category were nodular inflammatory cell aggregates that were present immediately beneath the pleura (Figure 1.3.20). Subpleural foci of interstitial pneumonia (i.e. obvious thickening of subpleural alveolar septa by inflammatory cell infiltrates) were excluded from this category, rather being considered part of the spectrum of lymphohistiocytic interstitial pneumonia.
The number of lesions within the lungs of each rat varied from one to five. These subpleural aggregates were seen in all age groups, including three week-old rats (including one rat with three separate foci), with maximum incidence at 12 weeks of age. There was no obvious strain (59 athymic nude and 56 Sprague Dawley) or housing (52 isolator and 63 barrier) predisposition for this lesion in ARC-housed rats. Slightly higher numbers of interstate facility rats (seven) compared to Murdoch University Animal House rats (four) were affected.

III. Interstitial nodular inflammatory cell aggregates

Small nodular inflammatory cell aggregates that focally expanded the pulmonary interstitium (but were not contiguous with the pleura or adjacent to a blood vessel) (Figure 1.3.21) were occasionally seen in 58/200 ARC-housed and 13/20
external facility-housed rats. The lesions were present in various areas of the lung and as with the subpleural nodular inflammatory infiltrates described, were predominately composed of lymphocytes and macrophages, with occasional plasma cells or eosinophils identified in some rats. These nodular foci were identified in rats of all strains, housing conditions and ages, including a single 3 week-old rat, reaching peak prevalence at 12 weeks of age.

![Figure 1.3.21: Nodular interstitial inflammatory cell aggregate (circled). H&E, bar = 50 μm.](image)

**IV. Other perivascular inflammatory cell infiltrates**

In addition to the dense perivascular cuffs and nodular interstitial infiltrates already described, several additional patterns of overlapping perivascular inflammatory cell infiltrates were also observed within the study population. Lesions in this category included the following:
i) Dense nodular perivascular inflammatory cell aggregates not obviously associated with an airway;

ii) Dense but incomplete perivascular inflammatory cell cuffing; and

iii) Loose perivascular inflammatory cell infiltrates.

The first lesion category comprised dense nodular perivascular aggregates of lymphocytes and macrophages (with occasional eosinophils and plasma cells) that were not obviously associated with an airway (Figure 1.3.22). Attempts were made to subclassify these lesions based on the size of the affected blood vessel i.e. large vessel versus small/medium sized vessel, although it was recognised that this distinction was subjective and there was very likely overlap between medium and small/large size vessels when recording these lesions.

![Figure 1.3.22: Dense focal nodular perivascular inflammatory cell infiltrate (asterisk). Note also the perivascular oedema (fixation artefact) characterised by increased white space (arrows). H&E, bar = 20 μm.](image)
Of the 200 ARC-housed rats, 152 rats had at least one perivascular inflammatory cell aggregate that was not obviously associated with an airway in the section examined. A total of 98 rats had these nodular inflammatory cell aggregates present around large diameter pulmonary blood vessels, while the lesion was identified adjacent to small or medium sized pulmonary blood vessels in 137 rats. The number of these lesions observed with the lungs of each rat was variable. For large diameter blood vessels, the number of lesions observed for each rat was between one and five, with a median of one. Lesions affecting small to medium-sized blood vessels were typically more common, ranging from one to twenty-two lesions, although the median number of lesions was still only four. Perivascular nodular inflammatory cell aggregates adjacent to both small/medium and large sized pulmonary blood vessels were seen in 83 rats, whilst 54 rats had this lesion adjacent to small/medium sized blood vessels only. In 15 rats, these perivascular nodular aggregates were found only in association with large diameter pulmonary blood vessels.

Rats of all age groups were affected, although the prevalence increased in the 6 week group and then again at 12 weeks, where it remained relatively constant for the remainder of the age groups examined. There was no overall association with housing, with 77 rats being isolator housed and 75 from the barrier. There was an increased prevalence in the athymic nude rats, with 92 of the affected rats being athymic nude rats and the remaining 60 Sprague Dawley. Rats from both external institutions were also affected with similar prevalence at each institution. Twelve of 20 of these rats had the nodular inflammatory cell aggregate near a large blood vessel, while in 19/20 rats small/medium blood vessels were affected.
The second lesion noted was dense, but incomplete, perivascular inflammatory cell cuffing. As in the previously described dense and complete perivascular inflammatory cell cuffs observed in many rats, these lesions were also characterised by dense perivascular inflammatory cell infiltration of lymphocytes and macrophages accompanied in some cases by plasma cells and/or eosinophils. The difference between this lesion and the previously described complete dense perivascular inflammatory cell cuffs is that in these lesions, the perivascular inflammatory cell infiltrate was incomplete, containing large gaps that often comprised >25% of the vessel wall. (Figure 1.3.23)

![Image](image.png)

Figure 1.3.23: Dense incomplete perivascular infiltrate (asterisk). *BV* = blood vessel. H&E, bar = 50 μm.

Classification of some lesions as dense nodular perivascular inflammatory cell infiltrates versus dense, incomplete perivascular inflammatory cell cuffs was subjective and sometimes difficult, with nodular infiltrates sometimes extending a significant
distance along the circumference of a vessel wall. Alternatively some lesions had a region of dense nodular perivascular inflammatory cell infiltration, as well as concurrent extensive but incomplete perivascular inflammatory cell cuffing (Figure 1.3.24).

![Image](image.png)

**Figure 1.3.24:** Dense nodular lymphohistiocytic infiltrate (asterisk) adjacent to large blood vessel (BV) with concurrent incomplete perivascular inflammatory cell cuffing and adjacent alveolar septal thickening (arrow) affecting adjacent alveolar septa. H&E, bar = 50 μm.

The third type of perivascular inflammatory cell infiltrate seen and included in the miscellaneous infiltrate category was loose perivascular cell infiltrates (Figures 1.3.25 and 1.3.26). These loose infiltrates were typically composed predominately of lymphocytes and/or eosinophils, admixed with occasional macrophages and plasma cells. These loose perivascular inflammatory cell infiltrates varied from 1-3 cells thick around a single vessel, often with a variable distance between the cells. As such, this cuffing was often incomplete and therefore overlapped with the incomplete perivascular
cuff category. Additionally, the thickness and density of the perivascular cuffing sometimes varied from loose to dense around the same vessel. This lesion was seen in all age groups except for the 3 week-old rats and the prevalence was relatively high, being 40% in the 6 week-old rats and over 75% in each of the 12 week, 18 week and 24 week old age groups. Both Sprague Dawley and athymic nude rats from both the barrier and isolator facility were affected and there was no obvious association of this lesion with strain or housing. This lesion was also seen in 100% of the 12 week-old Sprague Dawley rats in both external facilities.

![Image](image_url)

Figure 1.3.25: Loose eosinophilic and lymphocytic perivascular infiltrates (arrows). H&E, bar = 100 μm.
Figure 1.3.26: Loose and incomplete lymphohistiocytic perivascular inflammatory cell infiltrate (arrow). BV = blood vessel. H&E, bar = 50 μm.

3.3.4 Bronchus-associated Lymphoid Tissue Hyperplasia

BALT hyperplasia was present in 31/200 ARC-housed rats. Of these rats, 12 were Sprague Dawley and 19 were athymic nude rats. As expected, the morphology of the BALT hyperplasia differed between strains. In the Sprague Dawley rats, BALT hyperplasia was characterised by an increase in the amount of peribronchial/peribronchiolar lymphoid tissue and the presence of germinal centres containing tingible body macrophages (Figure 1.3.27 A & B). In the nude rats, secondary follicle formation was absent, and the morphologic change considered consistent with lymphoid hyperplasia was a noteable overall increase in the amount of peribronchial/peribronchiolar lymphoid tissue compared to other rats in the group. In
both Sprague Dawley and athymic nude rats, occasional lymphatic vessels were
distended and filled with lymphocytes. There was no apparent association between
housing and the presence of BALT hyperplasia, with 16 of the affected rats being
isolator housed and the other 15 from the barrier. Overall, the prevalence of BALT
hyperplasia increased with rat age, with no changes in BALT being observed in the 3
and 6 week old groups. The lesion was first noted in the 12 week-old groups (n = 8).
Eleven 18 week-old rats were affected with the maximum prevalence occurring in the
24 week old groups, with a total of 12/20 rats affected. Concurrent peribronchiolar
inflammation, in the form of submucosal lymphohistiocytic infiltrates (as outlined
above), was seen in 14/31 rats. The BALT hyperplasia was also common within 12
week old rats in the Murdoch University Animal House and in the interstate facility
group, at a prevalence (70%) that was higher than that seen in the combined ARC-
housed groups (45%), however the prevalence of this lesion was highly variable
between groups. In ARC-housed animals, 5/8 affected rats were barrier-housed Sprague
Dawleys, and the other were 3/8-athymic nude rats within the barrier. Similarly, the
prevalence in the interstate facility group (90%) was higher than that in the Murdoch
University Animal House (50%). Within ARC-housed 18 week old rats, this lesion was
relatively evenly scattered across groups of rats of different strains and housing
conditions, whereas in 24 week old rat groups, lesion prevalence was again markedly
uneven between groups, with 10/12 24 week-old rats being athymic nude and 8/12
being housed within the isolator, including both affected Sprague Dawley rats.
Figure 1.3.27A: BALT follicular hyperplasia in a Sprague Dawley rat. Note the germinal centres (asterisks), H&E, bar = 100 μm.

Figure 1.3.27B: Higher magnification of Figure 1.3.30A. BALT hyperplasia in a Sprague Dawley rat. Note the prominent germinal centre (asterisk) containing numerous tingible body macrophages (arrows). H&E, bar = 50 μm.
3.3.5 Non-cellular deposits

I. Eosinophilic crystals

Focal, brightly eosinophilic, variably sized crystals were found sporadically in lung section(s) from 12/200 ARC-housed rats. These crystals were not seen in any of the externally housed rats. Often found within the cytoplasm of alveolar macrophages, these crystals were also found free within alveoli. These crystals were rectangular, 10 – 30μm in length and sometimes had an angular notch at one end (Figure 1.3.28).

![Figure 1.3.28: Eosinophilic crystals (asterisks) within the cytoplasm of alveolar macrophages, within a focus of interstitial pneumonia. H & E, bar = 10 um.](image)

Both nude (n = 6) and SD (n = 6) rats of varying ages in both isolator and barrier housing were affected, including 6 week (n = 3), 12 week (n = 5), 18 week (n = 3) and 24 week (n = 1) old rats. All but one rat had concurrent foci of alveolar histiocytosis,
and in the majority of these rats, a varying proportion of these macrophages had abundant foamy cytoplasm. Multinucleated macrophages, macrophage cytophagia, pigment-laden macrophages and alveolar neutrophils were also present in a proportion of affected rats.

II. Alveolar eosinophilic material

Small focal (occasionally multifocal) accumulations of finely granular to amorphous eosinophilic material were seen in within alveoli in 15 rats (Figure 1.3.29).

All affected rats were nude rats and both rats from the barrier and isolator
housing groups were represented. These rats were typically older, with 12/15 being 24 weeks-old. All had multiple concurrent foci of alveolar histiocytosis, including macrophages with abundant foamy cytoplasm and/or displaying evidence of cytophagia. Many rats (12/15) also had multinucleate macrophages within the alveoli and neutrophils were observed within the alveoli of all but one rat. Scattered haemosiderophages were seen in four rats but erythrophagocytosis and eosinophilic crystals were not seen.

II. **Acicular clefts (cholesterol crystals):**

Low numbers of non-staining acicular clefts (Figure 1.3.30), interpreted as cholesterol crystals, were found within the alveolar eosinophilic material in one-third (5/15) of the rats in which it was found.

**Figure 1.3.30:** Foamy macrophages (short arrows) and granulocytes (arrowhead) in a background of amorphous eosinophilic material containing numerous acicular clefts (long arrows). Also note the interstitial pneumonia with prominent type II pneumocyte hyperplasia (asterisks). *Bar = 20 μm.*
3.2.6 Miscellaneous findings

I. Infectious organisms

In addition to the finely amorphous eosinophilic alveolar deposits described in section 3.2.5, abundant eosinophilic foamy to flocculent material was seen within rare alveoli in H&E stained lung sections from 2/200 ARC-housed athymic nude rats (Figures 1.3.31 & 1.3.32). Rarely, this material was also seen adjacent to (“hugging”) alveolar septa and both affected rats had concurrent lymphohistiocytic interstitial pneumonia. This lesion was not seen in any Sprague Dawley rats, including those housed within the external facilities. Both affected athymic nude rats were 24 week-old athymic nude rats, one each housed within the ARC barrier and isolator. This lesion was compatible with descriptions of *Pneumocystis carinii* infection (see Chapter 1.8.1:III).

![Image](image.jpg)

*Figure 1.3.31: Abundant eosinophilic flocculent material suggestive of *Pneumocystis* sp. cysts (asterisks) seen with the alveoli of a 24 week old athymic nude rat. The surrounding alveolar septa are thickened by an inflammatory cell infiltrate and type II pneumocyte hyperplasia. H & E, bar = 20 μm.*
II. Presumed collection or processing artefacts

Circulatory disturbances

Acute alveolar haemorrhage was seen in 79/200 ARC-housed rats and in 7/20 external facility-housed rats. This haemorrhage was typically mild and patchy, but rarely, in some rats, there were regionally extensive foci of severe acute haemorrhage. Concurrent pulmonary congestion (Figure 1.3.33) was present in 30 rats, approximately two-thirds of which had concurrent acute alveolar haemorrhage.
Perivascular oedema

Almost all rats (193/200 ARC-housed and 19/20 external-facility-housed) had some degree of interstitial (perivascular) oedema, characterised by increased non-staining space expanding the perivascular connective tissues of all sized blood vessels within the lungs (Figure 1.3.34). In 47 of these rats, the perivascular oedema was severe and affected all blood vessels within the lung sections examined. This change was considered an artefact associated with the intratracheal infusion of formalin.
Other artefacts

In low numbers of rats, a variety of other changes were seen on histological evaluation of the lungs that were interpreted as artefacts of euthanasia, sample collection, fixation or processing. These included:

i) Fading and loss of cellular detail of either the subpleural and/or perivascular parenchyma (Figure 1.3.35),

ii) “Wash-in” material such as aggregates of enterocytes adjacent to lung tissue on the slide,

iii) Intravascular emboli composed of hepatocytes in one rat and,

iv) Sloughing of large airway epithelium into the airway lumen.

Figure 1.3.34: Perivascular oedema (arrows). Also note the nodular perivascular lymphohistiocytic aggregate in the absence of a visible airway (asterisk). H&E, bar = 100 μm.
3.4 Ancillary diagnostics

3.4.1 ARC colony health monitoring

Routine colony health monitoring in the barrier rooms and isolators was negative for pneumonia virus of mice, Theiler’s encephalomyelitis virus (GD VII), Hantaan virus (Korean hemorrhagic fever), lymphocytic choriomeningitis virus, Sendai virus, reovirus-3, sialodacryoadenitis (rat coronavirus), rat parvoviruses (rat virus, rat parvovirus-1, Toolan H1 virus), Corynebacterium kutscheri, Bordetella bronchiseptica, Pasteurella multocida, Streptococcus pneumonia, Streptococcus moniliformis, Staphylococcus aureus, Mycoplasma pulmonis, CAR bacillus, Clostridium piliforme, Salmonella enteriditis, Helicobacter sp., nematodes, cestodes, intestinal protozoa
(pathogenic species), *Encephalitozoon cuniculi* and arthropods. The only positive results obtained during this period were *Pasteurella* spp. (*P. pneumotropica* and *P. aerogenes*) and *Staphylococcus aureus* in some rooms within the facility.

### 3.4.2 Serology

Paired sera samples from four arbitrarily selected rats from the 12 week, 18 week and 24 week old Sprague Dawley isolator rat groups (not housed with ARC sentinels used for routine colony health monitoring) were negative for antibodies to rat coronavirus, pneumonia virus of mice, *Mycoplasma pulmonis*, Sendai virus, reovirus type 3, hantavirus, CAR bacillus and rat paroviruses (Kilham rat virus, rat parvovirus, Toolan’s H1) (Cerberus Sciences, Adelaide).

### 3.4.3 Microbiological culture

Lung culture samples from 197/200 rats yielded no growth after seven days of aerobic and anaerobic incubation. Additionally, in the 6 and 18 week-old athymic nude barrier-housed rats, a high prevalence of macroscopic lung lesions at necropsy prompted fungal culture in addition to routine aerobic and anaerobic culture, which yielded no growth of fungal elements after four weeks incubation. A light to moderate, pure growth of a coagulase negative *Staphylococcus* sp. was cultured from the lungs of two 3 week-old Sprague Dawley isolator housed rats and a moderate growth of a possible *Pseudomonas* sp. was cultured from a 12 week-old isolator-housed Sprague Dawley rat.
Aerobic, anaerobic and fungal culture of samples collected from the Murdoch Animal House group yielded no growth after 7 days incubation. As outlined previously, lung culture of the interstate facility group was not performed.

3.5 Summary and conclusions

The results of the gross and histopathological survey of the 200 ARC-housed rats demonstrated the presence of background lung lesions in rats maintained exclusively at the ARC colony. A similar, but smaller analysis of 20 ARC-sourced rats held under routine husbandry conditions at one of two external facilities revealed similar background lesions in these animals. A variety of lesions were noted within the lungs of rats within the study population, however the lesions of lymphohistiocytic interstitial pneumonia and dense perivascular inflammatory cell cuffing predominated. The number and extent of these lesions was highly variable between rats and in many cases, there was also marked variability between individual lung lobes from the same individual. In athymic nude rats, lesion prevalence and severity tended to increase with age, while the prevalence and severity trends in Sprague Dawley rats tended to be more variable, and in some cases, decreased with advancing age. There were no consistent trends with lesion prevalence and severity between rats of different housing conditions, particularly between ARC-housed rats from the isolator and barrier. In 12 week old rats, however, rats from the externally housed groups (Murdoch University Animal House and the interstate facility) often had increased lesion prevalence and/or severity compared to rats of the same age housed within the ARC isolator or barrier.
Part one of this study fulfilled the aim of surveying the ARC population for evidence of lung disease, with a variety of lesions being noted within the study population, including the lymphohistiocytic interstitial pneumonia and dense perivascular inflammatory cell cuffing that predominated. Similar lesions were also identified at a high prevalence in both groups of rats that were raised at the ARC but then maintained under routine experimental husbandry conditions at one of two external research facilities. Review of the prevalence and severity scoring data of the most prevalent lesions (lymphohistiocytic interstitial pneumonia and dense perivascular inflammatory cell cuffing as well as alveolar histiocytosis and alveolar neutrophils) revealed some trends in lesion prevalence and severity between rats of different ages, strains and housing conditions, including a trend for increasing prevalence and severity with age, especially in athymic nude rats. As the sample size for each group was relatively small, however, these results were considered preliminary indicators, which would require additional investigation for confirmation and associated statistical analysis.

Upon review of the historical and recent literature relevant to inflammatory lung lesions in rats, it became clear that the combination and nature of the lesions of lymphohistiocytic interstitial pneumonia and dense perivascular inflammatory cell cuffing that predominated in the study were not consistent with any of the historical and well-recognised lung diseases of rats. Rather, these lesions were consistent with an emerging and at the time, poorly characterised disease of rats known as rat respiratory virus (and later, infectious interstitial pneumonia) (Albers, Simon et al. 2009). This was considered an important finding, as the combination of lesions compatible with this disease had not, to the author’s knowledge, been previously recognised in Australia.
Given the possible confounding effects of this disease on research, particularly on experiments focused on the lower respiratory tract, it is essential that this disease be recognised and taken into consideration in both the planning stages of an experiment and when interpreting experimental data. An example of this is outlined in Elmore et al (2013), which includes a discussion of the difficulties in distinguishing the lesions of infectious interstitial pneumonia from those induced during inhalation toxicology studies (Elmore, Boyle et al. 2014)

As outlined in the initial aims of this study (Chapter 1.10), in the event that lung lesions were identified within the study population, additional investigations would be launched aimed at determining the aetiology/aetiologies of the lesion(s). The methodology and rationale for this second part of the study are outlined in the next part of this document, designated Part two, while the discussion of the results for both Part one and Part two is provided in the final part of this document, namely Part three.
Part Two: Evaluation of Infectious Interstitial Pneumonia within the ARC Colony and in Rats

Sourced from the ARC Colony
1. Introduction

1.1 Guidelines for the histopathological diagnosis of rat respiratory virus

During examination of the lungs outlined in part one of this study, a pattern of distinctive interstitial inflammatory lesions was noted in both Sprague Dawley and athymic nude rats of various ages from a range of different housing conditions. These inflammatory lesions, which comprised lymphohistiocytic interstitial pneumonia and/or dense perivascular inflammatory cell cuffing, were noted to be consistent with those described in the literature for an emerging disease known at this time as “Rat respiratory virus” (RRV), which had been reported in various countries within the northern hemisphere but had not, to the author’s knowledge, been previously described in rats in Australia. In 2009 and 2010 when the bulk of this study was being performed, the aetiology of this disease remained unknown and histopathology was considered the only method of diagnosis (Besselsen, Franklin et al. 2008, Albers, Simon et al. 2009).

Despite the reliance of histopathology for diagnosis, it was not until mid 2009, when the bulk of the histopathology performed in part one of this study was complete, that peer-reviewed guidelines were published for the histopathological diagnosis of RRV (Albers, Simon et al. 2009). The authors of this paper noted the relative importance of this disease in contemporary laboratory rats; pointing out that not only does it cause structural damage to the lung, it also has a prevalence rate much higher than for many of the viruses routinely screened for via serological testing. For these reasons, the authors of this paper considered it essential that the diagnostic criteria for
RRV be refined, particularly in regards to lesion development and progression. To do this, they introduced naïve rats from a caesarean-derived, isolator maintained and historically RRV-negative colony, into a known RRV-positive colony. Histopathological examination was then carried out on multiple rats, weekly over a period of 13 weeks. The characteristic pulmonary lesions of multifocal lymphoid perivascular cuffing and/or foci of lymphohistiocytic pneumonia were evident by week 5 and persisted through to the end of the study period (13 weeks), reaching maximum severity between 8 and 12 weeks following exposure. Based on the results of this evaluation, the authors proposed a set of diagnostic criteria for the histopathological diagnosis of RRV (Figure 2.1.1). An additional equivocal category was also proposed, and was kept purposefully broad to maximise diagnostic sensitivity and minimise the chances of obtaining false negative results. Negative diagnostic criteria included “no lesions” but also included several lesions, noted within proportion of lungs in the study, that were considered non-specific. These non-specific lesions included a minimal increase in perivascular eosinophils, intra-alveolar haemorrhage and low numbers of perivascular lymphoid infiltrates that are loosely arranged and incompletely surround vessels. Alveolar histiocytosis or increased BALT were not a feature within the study population (Albers, Simon et al. 2009).
### Table 3. Suggested criteria for the diagnosis of RRV infection based on pulmonary histopathology.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRV positive</td>
<td>1. Dense cuffs of lymphocytes, often with some plasma cells and macrophages around multiple blood vessels in multiple areas of the lung. AND/OR</td>
</tr>
<tr>
<td></td>
<td>2. Lymphohistiocytic interstitial pneumonia</td>
</tr>
<tr>
<td>RRV equivocal*</td>
<td>1. A single dense perivascular cuff of lymphocytes.</td>
</tr>
<tr>
<td></td>
<td>2. Unexplained (e.g., no foreign body visible, inflammatory lesions that in any way resemble RRV lesions).</td>
</tr>
<tr>
<td>RRV negative</td>
<td>1. No lesions.</td>
</tr>
<tr>
<td></td>
<td>2. Alveolar histiocytosis: intra-alveolar aggregates of foamy macrophages in one or a cluster of alveoli without significant lymphocyte infiltration.</td>
</tr>
<tr>
<td></td>
<td>3. Perivascular eosinophil infiltration. Usually one or two areas. The perivascular infiltration is not dense. Lymphocytes may be present.</td>
</tr>
<tr>
<td></td>
<td>4. Perivascular lymphoid infiltrates that are loosely arranged around the vessels, in low numbers, and incompletely surrounding the vessel.</td>
</tr>
<tr>
<td></td>
<td>5. Increased bronchus-associated lymphoid tissue (BALT) without any inflammation or other lesion.</td>
</tr>
<tr>
<td></td>
<td>6. Intra-alveolar hemorrhage. This may incite a local eosinophilic response.</td>
</tr>
<tr>
<td></td>
<td>7. Eosinophilic granulomatous pneumonia of the Brown Norway rat. We recommend that the Brown Norway rat NOT be used for histologic screening for RRV.</td>
</tr>
<tr>
<td></td>
<td>Nonetheless, the dense lymphoid cuffs of RRV are not seen in the strain-related Brown Norway pneumonia.</td>
</tr>
</tbody>
</table>

*The criteria for an equivocal diagnosis are intentionally broad to maximize sensitivity, despite the necessity of resampling populations to further investigate equivocal results.*
1.2 Search for an aetiologic agent

During the initial evaluation in the first part of this study, attempts at identifying infectious agents were restricted to light microscopic examination of H&E sections and microbiological culture, the methodology and results of which have been previously reported above. Other than flocculent eosinophilic material suggestive of *Pneumocytis* spp. that was noted in two 24 week old athymic nude rats, no infectious agents were identified on light microscopic examination of H&E stained sections. As *Pneumocystis* sp. infection is a relatively common finding on histopathological examination of the lung in immunosuppressed animals and given that this organism was not eliminated from the ARC colony, this was considered an incidental finding of no significance at the time.

This view, however changed partway through this study, when two independent overseas research groups, released abstracts within weeks of each other containing convincing evidence that the fungal agent *Pneumocystis carinii* is the causative agent of the distinctive lung lesions known as rat respiratory virus or infectious interstitial pneumonia. Techniques used to demonstrate the correlation between the presence of *P. carinii* and the presence of the characteristic lung lesions included PCR, serology and experimental infection studies (Livingston, Besch-Williford et al. 2011, Henderson, Dole et al. 2012). Subsequently, Charles River Research Animal Diagnostic Services released a serological test (indirect fluorescent antibody test) for *P. carinii*, which they advocated should be used in conjunction with TaqMan® PCR for diagnosis of infectious interstitial pneumonia infection in lieu of histopathological examination of lung tissue (Charles River 2010).
The results of these studies were surprising for a number of reasons. The possibility of a fungal aetiology had not been considered for many years, particularly as early research had suggested causative agent to be a virus (Riley, Purdy et al. 1997, Riley, Simmons et al. 1999). Renewed interest in identifying a non-viral aetiology, however, came several years later when application of advanced molecular technologies failed to identify the presence of viral sequences in affected tissues (Henderson, Dole et al. 2012). Renewed interest in a potential role for *P. carinii* in the pathogenesis of RRV followed the identification of large numbers of *P. carinii* organisms in the lungs of RRV-exposed athymic nude rats. This finding was considered particularly interesting given that RRV-negative rat colonies at the study institution were known to be concurrently negative for *P. carinii*. Subsequently, retrospective PCR studies demonstrated the presence of *P. carinii* antigen in archived RRV-positive paraffin-embedded lung tissue (Henderson, Dole et al. 2012).

### 1.3 Aims

The publication of criteria for the histopathological diagnosis of rat respiratory virus within the peer-reviewed literature, followed by the revelation this entity was caused by *Pneumocystis carinii*, provided new leads and changed the focus of the search for an aetiologic agent in the current study. Firstly, given the publication of peer-reviewed guidelines for the histopathological diagnosis of rat respiratory virus, which will henceforth be used in this document be referred to as infectious interstitial pneumonia (IIP) due to its non-viral aetiology (Henderson, Dole et al. 2012), plans were made to re-examine the original H&E slides from the 220 rats evaluated in Part one of this study, and to classify each rat as IIP-positive, negative or equivocal based on
histopathological findings as outlined in these guidelines (Albers, Simon et al. 2009).

Secondly, in order to further investigate lesion development and progression in the study population, a grading scheme was developed to assess the relative severity of the IIP in rats classified as positive on histopathological evaluation. The results of infectious interstitial pneumonia classification and severity grading would then be used to investigate IIP-lesion development and progression, including assessing if there were trends in IIP-lesion prevalence and severity between rats of different strain, age, and housing conditions.

Lastly, with the new information regarding a causal role for \textit{P. carinii} in the lesions of IIP, a final aim was to undertake efforts to demonstrate \textit{P. carinii} infection in rats from the current study. As described previously, the presence of eosinophilic flocculent material in rare alveoli in two 24 week old athymic nude rats was compatible with \textit{P. carinii} infection in this population, however as routine histopathology is an insensitive indicator of infection, especially in immunocompetent animals (Weisbroth, Kohn et al. 2006), clearly additional techniques needed to be employed to more accurately assess for the presence of \textit{P. carinii} infection.
2. Materials and Methods

2.1 Infectious interstitial pneumonia classification

The original lung slides from the 220 rats, each containing five standard sections through each lung lobe, were re-evaluated and classified as negative, equivocal or positive for infectious interstitial pneumonia using a modified version of a the previously published classification scheme (Table 2.2.1) (Albers, Simon et al. 2009). A second pathologist, who is board certified by the American College of Veterinary Pathologists, also evaluated and classified any cases classified as equivocal on initial evaluation. A small number of slides were submitted for a diagnostic opinion regarding IIP status to Charles River Research Animal Diagnostic Services (Wilmington, MA, USA) and to the Research Animal Diagnostic Laboratory (RADIL) at the University of Missouri (Columbia, MO, USA).

The reasons behind the decision to make modifications to the original guidelines for the histopathological classification of IIP (Albers, Simon et al. 2009) relate to difficulties that were encountered during initial attempts to use this classification scheme. Firstly, it was noted that a range of miscellaneous interstitial inflammatory lesions, that were outlined in Part 1 (Chapter 3.3.3) were not specifically mentioned in the Albers et al paper, but had potential to confound efforts to diagnose subtle cases of IIP. As these lesions were considered non-specific for IIP following review of the 220 rats slides in consultation with the literature, it was decided to specifically add these lesions to the list of IIP-negative criteria.
Additionally, it was considered that the short description of “lymphohistiocytic interstitial pneumonia” under the IIP-positive classification could lead to confusion and misdiagnosis. The reason for this is that very small foci of lymphohistiocytic pneumonia (<25% of a 40x objective field) were seen in a small proportion of rats, often in association with haemoglobin crystals. Initial concerns that these small foci of interstitial pneumonia were not specific for IIP were later supported by descriptions of a very similar lesion as a background lesion in laboratory rats (McInnes 2012). Therefore, this lesion was considered insufficient evidence for a diagnosis of IIP alone without any other evidence of IIP and it was decided that rats with this lesion alone, and lacking any other evidence of IIP, should be classified as equivocal for IIP. In order to minimize the chances of false-positives in this regard, rats were only classified as IIP-positive if: 1) two or more foci of lymphohistiocytic interstitial pneumonia were identified or 2) a single, large (>25% of a 40x objective field) focus of lymphohistiocytic interstitial pneumonia was identified. Therefore, the revised guidelines utilised for IIP classification in this study are as follows:
Table 2.2.1 Infectious interstitial pneumonia (IIP) classification scheme, adapted from Albers et al 2009.

| Positive | 1. Multiple (≥2) complete dense perivascular lymphohistiocytic cuffs AND/OR multiple (≥2) foci of interstitial pneumonia  
|          | 2. A single large focus of lymphohistiocytic interstitial pneumonia ≥25% of a 40x objective field |
| Equivocal | 1. A single dense lymphohistiocytic perivascular cuff  
|          | 2. A single small focus of interstitial pneumonia <25% of a 40x objective field  
|          | 3. Unexplained (i.e. no foreign body visible lesions that in any way resemble IIP lesions) |
| Negative | 1. No lesions  
|          | 2. Alveolar histiocytosis in the absence of interstitial pneumonia or dense lymphohistiocytic perivascular cuffs  
|          | 3. Loose perivascular lymphohistiocytic or eosinophilic infiltration  
|          | 4. Dense nodular perivascular lymphohistiocytic infiltrates (presumptive BALT)  
|          | 5. Dense nodular interstitial or subpleural lymphohistiocytic infiltrates  
|          | 6. Peribronchial or peribronchiolar lymphohistiocytic cuffing in athymic nude rats  
|          | 7. Intra-alveolar hemorrhage |

2.2 Infectious interstitial pneumonia severity grading

Following histopathological classification for IIP, lung sections from rats classified as positive were then scored using a grading scheme developed to assess the relative severity of the IIP-lesions in each rat using the criteria outlined in Table 2.2.2. For each rat, the score for each category was added to give an overall severity score, which ranged from a minimum of three (least severe) to a maximum (most severe) of nine:
<table>
<thead>
<tr>
<th>Score</th>
<th>Total number of IIP lesions</th>
<th>Proportion of 10x objective fields with IIP lesions</th>
<th>Maximum size of IIP lesions (perivascular cuffs and foci of interstitial pneumonia)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;10 lesions</td>
<td>&lt;25% total 10x objective fields affected</td>
<td>All lesions occupy &lt;25% of a 10x objective field</td>
</tr>
<tr>
<td>2</td>
<td>10 – 100 lesions</td>
<td>25% – 75% of total 10x objective fields affected</td>
<td>At least one lesion occupies 25 – 75% of a 10x objective field</td>
</tr>
<tr>
<td>3</td>
<td>&gt;100 lesions</td>
<td>≥75% of total 10x objective fields affected</td>
<td>At least one lesion occupies &gt;75% a 10x objective field</td>
</tr>
</tbody>
</table>

Table 2.2.2. Histopathologic lesion severity grading for infectious interstitial pneumonia.

2.3 Histochemical staining

For lungs (rats) classified as IIP-positive based on the histopathological classification system described above in section 2.1, additional recuts were stained with a modified periodic acid ammoniacal silver (PAAS) histochemical staining method for the detection of fungal organisms (see Appendix D). Stained slides were then evaluated via light microscopy to identify fungal cysts (asci) consistent with *P. carinii*.

2.4 Pneumocystis carinii PCR

Samples of right middle lung lobe, collected at the time of autopsy and stored frozen at -20 °C in RNAlater®, were submitted for *Pneumocystis carinii* PCR testing to a commercial laboratory (Cerberus Sciences, Adelaide, South Australia). Due to financial constraints, comprehensive PCR testing of all samples was not viable. A decision was made...
to test 5/10, arbitrarily chosen rats from each of the 3, 6 and 12 week-old ARC-housed rat groups (SD barrier, SD isolator, athymic nude barrier and athymic nude isolator). The PCR assay utilised a nested PCR format coupled with SYT09 real time PCR. The outer primer set used in the nested set up utilised two sets of primers using a protocol previously described (Jacoby and Lindsey 1997). Specifically, the primers pAZ102-E (5'-GATGGCTGTTCCAAGCCCA-3') and pAZ102-H (5'-GTGTACGGTTGCAAAGTACT - C-3') were used for amplification of a portion of the gene encoding mitochondrial rRNA of *Pneumocystis carinii*. Where applicable, positive PCR products were selected at random and sequenced to confirm the results, with the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) being used to assess these sequences for homology with the expected mitochondrial sequence of *P. carinii*. 
3. Results

3.1 Infectious interstitial pneumonia classification

As was expected given the high prevalence of lymphohistiocytic interstitial pneumonia and perivascular inflammatory cell cuffing described in Part one, application of the criteria for the histopathological diagnosis of rat respiratory virus (infectious interstitial pneumonia) (Albers, Simon et al. 2009), modified as outlined above, demonstrated a high overall prevalence of this disease in the study population. Overall in the ARC-housed rats, 69/200 rats were classified as positive (34.5%), 28/200 (14%) as equivocal, and 103/200 as negative (51.5%). Additionally, there was a very high prevalence of IIP in the externally housed 12 week-old rat groups (85%), with 8/10 Murdoch University animal house and 9/10 interstate facility rats classified as positive, with the remaining rats being equivocal.

3.1.1 Effect of age, strain and housing on prevalence of IIP

In ARC-housed rats, the overall prevalence of IIP increased with age and was higher in athymic nude rats (44/100) than in Sprague Dawley rats (25/100). Overall, the prevalence in the barrier-housed rats (34/100) being very similar to that seen in isolator housed-rats (35/100), although the overall prevalence within each housing type varied with strain, being higher in the isolator for Sprague Dawley rats (15 isolator/10 barrier) and higher in the barrier for athymic nude rats (24 barrier/20 isolator). Despite these overall trends, however, there were often considerable differences in prevalence between groups of rats of different strain and housing types (Figure 2.3.1).
Figure 2.3.1: Prevalence of infectious interstitial pneumonia (IIP) by rat age (weeks), strain and housing. SD: Sprague Dawley; RNU: athymic nude; B: barrier-housed (ARC); Is: isolator-housed (ARC); AH: Murdoch University animal house-housed; IF: interstate facility-housed.
No lesions compatible with IIP were noted in any of the 3 week-old groups. Microscopic lung lesions consistent with an IIP-equivocal classification were first identified in the 6 week groups of all strains and housing types, although the prevalence was higher in barrier-housed rats and in athymic nude rats (6/20, four barrier/two isolator) compared to Sprague Dawley rats (2/20, one barrier/one isolator). IIP-positive rats were first identified in 12 week-old rats, with compatible lesions being present in rats of both strains and housing types, with the exception of the isolator housed athymic nude group. In contrast to the other age groups, in 12 week-old rats, the IIP prevalence was highest in Sprague Dawley rats, both when the results of the external rat groups are included and when the ARC-housed rats are considered in isolation. However, the prevalence within each strain also varied between rats in different housing conditions. Within the ARC-housed groups, the prevalence of IIP was slightly higher in Sprague Dawley rats (8/20) than in athymic nude rats (5/20) and was highest in barrier-housed groups (6/10 Sprague Dawley and 5/10 athymic nude) compared to isolator-housed groups (2/10 Sprague Dawley and 0/10 athymic nude). In contrast, the 12 week-old isolator-housed groups, however, had a higher prevalence of rats classified as equivocal for IIP (4/10 Sprague Dawley and 5/10 athymic nude) than those in the barriers (0/10 Sprague Dawley and 1/10 athymic nude). Lastly, the prevalence of IIP was higher in the externally housed 12 week-old Sprague Dawley rats (9/10 interstate facility and 8/10 Murdoch University animal house) than in those housed exclusively at the ARC (6/10 barrier and 2/10 isolator).

Overall IIP-prevalence increased at 18 weeks and peaked at 24 weeks, however again there were differences in prevalence within age groups in rats of differing strain and housing conditions. The prevalence of IIP increased with age in the barrier and isolator-
housed athymic nude rats and in the isolator-housed SD rats. In the barrier-housed SD rats, however, the prevalence of IIP peaked at 12 weeks and decreased at 18 weeks, returning to zero prevalence in 24 week-old rats. Within the 18 week-old groups, the prevalence of IIP in athymic nude rats (19/20, 9 barrier and 10 isolator) was twice that seen in Sprague Dawley rats (8/20, 4 barrier and 4 isolator). From these data, it is also evidence that the prevalence of IIP in 18 week-old rats was relatively similar across rats in different housing conditions. In the 24 week-old rats, the prevalence of IIP in athymic rats was 100% (20/20, 10 barrier and 10 isolator) and was over twice that seen in Sprague Dawley rats of the same age (9/20, 0 barrier, 9 isolator), although 3/10 of the 24 week-old barrier-housed Sprague Dawley rats were classified as equivocal. From these data, however, it is clear that there was a marked difference in IIP-prevalence in 24 week-old Sprague Dawley rats depending on housing type, in contrast to the athymic nude rats in which the prevalence was the same regardless of housing type.

The pathology reports from the lung recuts submitted to Charles River Research Animal Diagnostic Services and the Research Animal Diagnostic Laboratory (RADIL) at the University of Missouri concurred with the interpretation (classification) of IIP status in all cases submitted. For the cases submitted to Charles River Research Animal Diagnostic Services, this included four positive, one equivocal and one negative rat. The same slides were submitted to RADIL, however three broke in transit, and so two positive and one equivocal case were evaluated.
3.1.4 Gross lesions

As previously described in Part one, gross lesions were identified during necropsy examination in 14/69 of the rats housed at the ARC. Of these 14 rats, all were athymic nude rats and all but one was subsequently classified as IIP-positive based on histopathological classification. In formalin-fixed lung tissue from ARC-housed rats, gross lesions were evident in 59/69 (86%) of rats that were subsequently classified as IIP-positive. Similarly, 60/65 (92%) rats with grossly visible lung lesions post-fixation were subsequently classified as IIP-positive. Of the remaining 5 rats, 2 were classified as equivocal and 3 (2 x 12 week-old Sprague Dawley rats - 1 barrier/1 isolator and 1 x 24 week-old barrier Sprague Dawley) were classified as negative on histopathological examination. The gross lesions in these rats were, however quite subtle, with only 10-15 <1 mm pinpoint foci on two or more lung lobes (Figure 2.3.2).

Figure 2.3.2: This 24 week-old, barrier housed Sprague Dawley rat was classified IIP-negative on histopathological examination despite subtle macroscopic white foci (arrowheads).
In the externally housed rat groups, 4/10 rats from the Murdoch University animal house had gross lesions at the time of necropsy. All of these rats were subsequently classified as IIP-positive on histopathology. No gross lesions at necropsy were reported in rats from the interstate facility. In both groups of externally housed rats, however, gross lesions were relatively common in formalin-fixed tissues, being noted in 5/8 rats (Murdoch University Animal House) and 6/9 rats (interstate facility) that were subsequently classified as IIP-positive on histopathology.

3.1.5 Histopathology

The majority of IIP-positive lungs in the ARC-housed rats (60/69) and the external facility-housed rats (14/17) contained at least one dense perivascular inflammatory cell cuff as well as one or more concurrent regions of lymphohistiocytic interstitial pneumonia. The remaining rats had either multiple (≥2) foci of dense perivascular inflammatory cell cuffing in the absence of concurrent lymphohistiocytic interstitial pneumonia, or lymphoplasticotic interstitial pneumonia without concurrent perivascular cell cuffing. Of the latter rats, all but one had at least two foci of interstitial pneumonia. The remaining rat, a 12 week-old barrier housed nude rat was classified as IIP-positive the basis of a single large (>25% of a 10x objective field) focus of interstitial pneumonia. Results were similar for the externally housed rats, where all but one IIP-positive rat had concurrent dense perivascular inflammatory cell cuff(s) and at least one focus of lymphohistiocytic interstitial pneumonia. The remaining rat in this group was classified as IIP-positive on the basis of having ≥2 foci of dense perivascular inflammatory cell cuffing in the absence of concurrent lymphohistiocytic interstitial pneumonia.
Of the 27 ARC-housed rats classified as equivocal for IIP, 17 were classified as such due to having a single dense perivascular inflammatory cell cuff. The remaining 11 rats lacked concurrent perivascular inflammatory cell cuffing, however, had a single small (<25% of a 40x objective field) focus of lymphohistiocytic interstitial pneumonia. In the externally housed rat groups, all IIP equivocal rats had a single dense perivascular inflammatory cell cuff in the absence of concurrent lymphohistiocytic interstitial pneumonia.

A number of other lesions were inconsistently seen on microscopic examination of lungs classified as IIP-positive, however, these lesions were not specific for IIP, as they were also being noted in lungs classified IIP-equivocal or negative (Table 2.3.1). The prevalence of several of these lesions, however, was higher in IIP-positive rats than in IIP-negative rats (including alveolar histiocytosis, alveolar neutrophils, BALT hyperplasia and bronchial/bronchiolar submucosal lymphohistiocytic infiltrates). This information is summarised below in Table 2.3.1.
<table>
<thead>
<tr>
<th>IIP Classification</th>
<th>Positive n = 69 (17)</th>
<th>Equivocal n = 28 (3)</th>
<th>Negative n = 103 (0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perivascular inflammatory cell cuffing</td>
<td>64 (16)</td>
<td>17 (3)</td>
<td>- (-)</td>
</tr>
<tr>
<td>Lymphohistiocytic interstitial pneumonia</td>
<td>65 (15)</td>
<td>11 (-)</td>
<td>- (-)</td>
</tr>
<tr>
<td>Alveolar histiocytosis</td>
<td>64 (17)</td>
<td>15 (-)</td>
<td>24 (-)</td>
</tr>
<tr>
<td>Multinucleated macrophages</td>
<td>31 (5)</td>
<td>5 (-)</td>
<td>1 (-)</td>
</tr>
<tr>
<td>Cytophagia (macrophages)</td>
<td>54 (13)</td>
<td>6 (-)</td>
<td>1 (-)</td>
</tr>
<tr>
<td>Pigment-laden macrophages</td>
<td>15 (5)</td>
<td>7 (-)</td>
<td>7 (-)</td>
</tr>
<tr>
<td>Interstitial nodular inflammatory infiltrates near large vessel</td>
<td>49 (9)</td>
<td>17 (3)</td>
<td>32 (-)</td>
</tr>
<tr>
<td>Interstitial nodular inflammatory infiltrates near small/medium vessel</td>
<td>62 (16)</td>
<td>21 (3)</td>
<td>54 (-)</td>
</tr>
<tr>
<td>Interstitial nodular inflammatory infiltrate associated with a vessel</td>
<td>33 (12)</td>
<td>7 (1)</td>
<td>17 (-)</td>
</tr>
<tr>
<td>Subpleural inflammatory infiltrate</td>
<td>47 (10)</td>
<td>23 (1)</td>
<td>45 (-)</td>
</tr>
<tr>
<td>Airway submucosal lymphohistiocytic infiltrate</td>
<td>29 (7)</td>
<td>1 (2)</td>
<td>1 (-)</td>
</tr>
<tr>
<td>BALT hyperplasia</td>
<td>27 (13)</td>
<td>2 (2)</td>
<td>2 (-)</td>
</tr>
<tr>
<td>Loose perivascular inflammatory cell infiltrate</td>
<td>65 (17)</td>
<td>21 (3)</td>
<td>36 (-)</td>
</tr>
<tr>
<td>Alveolar neutrophils</td>
<td>55 (10)</td>
<td>7 (-)</td>
<td>2 (-)</td>
</tr>
<tr>
<td>Eosinophilic crystals</td>
<td>6 (-)</td>
<td>6 (-)</td>
<td>- (-)</td>
</tr>
<tr>
<td>Acicular clefts</td>
<td>5 (-)</td>
<td>- (-)</td>
<td>- (-)</td>
</tr>
</tbody>
</table>

**Table 2.3.1:** Histological lesions in rats categorised as being positive, equivocal or negative for infectious interstitial pneumonia based on the histological lesions. Numbers for ARC-housed rats (barrier + isolator) with combined external facility rats (Murdoch University animal house and interstate facility) in parentheses.
3.2 Infectious interstitial pneumonia severity grading

3.2.1 Effect of age, strain and housing conditions on the severity of IIP

Overall, IIP severity scores increased with age, although as with disease prevalence, there was considerable variability, both within groups and overall between rats of different age, strain and housing. As with overall prevalence, however, the severity scores of the infectious interstitial pneumonia in IIP-positive rats tended to increase with age (Figure 2.3.3). There were, however, some general trends. While the majority of 12 week-old rats (8/13) had the lowest possible IIP severity score (3), only (2/31) of the 24 week-old rats had a severity score of 3. In contrast, at the other end of the severity spectrum, the majority of the 24 week-old IIP-positive rats (22/31) had a severity score >5, while only 1/13 12 week old IIP-positive rats had a severity score >5. This trend is also illustrated by the median severity scores for each age group, which were 3, 5 and 7 in the 12, 18 and 24 week-old rat groups, respectively. Across all ages and housing conditions, the median severity score was slightly higher in athymic nude rats (6) than in SD rats (5), however the proportion of rats with a mid to high range severity score (scores 6-9) was considerably higher in athymic nude rats (25/44) than in SD rats (15/42). Similarly, 17/42 (40%) of IIP-positive SD rats had the lowest possible IIP-severity score (score 3) compared to only 7/44 (16%) athymic nude rats. Lastly, the median severity score was slightly higher in isolator-housed rats (6) than for those in the barrier (5). Overall, fewer isolator-housed rats had a severity score of 3 (20%, 7/35) compared to the barrier-housed rats (32%, 15/34) and similarly, a greater proportion of isolator-housed rats (51%, 18/35) had a mid to high range severity score (scores 6-9) than did the barrier-housed animals (44%, 15/34).
Figure 2.3.3: The prevalence and severity of IIP in rats aged 12, 18 and 24 weeks, categorised according to rat strain and housing. SD: Sprague Dawley; RNU: athymic nude; B: barrier-housed (ARC); Is: isolator-housed (ARC); AH: Murdoch University animal house-housed; IF: interstate facility-housed.
In 12 week-old rats, severity scores were highly variable, both within and between groups of different strain and housing conditions. For ARC-housed rats, severity scores seen ranged from 3 – 7; while in the externally housed groups, all severity scores were represented, including 8 and 9 on the severe end of the spectrum. Overall, 12 week-old rats had the greatest proportion of rats with the lowest possible severity score, with almost 50% (14/30) of IIP-positive rats of this age having a severity score of 3. Overall in the 12 week-old rats, the highest severity scores were seen in the SD rats. No IIP-positive rats were present in the 12 week-old isolator housed athymic nude rats and all five IIP-positive rats in the barrier housed athymic nude group had the lowest possible severity score of 3. The two IIP-positive 12 week-old Sprague Dawley isolator group rats also scored 3 for IIP-severity, however their barrier-housed Sprague Dawley counterparts had a higher median severity score of 5. Higher median severity scores were also evident in rats of the same age (12 weeks) from the externally housed groups in the interstate facility group (5) and the Murdoch University Animal House (4). It is worth noting, however, that although the median severity scores in the ARC barrier and two external facility groups are similar, the severity score distributions for the two externally housed rat groups were notably skewed to the right compared to the barrier-housed group. Not only did the external groups contain at least one rat with severity scores of 8 and/or 9, but 4/9 of rats in the interstate facility group and 3/8 in the Murdoch University animal house had a mid to high range severity score (scores 6 – 9). In contrast, such high severity scores were not seen in any of the ARC-housed groups. In these rats, only the barrier-housed SD group contained any rats with a severity score greater than 3, and in this group, only a single rat (score 7) had a score over 5.
IIP-severity scores in the 18 week old rats ranged from 3 – 8, with approximately 30% (8/27) having the lowest possible severity score (3). In the SD rats, despite equal number of rats (4) being IIP-positive in the barrier and isolator, severity scores were overall slightly higher in rats housed in the isolator (median 4) than in the barrier (median 3). There were also differences between groups of different strains. Not only were over twice as many 18 week-old athymic nude rats IIP-positive compared to their SD counterparts, but athymic nude rats also tended to have slightly higher IIP-severity scores, indicated by higher median severity scores in the barrier (6) and isolator (5) housed athymic nude rats compared to SD rats in the barrier and isolator (3 and 4, respectively).

Overall, while 24 week-old IIP-positive rats tended to have the highest IIP-severity scores compared to other age groups, again there was considerable variation between rats of different strains and housing conditions. Overall, approximately 72% of 24 week-old rats had a severity score of 6 or higher. The majority of IIP-positive rats in this group were athymic nude rats, largely due to the fact that no rats in the 24 week-old SD barrier group were IIP-positive. Although the prevalence of IIP was similar in the 24 week-old SD isolator rats (9) and in the two 24 week-old athymic nude (barrier and isolator) groups (10 each), the severity scores of the athymic nude rats had a right-skewed distribution, ranging from 5-9 with a median severity score of 8 and 6.5 in the barrier and isolator housed groups, respectively. In contrast, while the median in the 24 week-old SD isolator rats was similar (7), the severity score distribution was more normalized for this group, with a range of 3 to 8, suggesting that the overall severity in the isolator-housed athymic nude rats was slightly higher.
3.3 **Histochemical staining**

Microscopic examination of PAAS-stained lung sections from the IIP-positive rats demonstrated the presence of fungal cysts (asci), morphologically consistent with *Pneumocystis carinii*, in 40/86 rats. There was marked strain variation, with all but one of these rats being athymic nude. All 40 were housed at the ARC (20 each in the barrier and isolator) and the only affected Sprague Dawley rat was 12 weeks-old and housed in the barrier. No fungal cysts were seen in any of the silver-stained sections from IIP-positive externally housed rats. Staining was not performed on lung sections from IIP-equivocal or IIP-negative rats.

The number of organisms seen varied markedly between individual rats, ranging from rare (<5 in the Sprague Dawley rat) to common (50+). Subjectively, the number of organisms identified on histochemical staining increased with age and in some of the worst affected IIP-positive 18 and 24 week-old athymic nude rats, multiple organisms were present in almost every 10x field. Where present, fungal cysts were usually, but not always, identified within lesion-affected regions. Most commonly, the fungal organisms were present within alveolar septa (Figures 2.3.4 – 2.3.5), but were also occasionally seen within the cytoplasm of alveolar macrophages (Figure 2.3.6).
Figure 2.3.4: Pneumocystis sp. organisms (arrows) in the alveolar septa in a region of interstitial pneumonia. PAAS, bar = 10 μm.
Figure 2.3.5: Pneumocystis organisms (arrows) within the alveolus as well as within the alveolar septa in a region of interstitial pneumonia. PAAS, bar = 10 μm.

Figure 2.3.6: Pneumocystis sp. organisms in the alveolar septa and within an alveolar macrophage (circled) in a region of interstitial pneumonia. PAAS, bar = 10 μm.
Overall, the IIP-severity scores of lungs in which lesions were identified using this method varied markedly, ranging from 3-9 with a median of 6. This median severity score was higher than that for rats in which fungal cysts could not be demonstrated this way. In ARC-housed rats without demonstrable organisms, the median severity score was 4.5 and when the external rats were also included, the median severity score decreased to 3.

### 3.4 Pneumocystis carinii PCR assay

*Pneumocystis carinii* was detected by PCR in the lungs of 11/60 rats tested. As outlined above in section 2.4, all rats tested were from the ARC housed groups (barrier and isolator) and included equal numbers (5) from each of the 3, 6 and 12 week-old age groups. There was imperfect agreement between the *Pneumocystis* sp. PCR results and the corresponding histopathological IIP classification (Figures 2.3.2 & 2.3.3). PCR-positive rats included individuals from all age groups and both housing types were relatively evenly represented, with five from the barrier and six from the isolator. There was a clear strain effect, with the overwhelming majority of PCR-positive rats being athymic nude (9/11). The two PCR-positive Sprague Dawley rats were both 12 weeks old and barrier-housed. The rate of *P. carinii* detection also increased with age, with over half (7/11) of the PCR-positive rats being 12 weeks of age. These results are summarised in Table 2.3.3.

<table>
<thead>
<tr>
<th>PCR-Positive</th>
<th>PCR-Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIP-Positive or Equivocal</td>
<td>3 positive, 2 equivocal</td>
</tr>
<tr>
<td>IIP-Negative</td>
<td>6</td>
</tr>
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</table>

*Table 2.3.2: Agreement between histopathological infectious interstitial pneumonia (IIP) classification and Pneumocystis carinii PCR results (n = 60 rats).*
<table>
<thead>
<tr>
<th>Age</th>
<th>Strain</th>
<th>PCR + / Histo +</th>
<th>PCR + / Histo E</th>
<th>PCR + / Histo -</th>
<th>PCR - / Histo +</th>
<th>PCR - / Histo E</th>
<th>PCR - / Histo -</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 weeks</td>
<td>SD</td>
<td>B</td>
<td>.</td>
<td>.</td>
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<td>.</td>
<td>5</td>
</tr>
<tr>
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<td></td>
<td>Is</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>RNU</td>
<td>B</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>5</td>
</tr>
<tr>
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<td>2</td>
<td>.</td>
<td>.</td>
<td>3</td>
</tr>
<tr>
<td>6 weeks</td>
<td>SD</td>
<td>B</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>5</td>
</tr>
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<td></td>
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<td>Is</td>
<td>.</td>
<td>.</td>
<td>.</td>
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<td>5</td>
</tr>
<tr>
<td></td>
<td>RNU</td>
<td>B</td>
<td>.</td>
<td>.</td>
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<td>3</td>
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<td></td>
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<td>Is</td>
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<td>2</td>
<td>.</td>
<td>.</td>
<td>3</td>
</tr>
<tr>
<td>12 weeks</td>
<td>SD</td>
<td>B</td>
<td>2</td>
<td>.</td>
<td>3</td>
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<td>Is</td>
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<td>.</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>RNU</td>
<td>B</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>.</td>
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<tr>
<td></td>
<td></td>
<td>Is</td>
<td>.</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.3.3: Number of rats positive and negative on Pneumocystis PCR testing categorised by age, strain and housing conditions. SD: Sprague Dawley; RNU: athymic nude; B: barrier-housed (ARC); Is: isolator-housed (ARC).
Fungal organisms morphologically compatible with *P. carinii* were seen on examination of PAAS-stained sections in one 12 week-old barrier-housed athymic nude rat and the 12 week-old barrier housed SD rat. Two other 12 week-old barrier-housed athymic nude rats were PCR negative for *P. carinii*, despite the detection of similar fungal organisms within silver-stained sections of lung. One of these latter rats also had some very subtle gross lesions (four black <1 mm circular foci on right middle and caudal lobes).
Part Three: Discussion
3.1 Introduction

The results of Part one of this study demonstrated the presence of both gross and histologic lung lesions in the study population, in both Sprague Dawley and athymic nude rats housed in barrier and isolator housing facilities at the ARC. Additionally, similar lung lesions were also seen in Sprague Dawley rats that were housed in the external conventional facilities. A variety of different lung lesions were observed, including interstitial and alveolar inflammation, BALT hyperplasia, non-cellular deposits (e.g. cholesterol clefts and eosinophilic crystals) and circulatory disturbances such as pulmonary congestion and perivascular oedema. While some of these lesions are previously described background lesions of laboratory rats or are attributable to euthanasia or sample processing artefacts, the most common and striking lesions seen in this rat population were foci of lymphohistiocytic interstitial pneumonia and lymphocyte-predominant perivascular inflammatory cell cuffs. These two lesions were seen concurrently in the vast majority of affected rats, and were qualitatively indistinguishable in Sprague Dawley and athymic nude rats. Although the prevalence and severity of these lesions was variable, both within and between groups of rats, this combination of lesions was distinctive, and compatible with the lesions reported in prior reports of a recently-recognised disease initially referred to in the literature as “rat respiratory virus”, then infectious interstitial pneumonia and most recently pneumocystis pneumonia (Elwell, Mahler et al. 1997, Farrar and LaRegina 1997, Gilbert, Black et al. 1997, Riley, Purdy et al. 1997, Slaoui, Dreef et al. 1998, Riley, Simmons et al. 1999, Albers, Simon et al. 2009, Livingston, Besch-Williford et al. 2011, Henderson, Dole et al. 2012).
For many years following the initial reports of these distinctive lesions in rats (Elwell, Mahler et al. 1997, Slaoui, Dreef et al. 1998), the aetiology of these lesions remained elusive. There was, however, strong circumstantial evidence that the disease was infectious, including eradication of lesions in rederived colonies, the presence of both affected and non-affected groups of animals within the same facility despite standardised husbandry procedures, and evidence that the lesions are transient, with a consistent time course, and resolve with time (Clifford 2010, Henderson, Dole et al. 2012). The lymphohistiocytic nature of the inflammatory infiltrate is suggestive of a viral aetiology (Henderson, Dole et al. 2012) and early investigations involving immunofluorescence assays, western blot analysis and electron microscopy of affected lung tissue implicated a putative member of the Hantavirus genus as the possible cause, however subsequent attempts to definitively characterise and identify this purported virus were ultimately unsuccessful (Riley, Purdy et al. 1997, Riley, Simmons et al. 1999, Livingston, Simmons et al. 2001) and the suspected agent was never reproducibly grown in vitro (Albers, Simon et al. 2009). Subsequently, PCR assays utilising Hantavirus-specific primers failed to establish any similarity to other known hantaviruses (Percy and Barthold 2007) and the results of a limited evaluation of an experimental RRV antibody assay developed based on these early investigations did not correspond with the presence or absence of RRV-associated lesions established by histopathology (Henderson, Dole et al. 2012). Similarly, application of advanced molecular technologies, including sequence-independent, single primer and random primer DNA amplification techniques and pan-viral DNA microarray technology (Virochip) also failed to identify the presence of viral sequences in affected tissues (Clifford 2010, Henderson, Dole et al. 2012).
As part of ongoing work to identify the cause of IIP lesions in rats, two independent research groups began to investigate the possibility of a role for *Pneumocystis carinii* infection in the development of IIP (Livingston, Besch-Williford et al. 2011, Henderson, Dole et al. 2012). For the first group, this investigation was prompted by detection of *Pneumocystis* DNA via PCR in several immunocompetent rats with histopathologic lesions of IIP (Livingston, Besch-Williford et al. 2011). Similarly, the other research group had identified *Pneumocystis* organisms in the lungs of an athymic nude rat used in IIP transmission experiments and subsequently detected *Pneumocystis* DNA via PCR in the lungs of immunocompetent CD rats from IIP-affected isolators (Henderson, Dole et al. 2012).

Following this initial detection of *Pneumocystis* in the lungs of IIP-positive rats, both groups conducted several follow up studies. The first study found a significant association between the presence of *Pneumocystis* DNA and IIP (RRV) lung lesions in archived formalin-fixed, paraffin embedded lung tissue. The authors then performed experimental transmission studies and successfully induced lung lesions compatible with IIP using intratracheal inoculation of *P. carinii*. No lesions were seen in sham-inoculated rats or in rats dosed with a 0.22 µm filtrate of the inoculum. Compatible lung lesions were also seen in rats cohoused with immunosuppressed rats inoculated with *P. carinii*, but not those cohoused with sham-inoculated rats. The results of these studies demonstrated a statistically significant association between exposure to *P. carinii* and the development of the lung lesions associated with IIP (Livingston, Besch-Williford et al. 2011).
A similar association was detected by the second research group, which analysed large numbers of samples from colonies and experimental groups of known IIP and *Pneumocystis* infection status and only detected *Pneumocystis* antibodies and DNA (using immunofluorescence assay and PCR) from colonies and experiments that had exhibited histopathological lesions of IIP. Retrospective PCR studies also demonstrated the presence of *P. carinii* antigen in archived RRV-positive paraffin-embedded lung tissue. Follow up experimental studies utilising immunocompetent rats showed that the prevalence and severity of IIP lesions correlated with the concentration of *Pneumocystis* in the lung. Additionally, these studies also showed clearance of *P. carinii* from lung tissue and resolution of the histopathological lesions of IIP were correlated with rising antibody titres against *Pneumocystis* (Henderson, Dole et al. 2012). The results of these studies led both groups to independently conclude that *Pneumocystis carinii* causes the distinctive lung lesions that had previously been attributed to “rat respiratory virus” (Livingston, Besch-Williford et al. 2011, Henderson, Dole et al. 2012).

### 3.2 Infectious interstitial pneumonia (IIP) characterisation

#### 3.2.1 Gross pathology

The nature of the gross lesions seen in the current study is consistent with the lesions documented in previous reports of infectious interstitial pneumonia (Farrar and LaRegina 1997, Riley, Purdy et al. 1997, Riley, Simmons et al. 1999, Albers, Simon et al. 2009). As in previous reports, gross lung lesions in this study were usually multifocal and randomly distributed across one or more lobes and the lesions were qualitatively similar in
both rat strains. Lesions were frequently raised and varied from white-tan, grey or less commonly red. It should be noted that the gross lesions of infectious interstitial pneumonia can resemble and therefore must be differentiated from the white subpleural foci commonly seen in alveolar histiocytosis, a common background lesion seen in the lungs of both young and old rats (McInnes 2012).

In the current study, gross lesions were first identified in 12 week-old rats and the prevalence generally increased steadily with age (in all groups except the barrier housed Sprague Dawley rats which showed an overall decrease in gross lesions between 12 and 24 weeks). The prevalence of gross lesions has been rarely documented in prior reports of the disease. In some cases, gross lesions in affected rats were not commented on at all (Slaoui, Dreef et al. 1998), whilst in other reports, gross lesions are described as being noted in an unspecified proportion of rats (Elwell, Mahler et al. 1997, Farrar and LaRegina 1997, Riley, Simmons et al. 1999). Elwell et al specifically noted that gross lesions were seen in the regions of “more extensive inflammatory lesions” in some rats (Elwell, Mahler et al. 1997). In studies where the incidence of gross lesions has been examined across more than one time-point, gross lesions were first noted in Sprague Dawley rats at 8 weeks of age (Riley, Simmons et al. 1999) and in an experimental transmission study using naïve Wistar Han rats (Albers, Simon et al. 2009) at 6 weeks post exposure to dirty bedding or animals from an historically RRV-positive colony. The latter study also provided prevalence data, with gross lesions being seen in 1/8 rats 6 week post exposure and reaching a prevalence of 100% at 8 and 11 week post exposure. At the end of the 13 week study period, the prevalence of gross lesions remained high (75%). It is difficult to directly compare the results of these prior studies to this study, as rats were not examined in this study between 6
and 12 weeks of age and the authors of prior studies have not noted whether the gross lesions were seen in fresh or fixed lung tissue. Nonetheless, the general trend of increasing gross lesion prevalence over time seen in the other studies was also seen in the barrier and isolator housed athymic nude rats and the isolator-housed Sprague Dawley rats in this study.

The presence of gross lesions in the lung at the time of necropsy was a poor predictor of IIP classification, with only 19% of IIP-positive rats having visible lesions at this time. In contrast, the presence of gross lung lesions in formalin-fixed tissue correlated relatively well with a positive IIP diagnosis, with 83% of rats with gross lung lesions post-fixation subsequently classified as IIP-positive on microscopic examination of lung sections. Of the ARC-housed rats, only three rats with gross lung lesions were subsequently classified as IIP-negative. As previously outlined, the lesions in these three rats were subtle (scattered pinpoint foci) and scattered across all lobes. Whilst the possibility that these foci represented lesions of alveolar histiocytosis, which can look similar grossly (Dungworth, Ernst et al. 1992), cannot be definitively excluded, given the relatively young age of the affected rats and the high prevalence of infectious interstitial pneumonia in the colony, it is highly likely that these lesions represented small foci of interstitial pneumonia that were not present in the standardised lung sections that were selected for histopathology. This finding highlights the relative lack of sensitivity of histopathology for detecting early or mild cases of IIP in rats. Additionally, this finding also highlights the importance of including any gross lesions seen in samples selected for histopathology, in addition to standard samples collected in order to maximize diagnostic sensitivity.
The pronounced enhancement and associated increase in prevalence of IIP-associated gross lung lesions in formalin-fixed lung tissue compared to fresh tissue was an unexpected finding of this study. The relative importance of fixation-status in assessing for gross lesions was also highlighted during examination of the lungs from the 10-12 week-old interstate facility rats examined as part of this study. As outlined previously, this was the only group not necropsied by the author. Following necropsy examination, the prosectors reported that no gross lesions were seen, however upon receipt of the fixed tissues at Murdoch University, prominent lung lesions (consistent with those seen in the other groups and within the literature for RRV/IIP) were seen in 6/10 rats. While the possibility that subtle gross lesions may have been present at the time of necropsy, but were overlooked cannot be completely ruled out, given the formalin-fixation effect noted with the other rats in this study, it is possible that these lesions simply were not evident until following formalin-fixation. This effect of formalin-fixation enhancing lesion detection has not been reported in previous studies of IIP. The results of the current study suggest that there is potential for markedly underestimating the prevalence of gross lesions in this disease unless lungs are examined for lesions post-formalin fixation.

### 3.2.2 Histopathology

The predominant and characteristic histological pattern seen in this study was dominated by interstitial inflammation, characterized by multifocal dense nonsuppurative perivascular inflammatory cell infiltrates accompanied by foci of interstitial pneumonia. The resemblance of these inflammatory lesions to those described previously in reports of
RRV/IIP (Elwell, Mahler et al. 1997, Farrar and LaRegina 1997, Gilbert, Black et al. 1997, Slaoui, Dreef et al. 1998, Steffen 1998, Riley, Simmons et al. 1999, Raffaella, Sara et al. 2003, Albers, Simon et al. 2009) was remarkable and subsequently, all rats with these lesions were classified as either positive or equivocal for IIP based on recently published guidelines (Albers, Simon et al. 2009). This was an important finding, as lesions of IIP had not, to the author’s knowledge, been previously documented in Australia or neighbouring countries.

As in previous reports of IIP, the inflammatory cell infiltrates were composed predominately of lymphocytes, with lesser numbers of other inflammatory cells including macrophages (Gilbert, Black et al. 1997, Steffen 1998, Albers, Simon et al. 2009), neutrophils (Gilbert, Black et al. 1997, Steffen 1998, Riley, Simmons et al. 1999) and plasma cells (Albers, Simon et al. 2009). Less commonly, small numbers of eosinophils were also a component of the inflammatory cell infiltrate, a feature also noted by others (Gore, Gower et al. 2004), although as noted previously eosinophilic perivascular infiltration has also been described as a background lesion in laboratory rats (McInnes 2012), and as such the eosinophilic infiltration seen here may not necessarily be related to IIP. Multifocal type II pneumocyte hyperplasia was also a common feature of the disease in many rats, particularly in those rats with extensive lesions of lymphohistiocytic interstitial pneumonia. This finding has also been previously documented in several reports of RRV/IIP-associated lung lesions (Gilbert, Black et al. 1997, Slaoui, Dreef et al. 1998, Riley, Simmons et al. 1999, Gore, Gower et al. 2004). Interestingly, there did not appear to be any overt differences in the characteristics of the IIP lesions between athymic nude and Sprague Dawley rats, despite the innate differences in the immune responses of these
rats strains. Similar histopathologic findings between rat strains have been documented by, but not specifically commented on, by others studying both immunocompetent and athymic nude rats (Henderson, Dole et al. 2012).

There was marked variation in lesion severity in this study, both across rats and within different lobes of the same rat. On the mild end of the spectrum, only very few and small inflammatory foci were seen, often subpleural and/or at lung lobe margins, occasionally extending to affect alveolar walls and the perivascular connective tissue. At the severe end of the spectrum, necrotic debris and inflammatory cell infiltrate were also present within alveolar lumina, leading to obliteration of alveolar walls and consolidation of the affected pulmonary parenchyma. This spectrum of findings, including the notable variability in lesion severity between rats, is compatible with that described in other reports of rat respiratory virus/infectious interstitial pneumonia within the literature (Farrar and LaRegina 1997, Riley, Simmons et al. 1999).

Notably, the combination of lesions seen with IIP is distinct from those seen in other infectious respiratory diseases of rats (Percy and Barthold 2007, Albers, Simon et al. 2009). Unlike the predominately nonsuppurative inflammation seen in this study and described in previous reports of IIP, infections with Mycoplasma pulmonis and other bacteria tend to produce an inflammatory picture dominated by neutrophils. Similarly, with the exception of a single 24 week-old athymic nude rat in the current study and reports of a mild, sporadic bronchiolitis in an early report of the disease (Farrar & Regina 1997), lesions have not been described in airways. This feature helps differentiate IIP from infections that damage bronchial and/or bronchiolar epithelium, including CAR bacillis, M. pulmonis, rat
coronaviruses and Sendai virus (Albers, Simon et al. 2009). Indeed, the only pathogen known to be capable of inducing similar lesions (specifically interstitial pneumonia and perivascular inflammatory cell cuffing) is pneumonia virus of mice (PVM) (Albers et al 2009). Although the reported prevalence rates of this virus vary dramatically, as was previously outlined in Chapter 1.8.1, relatively recent studies support a low prevalence of this virus in current research establishments around the world. The documented prevalence of this virus on serologic testing of 2,605 rats from a variety of Australasian institutions was 1.1%. In the same study, the prevalence in over 23,000 mice was 0% (McInnes, Rasmussen et al. 2011). In a similar, larger serological survey of over 500,000 mice and 80,000 rats from a diverse variety of institutions in North America and Europe, demonstrated extremely low prevalence rats (0.01% in mice and 0.1% in rats) (Pritchett-Corning, Cosentino et al. 2009). In contrast, this same study reported the prevalence rate of RRV in the rat population to be much higher, at 6.36%. Additionally, while gross and histopathological lesions have been described in natural PVM infection in rats (Brownstein 1996), other authors describe a lack of lesions in naturally infected immunocompetent rats (Baker 2003, Jacoby and Gaertner 2006). This discrepancy seems to suggest that even if lung lesions are occasionally observed in immunocompetent rats, they tend to be relatively mild and transient (Domachowske, Bonville et al. 2002, Baker 2003), which is in contrast to the striking inflammatory lesions frequently seen in IIP affected lung tissue.

The strongest evidence disproving a role for PVM in lesions of IIP has been the inability to demonstrate PVM or any other viral infection in affected rats. Serological studies of affected rat colonies have consistently failed to demonstrate the presence of antibodies against PVM or any other known viral respiratory pathogens of rats (Farrar and
LaRegina 1997, Riley, Simmons et al. 1999, Albers, Simon et al. 2009). In the current study, the results of serological testing of ARC-housed rats (including routine health monitoring data of sentinels and pooled sera samples from those rat groups housed separately), was similarly negative. However, even stronger evidence against a viral aetiology for the distinctive inflammatory lesions came when, given the nonsuppurative nature of the inflammation and a proposed viral aetiology (Riley, Purdy et al. 1997, Riley, Simmons et al. 1999), sophisticated efforts (including immunoassays and PCR techniques using conserved primers) to identify a novel virus failed to reveal any evidence of viral antigens or genomic sequences. Lastly, definitive evidence against a viral aetiology came when a *P. carinii* inoculum, filtered to allow viral particles to remain but exclude most other pathogens (including *P. carinii*) through, failed to reproduce the lung lesions associated with IIP (Livingston, Besch-Williford et al. 2011).

It is also important to note the striking differences between IIP associated with *P. carinii* infection and the lesions of classical pneumocystosis recognised primarily in rats with an inherited immunodeficiency or naturally acquired or experimentally induced immunosuppression. Two main species of *Pneumocystis* are recognised in laboratory rats (*P. carinii* and *P. wakefieldi*), although to date only *P. carinii*, the most common species, has been associated with lesions of IIP. Historically, *Pneumocystis* sp. infection in immunocompetent rats has generally been of little or no concern, being considered an opportunistic pathogen that requires immunodeficiency in order to cause clinical disease, (Konish and Higashiguchi 1996, Baker 1998, Weisbroth 2006). As noted by Weisbroth in 2006, however, pneumocystosis more commonly occurs as a subclinical infection of rat (and mouse) stocks that because of its “symptomalogic silence” and difficulties associated
with diagnosis, has historically seemed to provide little incentive for eradication efforts within commercial stocks (Weisbroth 2006), despite the fact that *Pneumocystis* sp. infection has been shown to alter a variety of physiological and immunological responses (Nicklas, Homberger et al. 1999). As such, the infection has often been overlooked (Waggie, Kagiyma et al. 1994) and led to frequent contamination of commercial rat stocks (Icenhour, Rebholz et al. 2001). Despite the subclinical nature of infection in immunocompetent animals, these animals will still shed the organism (Menotti, Emmanuel et al. 2013) and as such act as a maintenance reservoir for the propagation of infection to immunocompromised hosts (Weisbroth 2006, Morris, Wei et al. 2008). In contrast to immunocompetent individuals, infection of immunodeficient and immunosuppressed animals has historically been associated with chronic progressive pneumonia characterised by high morbidity and mortality (Nicklas, Homberger et al. 1999) and associated with a variety of clinical signs including emaciation, cough, dyspnoea, and cyanosis (Weisbroth 2006). In contrast, while there have been some anecdotal reports of sneezing in rats (strain not indicated) later confirmed to have IIP-associated lesions (Clifford 2010), clinical signs have not been described in immunocompetent or immunodeficient (athymic nude) rats with IIP, including in the current study. Importantly, clinical signs of illness were not noted in athymic nude rats despite demonstration and detection of *Pneumocystis* infection via histochemical silver staining and/or PCR. There are also striking differences between the histopathologic lesions of classical pneumocystosis of immunodeficient / immunosuppressed animals and IIP in both immunodeficient and immunocompetent rats. In clinically affected immunodeficient and immunosuppressed animals, the classic lesion of pneumocystosis is alveolar thickening and alveolar filling with amorphous, eosinophilic foamy to honeycombed material (Baker 1998). While affecting multiple contiguous alveoli, this
lesion is typically associated with minimal concurrent inflammation (Boorman and Eustis 1990) and septal thickening associated with type II pneumocyte proliferation and interstitial fibrosis (Nicklas, Homberger et al. 1999). Special stains (e.g. GMS) reveal multiple discoid cysts (asci) within alveoli (Weisbroth, Kohn et al. 2006). These findings are in contrast to the lesions of IIP, in which alveolar filling with eosinophilic material is generally not described (Albers, Simon et al. 2009, Livingston, Besch-Williford et al. 2011, Henderson, Dole et al. 2012) or uncommon (current study). Additionally, on application of silver staining in the current study, cysts (asci) when seen tended to be present within the alveolar septa, rather than the alveoli. Additionally, unlike the classical lesions of pneumocystosis in immunodeficient or immunosuppressed individuals, the lesions of IIP are characterised by significant inflammatory cell infiltrates, in the form of dense perivascular inflammatory cell cuffing and/or foci of lymphohistiocytic pneumonia (Albers, Simon et al. 2009).

Historically, only mild lung lesions have been documented in immunocompetent hosts, both animal and human, in association with Pneumocystis sp. infection (Henderson, Dole et al. 2012). The reason(s) behind the sudden emergence of significant lesions associated with P. carinii infection in immunocompetent rats have not been elucidated. Given the striking nature of the lesions and the frequent use of rats in biomedical research, it seems unlikely that similar lesions could have been occurring for a prolonged period of time but left unrecognised. Similarly, the reasons behind the emergence of a new pattern of lesions in immunodeficient rats infected with P. carinii not associated with clinical disease and high mortality are similarly unknown. As Pneumocystis sp. have not been able to be continuously propagated in culture, pure cultures have not been available for inoculation for strict fulfilment of Koch’s postulates, raising the possibility of a co-infecting organism
being involved in the pathogenesis of IIP (Henderson, Dole et al. 2012). That said, no other pathogens have been identified despite intensive investigations outlined previously in section 1.8.1 and additional molecular diagnostics performed by Henderson et al (2012).

As with *P. carinii* in rats, *P. jirovecii* infection has long been recognised as a cause of severe clinical disease and death in immunosuppressed humans, but more recently, evidence has emerged suggesting an association between subclinical *P. jirovecii* infections and several diseases affecting immunocompetent humans, including chronic obstructive pulmonary disease (COPD) (Morris, Sciurba et al. 2008, Morris, Wei et al. 2008) and sudden infant death syndrome (SIDS) (Vargas, Ponce et al. 2013). As noted by others, the presence of predictable and striking inflammatory lung lesions in immunocompetent rats experimentally infected with *P. carinii* provides a promising potential animal model of *P. jirovecii* infection in immunocompetent humans (Livingston, Besch-Williford et al. 2011).

### 3.3 Detection of Pneumocystis carinii

Although the extent of the ancillary diagnostic testing in the current study was limited, *Pneumocystis* organisms were documented in the lungs of both affected immunocompetent (Sprague Dawley) and immunodeficient (athymic nude) rats, which supports previous research establishing *P. carinii* as the cause of IIP-associated lung lesions. In the initial evaluation of H&E stained lung tissue in this study, abundant eosinophilic flocculent material compatible with *Pneumocystis* spp. cysts (asci) were
detected in the alveoli of two IIP-positive 24 week-old athymic nude rats. This material was not found in any of the SD rats, including the 20 rats housed at external institutions. As has been the case with other researchers investigating the lesions of IIP in rats (Livingston, Besch-Williford et al. 2011, Henderson, Dole et al. 2012), the presence of this organism was originally dismissed as being purely incidental. This is primarily because while *Pneumocystis* has long been recognised as an opportunistic respiratory pathogen in numerous mammalian hosts, including rats, the development of pneumonia was thought to only occur in immunocompromised hosts (i.e. immunodeficient mutants or as a result of induced immunosuppression) (National Research Council 1991, Weisbroth, Kohn et al. 2006). In contrast, striking pulmonary lesions associated with RRV have been described in wide variety of immunocompetent rat strains and therefore the idea that *Pneumocystis* spp. could be responsible for both gross and microscopically evident pneumonia in fully immunocompetent adult rats seemed improbable. Another initial argument against a role for *Pneumocystis* in IIP was that literature searches conducted both as part of this study and by Henderson *et al* failed to identify any descriptions of lesions consistent with IIP among the many studies of immunosuppressed rats used as an animal model of human pneumocystosis (Henderson, Dole et al. 2012), despite *Pneumocystis carinii* being common and widespread within major commercial rat colonies (Icenhour, Rebholz et al. 2001). Lastly, early studies of IIP affected lungs had failed to consistently detect *Pneumocystis* spp. DNA on PCR examination and fungal cysts had not been detected on microscopic examination of routine and Gomori methenamine silver +/- periodic acid-Schiff stained sections. (Farrar and LaRegina 1997, Riley, Simmons et al. 1999). Following the publication of the previously outlined studies documenting a causal relationship between *P. carinii* infection and the lesions of IIP (Livingston, Besch-Williford et al. 2011, Henderson,
Dole et al. 2012), additional efforts were undertaken to search for evidence of \textit{P. carinii} infection in IIP-affected rats from the current study.

In the current study silver staining, which was performed on all rats diagnosed as IIP-positive based on histopathology (from ARC and externally housed groups), demonstrated low numbers of fungal cysts (asci), morphologically consistent with \textit{P. carinii}, in 40/86 rats. There was, however, marked strain variation, with the organism being detected in the lungs of 40/44 (>90%) of athymic nude rats but in only 1/42 of affected Sprague Dawley rats. Although quantitative PCR was not performed in the current study for confirmation, the discrepancy between the staining results in the two strains is almost certainly due to a markedly increased organism load in the athymic nude rats compared to the SD rats, a finding documented in a previous study that documented 10- to 100-fold higher \textit{P. carinii} organism concentrations (DNA copies/mg lung) in athymic nude rats compared to their immunocompetent counterparts (Wistar Han and CD rats) (Henderson, Dole et al. 2012). The poor diagnostic sensitivity of evaluating lungs for fungal cysts to diagnose \textit{P. carinii} infection in immunocompetent rats has also been noted by others (Livingston, Besch-Williford et al. 2011) and as noted by Livingston \textit{et al}, is also consistent with reports of \textit{P. carinii} infection in immunocompetent mice (Chabe, Dei-Cas et al. 2004). In immunocompetent rats, the rate of detection of fungal organisms via this method may also be associated with the infective dose and route of exposure, as in one study (Livingston, Besch-Williford et al. 2011). infrequent \textit{Pneumocystis} cysts were seen in 40% (4/10) immunocompetent CD rats inoculated with \textit{P. carinii} and in 31% (5/16) of immunocompetent CD rats co-housed with an immunosuppressed (using methylprednisolone acetate), \textit{P. carinii}-inoculated rat of the same strain. In contrast, during
a retrospective analysis of archived lung tissue with lesions of infectious interstitial pneumonia, evaluation of silver-stained lung sections revealed *Pneumocystis* cysts in less than 0.05% of archived lung sections. Although qPCR was not performed for confirmation, the authors of this study concluded that the data suggested a higher organism load within the lungs of experimentally infected immunocompetent rats, than in naturally infected immunocompetent rats, thereby allowing easier detection of the *Pneumocystis* cysts within silver-stained lung sections of experimentally infected immunocompetent rats compared to their naturally infected immunocompetent counterparts.

In contrast with previous studies that have shown a significant association between the presence of IIP lung lesions and *P. carinii* DNA (Livingston, Besch-Williford et al. 2011, Henderson, Dole et al. 2012), the results of the limited PCR assays performed as part of this study showed imperfect agreement, with *P. carinii* DNA being detected in only 3/8 rats diagnosed as IIP-positive and in 6/43 rats diagnosed as IIP-negative on histopathology. Of particular note is that in two of the PCR-negative rats, fungal cysts were identified microscopically in silver-stained lung sections, which was an unexpected finding. Possible reasons for this apparent lack of sensitivity in the PCR assay are varied and are discussed in more detail later in this section. Both of these PCR-negative, fungal-cyst positive rats were in the same group (12 week-old, barrier-housed, athymic nude rats) and had very mild histologic lesions comprising small foci of lymphohistiocytic interstitial pneumonia only with no perivascular cuffs, culminating in the lowest total possible IIP severity score (3). Given this small amount of tissue submitted and minimal nature of the histopathological lesions in these rats, it could be that no organisms were present in the samples tested for Pneumocystis by PCR. Alternatively, especially given that both rats were from the same
group, there could have been an unidentified error in sample handling or storage leading to degradation of *Pneumocystis* sp. DNA and therefore a false negative PCR result. Immunohistochemistry to confirm that the apparent fungal organisms noted on light microscopy were indeed *Pneumocystis* sp. would also have been useful to further investigate this result, however such testing was not readily available to the author at the time and as such was not able to be pursued further.

Although the sample size was relatively small, there was an apparent strain effect, with 9/11 PCR-positive rats being athymic nude. Again, while comprehensive testing (including histochemical staining and PCR testing) of all rats in this study would have been helpful to confirm this apparent trend, as discussed above for histochemical staining, this result may reflect a markedly increased organism load in the athymic nude rats compared to the SD rats. The rate of *P. carinii* detection by PCR also increased with age, with 7/11 PCR-positive rats being 12 weeks of age. As outlined in section 2.4, the rationale for choosing the youngest groups of rats (3, 6 and 12 weeks of age) to perform PCR on was to see if *P. carinii* could be detected using PCR earlier than lung lesions of IIP could be detected on histopathological evaluation. The small sample size however was a major limitation in this study and makes it difficult to draw any definitive conclusions from these results. *Pneumocystis carinii* was detected in two 3 week-old athymic nude rats from the isolator, compatible with exposure early in life and confirming the presence of *P. carinii* within the lung tissue prior to the development of histopathological lesions of IIP, as has been demonstrated by others (Henderson, Dole et al. 2012). Assuming infection at birth, the lag time between infection and lesion manifestation is compatible with the time needed for replication of the organism, which eventually induces an immune response and the
associated inflammatory lesions. In endemically infected immunocompetent rat colonies, maternal antibodies may also provide protection against productive infection for the first weeks of life (Weisbroth, Kohn et al. 2006), but this protective effect would clearly be dependent on the immune status of the dam.

Interpretation of the poor overall agreement between the histopathology and PCR in the current study is limited by the lack of comprehensive PCR testing of all samples and the associated small sample size. It is possible that had all rats been tested, the overall agreement may have been higher, particularly given that the rate of detection by PCR did increase with age, and that the number of DNA copies/mg lung has shown to increase with time post exposure to *Pneumocystis* (Henderson, Dole et al. 2012). That said, the fact that qPCR in prior studies was able to detect *Pneumocystis* in the lungs at one week post exposure (Henderson, Dole et al. 2012) brings into question the sensitivity of the PCR assay in the current study, especially given that the sensitivity of the commercial *Pneumocystis* PCR assay offered by Charles River Laboratories, the company behind the research outlined in the Henderson & Dole et al paper, is reportedly to be within the single copy range (C. Clifford, personal communication, September 9, 2010). Although the positive PCR products in this study were sequenced and confirmed 100% identical to the expected mitochondrial sequence of *P. carinii* with 100% coverage, the sensitivity and specificity of the commercial PCR assay used in the current study was untested (B. Stevenson, personal communication, November 30, 2010).

There are also several factors that could reduce the sensitivity of the PCR assay which are completely outside the control of the diagnostic laboratory. These include
prolonged sample storage or inadequate storage conditions. Attempts were made to estimate the volume of the tissue so as to use an adequate amount of RNAlater® RNA Stabilization Reagent and to process and store them as per manufacturer instructions, however any inadvertent inaccuracy here could have had a negative impact on assay sensitivity. Failure of lung samples to fully submerge within the reagent (e.g. floating on the surface or small samples sticking to the lid or side of the container) could have had a similar impact. In retrospect, storage at -80°C rather than -20°C may have also been better for optimal long-term sample preservation. Another plausible factor that could have contributed to the reduced sensitivity in this study was the sampling methodology used – only the distal half to one-third of the right middle lung lobe was submitted for PCR analysis from each tested rat, following storage in RNA later for approximately one year at -20°C. Collection and pooling of additional tissue for PCR, particularly lesional tissue where noted grossly as was the case in one of the rats, may have increased PCR assay sensitivity.

Despite the convincing nature of reports documenting an strong association between the lesions of IIP and Pneumocystis infection (Livingston, Besch-Williford et al. 2011, Henderson, Dole et al. 2012), the imperfect correlation of P. carinii PCR and the histopathological classification in in this study is particularly interesting given the results of early studies in which P. carinii PCR of IIP affected lungs was either negative (Riley, Simmons et al. 1999) or inconsistently positive (Farrar and LaRegina 1997). The cause of the negative or inconsistent PCR results in these prior studies is similarly uncertain, but this apparent lack of sensitivity led to researchers seeking alternate aetiologies, thereby contributing to the long delay in establishing the cause of IIP following initial
documentation of the distinctive lesions. It is also worth noting that even the studies that demonstrated excellent agreement between the presence of the histopathological lesions of IIP and detection of Pneumocystis via PCR results did not have perfect agreement, as Pneumocystis DNA was not detected in a small proportion of rats that either had histopathological lung lesions and/or had been inoculated with P. carinii (Livingston, Besch-Williford et al. 2011, Henderson, Dole et al. 2012). The precise method and size of lung samples for PCR testing in these studies was not described, but other possible contributing factors could include patchy distribution of Pneumocystis organisms throughout the lung (in the current study only the distal half to one-third of the right middle lung lobe was submitted for PCR testing), although it is hard to see given the random distribution of organisms noted within the lung (Livingston, Besch-Williford et al. 2011), that relatively large portions of lung would not contain low numbers of organisms that should be detectable by highly sensitive PCR methodology.

Differences in the sensitivity and/or specificity of different diagnostic tests for the detection of Pneumocystis infection in rats has been described by others, who have noted that immunocompetent rats are positive for Pneumocystis by combined rtPCR and IFA at a higher rate than when using either of these tests alone. Similarly, these studies have shown that both PCR and IFA alone have much higher diagnostic sensitivity than does pulmonary histopathology for the detection of infectious interstitial pneumonia lesions (Dole, Wunderlich et al. 2011, Henderson, Dole et al. 2012). These results highlight the limitations of relying on the results of a single diagnostic test for this condition and stress the importance of using multiple detection methods when screening for Pneumocystis in immunocompetent rats. These differences in diagnostic sensitivity between tests are
supported by the results of the current study, where there was poor agreement between the histopathological IIP classification and Pneumocystis PCR results. As discussed above, this may be due to poor sensitivity of the Pneumocystis PCR assay, but again, the lack of comprehensive PCR testing of all rats in the study, in particular the older age groups which had a high prevalence of pulmonary lesions of infectious interstitial pneumonia, limits interpretation of the significance of this finding.

### 3.4 Effects of age, strain and housing on prevalence and severity of IIP

Given that the vast majority of rats with lymphohistiocytic interstitial pneumonia had concurrent perivascular inflammatory cell cuffing, and that these lesions form the criteria for the histopathological diagnosis of IIP, the prevalence and severity data for each of these lesions closely parallels the prevalence and severity data of IIP. Statistical analysis was not performed, as statistical advice obtained was that the data are too sparse for meaningful statistical analysis, however subjective variation in the prevalence and severity score of IIP was noted both within and between rat groups of different age, strain and housing, revealing some trends that warrant further investigation.

In the ARC-housed rats that were examined across multiple time points, the prevalence and severity scores of IIP tended to increase with age, particularly in the athymic nude rats. In both rat strains, equivocal and positive cases of IIP were first detected at 6 and 12 weeks, respectively. The prevalence of IIP in athymic nude rats was variable between housing groups at 12 weeks, but increased to near 100% prevalence at 18 weeks.
and to 100% prevalence at 24 weeks. Similarly, the IIP severity scores within each group also tended to increase with age. A similar trend was evident in the isolator-housed Sprague-Dawley rats. While the overall prevalence (including rats in both the barrier and isolator) of IIP-positive rats was the same at 12 and 18 weeks, if equivocal rats are included, then the prevalence was marginally higher at 18 weeks. There was, however, marked variation in the 24 week-old Sprague Dawley groups, with the prevalence dropping dramatically in the barrier-housed group, but increasing in the isolator-housed group to high levels approaching that seen in the 24 week-old athymic nude groups. The reason(s) behind the marked group variation seen at this age is/are unknown, however variability in lesion prevalence within groups of rats has been previously reported (Riley, Simmons et al. 1999, Albers, Simon et al. 2009, Henderson, Dole et al. 2012). This variability in lesion prevalence within groups suggests that not all rats exposed to P. carinii go on to develop detectable histopathological lesions of infectious interstitial pneumonia, a conclusion supported by the results of previous experimental transmission studies utilising contact-exposed rats (exposed via contaminated bedding or cohoused with infected rats) (Albers, Simon et al. 2009, Henderson, Dole et al. 2012). Indeed, a small proportion of exposed rats in transmission studies also failed to demonstrate any evidence of Pneumocystis infection, with both IFA and qPCR being negative for serum antibodies and Pneumocystis DNA, respectively (Henderson, Dole et al. 2012). In another study, Pneumocystis DNA was not detected in 1/10 rats that were inoculated intratracheally with P. carinii. Therefore, these data support that for unknown reasons, some rats will not develop Pneumocystis infection and/or infectious interstitial pneumonia following exposure and therefore that Pneumocystis infection and infectious interstitial pneumonia do not have 100% morbidity within infected populations.
The prevalence and progression of IIP within a population over time has also been variable in previous studies and comparison between studies is complicated by differences in rat strain and experimental conditions, most notably in method of exposure. Additionally, as in the current study, many studies have gaps of up to several weeks between examination time points, during which time changes in lesion prevalence or severity are unknown. Lastly, not all studies that have looked at lesion occurrence over time have documented both lesion prevalence and severity, further complicating direct comparisons between different studies. As in this study, previous studies have also failed to identify IIP lesions in rats <4 weeks old (Farrar and LaRegina 1997, Riley, Simmons et al. 1999, Albers, Simon et al. 2009), although in experimental transmission studies utilising rats obtained at weaning or later from IIP/Pneumocystis-free colonies, histopathological lesions compatible with IIP have been identified in some rats 3 weeks after the initiation of cohousing with either immunosuppressed (Livingston, Besch-Williford et al. 2011) or immunodeficient (athymic nude) (Henderson, Dole et al. 2012) rats.

As reported by Henderson et al, the method of exposure also seems to influence lesion onset and progression. In one transmission study, IIP lesion severity peaked several weeks later and lesions persisted longer in Wistar Han rats that were exposed to Pneumocystis via soiled bedding material (Albers, Simon et al. 2009) compared to CD rats exposed via direct contact with Pneumocystis-infected rats. A duplicate study in which CD rats were exposed to soiled bedding demonstrated a similarly slower rate, suggesting that this difference was not due to a strain difference. This result led the authors of the study to conclude that contact-exposed rats are likely to develop and recover from IIP faster than rats exposed to soiled bedding due to a higher infective dose of P. carinii received with
contact compared to soiled bedding (Henderson, Dole et al. 2012). The authors also noted that this pronounced dose effect was, in turn, likely the result of the slow doubling time of *P. carinii*, which has been reported to be 4.5 days in rats (Burn, Peters et al. 2006, Henderson, Dole et al. 2012).

In this study, peak IIP lesion prevalence in the Sprague Dawley rats housed at the ARC was equal at 12 and 18 weeks (and if equivocal rats are included, peaked at 18 weeks). If the externally housed Sprague Dawley rats are included, then lesion prevalence amongst the Sprague Dawley rats evaluated peaked at 12 weeks. Interestingly, in the 24 week-old Sprague Dawley rats, in the barrier-housed rats, lesion prevalence and severity was markedly decreased, to the point that only equivocal lesions were seen in a small proportion of rats. This finding is compatible with previous studies, which have noted a decrease in the prevalence and/or severity of lesions over time (Slaoui, Dreef et al. 1998, Riley, Simmons et al. 1999, Albers, Simon et al. 2009, Livingston, Besch-Williford et al. 2011, Henderson, Dole et al. 2012). In contrast, the 24 week-old isolator group had peak IIP lesion prevalence and severity of all Sprague Dawley rats housed at the ARC. Although the exact reason(s) behind the prominent differences between these two groups of rats is unknown, in light of the previously described studies demonstrating an association between exposure method/infective dose and the rat of lesion progression/resolution, this finding suggests that the infective dose received by the rats in the isolator group was much higher than that received by rats of the same age and strain in the barrier. As the only likely infective source in the current study was the dams, particularly for rats in the isolators, this in turn suggests that the dam of the isolator group was more infectious, likely excreting more organisms into the air. In Sprague Dawley rats, this likely occurred prior to the dam
mounting a marked serum antibody response, which has been shown to correlate with decreased Pneumocystis DNA copies/mg lung and the resolution of histopathological lesions of IIP (Henderson, Dole et al. 2012). Thereafter, Pneumocystis populations eventually decline until eliminated, at which time the host becomes an immune non-carrier (Weisbroth 2006, Weisbroth, Kohn et al. 2006). The conclusion that the likely infective source for rats in this study was their dam is supported by the fact the ARC-housed rats received only acidified water, autoclaved feed and bedding, and isolator housed rat groups received only HEPA-filtered air. Pneumocystis carinii was not on the list of organisms monitored for and excluded at the ARC, and the widespread occurrence of this organism in commercial rat colonies has been previously documented (Icenhour, Rebholz et al. 2001). P Pneumocystis carinii has been demonstrated to be acquired via airborne transmission for de novo infection (Hughes 1982) and evidence of airborne excretion of P.carinii has been documented in the surrounding air of experimentally infected immunocompetent rats (Menotti, Emmanuel et al. 2013). In a similar experiment utilising immunosuppressed rats, a significant correlation was demonstrated between lung burdens and corresponding airborne levels measured by quantitative PCR (Prager, Bergstrom et al. 2011). Lastly, it has been shown that P. carinii infection is acquired very early in life, with one study detecting P. carinii DNA in oral swabs in the majority of neonatal rats within 2 hours of birth (Icenhour, Rebholz et al. 2002).

Assuming, therefore, that P. carinii infection was acquired by IIP-affected rats in the current study shortly after birth, peak lesion prevalence and severity for each group typically occurred later than in previous studies that have examined IIP lesion progression and/or lesion severity over time. In the current study, unequivocal lesions of IIP were first
noted in 12 week-old rats of both strains, reaching peak prevalence at 12 and 18 weeks in Sprague Dawley rats and at 24 weeks in athymic nude rats. In ARC housed Sprague Dawley rats, maximum severity was noted in the 24 week old isolator housed group. Peak severity in the athymic nude rats was similarly noted at 24 weeks. In contrast, one transmission study using cohabiting and dirty bedding exposure to transmit the disease first noted unequivocal lesions of IIP at 5-6 weeks post exposure, reaching maximum prevalence 8-12 weeks post exposure (Albers, Simon et al. 2009). In a second transmission study utilising cohabited exposure in immunocompetent rats, lesions were first noted at three weeks post exposure, reaching maximum severity before tapering off at seven weeks (Livingston, Besch-Williford et al. 2011). In a third study with an experimental design most similar to the current study (transmission was not purposefully induced, but rather animals were obtained from a facility in which characteristic lung lesions had been reported), pulmonary lesions consistent with IIP were first identified in 6 week-old rats with lesion severity peaking at 10-12 weeks of age (Riley, Simmons et al. 1999). These results highlight the variability in lesion progression across different studies, and as discussed earlier, this variability is likely reflective, at least in part, of differences in the infective dose.

As was seen in the ARC-isolator housed Sprague Dawley rats, in athymic nude rats the lesions of IIP in athymic nude rats also tended to increase in both prevalence and severity with age. Additionally, IIP prevalence and median severity scores also tended to be higher in athymic nude rats than in their Sprague Dawley counterparts. Unlike in the ARC-barrier housed Sprague Dawley rats, there was no evidence of lesion regression during the study period, with lesion prevalence and severity both reaching maximum levels at 24
This finding is consistent with the immunodeficient nature of the strain and suggests that the acquired immune system plays an important role in mediating lesion clearance. In contact transmission studies utilising immunocompetent rats conducted by Henderson et al., the detection of *Pneumocystis* antibodies (via IFA) was followed by the concurrent clearance of *P. carinii* DNA from the lungs and resolution of the distinctive histopathological lung lesions. In athymic nude rats, however, IFA failed to detect *Pneumocystis* antibodies in serum samples from any of the 40 rats tested (Henderson, Dole et al. 2012). Interestingly, and in contrast to the results of the current study, there was a low prevalence of histological lung lesions consistent with IIP in athymic nude rats in the Henderson et al study, with lesions not being seen until 10 weeks postexposure (rats were sampled between 1-10 weeks post exposure), and then in only half (2/4) of the rats examined. In contrast, in the immunocompetent rat strains evaluated, IIP lesions were readily detected in a variable proportion of rats at multiple time points throughout the 10 week study period, with the prevalence peaking at approximately 80% at around 4-5 weeks post exposure and resolving between 9-10 weeks. The contrasting low lesion prevalence in the athymic nude rats is surprising, not only given the high lesion prevalence of IIP lesions in athymic nude rats in the current study, but also given that *Pneumocystis* was detected by qPCR from 2 weeks post exposure in the Henderson et al study, with the organism load continuing to rise throughout the study and reaching $10^6$ DNA copies/mg lung. This concentration of *Pneumocystis* DNA was 10- to 100x higher than the peaks seen in the immunocompetent rat strains. (Henderson, Dole et al. 2012). Based on the findings in the current study, however, it could be that the low prevalence of IIP lesions documented in athymic nude rats in the Henderson et al (2012) was due to a relatively short experimental time period (four rats were samples each week from 1-10 weeks post exposure) and group
sample size. In the current study, IIP lesion prevalence in the 12 week-old athymic nudes was highly variable, ranging from 0% (with 50% equivocal) in the isolator-housed group and 50% (with 10%) equivocal in the barrier-housed group. Over a longer time course in the current study, lesion prevalence in athymic nude rats went on to peak at 100% in both 24 week-old groups, and given the lack of evaluation in older rats, the question remains when (and if) these lesions would have gone on to completely resolve in these immunodeficient rats. The Henderson et al study did not evaluate any athymic nude rats beyond 10 weeks post exposure and given the high prevalence in older athymic nude rats in the current study, one wonders if a similarly high prevalence would have been documented.

Additional studies using larger numbers of athymic nude rats would be needed to confirm a consistently high prevalence in older rats and to investigate the natural progression of IIP lesions in this immunodeficient strain that is less able to respond to the presence of the *Pneumocystis* organism compared to their immunocompetent counterparts. The authors of the Henderson *et al* study concluded that the lack of seroconversion and differences in lesion progression (lack of lesion resolution) noted in athymic nude rats compared to immunocompetent hosts are consistent with the resolution of IIP being mediated by an adaptive immune response to infection. While this is highly likely, further research into the immune response to *Pneumocystis* in IIP is required, in both immunocompetent and immunodeficient rat strains, for confirmation.

The prevalence of IIP in different housing environments was variable. Overall, the prevalence of IIP was very similar in barrier housed and isolator housed rats, although this varied with strain, with disease prevalence being higher in the isolator for Sprague Dawley rats and slightly higher in the barrier for athymic nude rats. A potential role for housing was
also evident when comparing the two 12 week-old ARC Sprague Dawley rats groups to the 12 week old Sprague Dawley rat groups housed within the interstate facility and the Murdoch University animal house. The prevalence of IIP was higher in both external facility groups compared to those at the ARC, and additionally severity scores tended to be slightly higher in the externally housed rats, with a severity score distribution skewed to the right.

There has been limited research on the role of environment in the manifestation of IIP, however we know that while not playing a causal role, that environmental factors can play a role in influencing the severity of other respiratory diseases in rats, including Mycoplasmosis (Lindsey, Baker et al. 1971). In an early study investigating the occurrence of RRV within a rat colony, analysis of lesion occurrence revealed no apparent correlation with housing type, with lesions being sporadically observed in animals from a variety of housing conditions, including open-top cages and micro-isolators in both negative and positive pressure rooms. Similarly, no association with bedding material (corncob, hardwood chip and pressed paper) was identified (Farrar and LaRegina 1997). In contrast, in another study investigating the long-term effects of cage-cleaning frequency and bedding type on a number of factors including rat health, a bedding effect was noticed, with immunocompetent Sprague Dawley and Wistar rats on aspen bedding having greater sneezing rates and lung pathology than those kept on paper bedding (48% having moderate-severe lesions versus 26%, respectively) (Burn, Peters et al. 2006). Interestingly the histopathological picture in this study was one of interstitial pneumonia dominated by macrophages with fewer neutrophils and eosinophils, typically mild and diffuse, however foci of increased lesion severity, including areas of consolidation were also described.
Bronchial lesions were notably absent, although prominent BALT was also noted. Given this spectrum of lesions, affected animals in this study may well have been infected with *P. carinii*, manifesting as infectious interstitial pneumonia. Interpretation is complicated by the presence of at least one known rat respiratory pathogen within the experimental colony (*Mycoplasma pulmonis* was isolated during a post-mortem screen of one of fourteen rats that died during the course of the study, along with concurrent infections with Kilham rat virus and a rat parvovirus) (Burn, Peters et al. 2006). Although the interstitial pneumonia and lack of bronchial involvement does not fit with the spectrum of lesions associated with *M. pulmonis*, without further information as to what pathogen screening was performed, contribution from other unidentified respiratory pathogens, including viruses, cannot be excluded.

### 3.5 Other histopathological findings

A variety of other histopathological lesions not characteristic for rat respiratory virus/infectious interstitial pneumonia were noted on examination of rats from both the ARC-housed and externally housed groups in this study. Many of these lesions have been previously described as background lesions in laboratory rats, including alveolar haemorrhage, pulmonary congestion, alveolar histiocytosis (including pigment laden macrophages), eosinophilic crystals, focal subpleural inflammation and perivascular eosinophil infiltrates (McInnes 2012).
3.5.1 Pulmonary congestion and haemorrhage

Pulmonary congestion and small foci of alveolar haemorrhage, as seen in many of the rats in this study, are not uncommon in rat lungs and because they are unassociated with any other subacute or chronic disorders of haemostasis (eg haemosiderosis, thrombosis etc), they are interpreted as agonal changes. These changes are considered particularly common in rats that die spontaneously or are euthanased with carbon dioxide (Renne, Brix et al. 2009, McInnes 2012), however as evidenced by this study, can also be seen in rats euthanased using pentobarbitone administered via intraperitoneal injection.

3.5.2 Alveolar histiocytosis

Multifocal aggregates of large mononuclear cells, morphologically compatible with macrophages, were a common finding in rats in this study, being noted in over half of the rats in this study. While immunohistochemical staining of these cells using F480 antibody may have been useful for confirming that these cells were macrophages, this was precluded due to cost constraints. Alveolar macrophage aggregates, also referred to as alveolar histiocytosis and alveolar macrophage aggregation, are well documented in rats (Renne, Brix et al. 2009, McInnes 2012). In the current study, alveolar macrophages comprising these aggregates frequently contained abundant foamy cytoplasm and in some rats, a proportion also contained brown granular pigment (predominately haemosiderin). Additionally in some rats, fewer alveolar macrophages contained cytoplasmic karyorrhectic debris or less commonly, intact granulocytes or erythrocytes.
Although noted within rats of all ages in this study, the prevalence of alveolar histiocytosis was highest at 18 and 24 weeks. As alveolar histiocytosis is described as a common incidental finding in the lungs of older rats, particularly subpleurally or in more peripheral areas of lung (Boorman and Eustis 1990, Renne, Brix et al. 2009), the increased prevalence with age may simply reflect advancing age. That said, at 6 months of age, even the oldest rats in this study are not considered “aged” and alveolar histiocytosis has also been described as a background lesion within the alveoli and terminal airways of young rats (McInnes 2012). Additionally, however, alveolar histiocytosis has also has been described previously as a feature in RRV/IIP-affected lungs (Gilbert, Black et al. 1997, Riley, Simmons et al. 1999, Gore, Gower et al. 2004, Albers, Simon et al. 2009).

The prevalence of alveolar histiocytosis in this study was certainly much higher in IIP-positive rats (n = 64) than in IIP-negative rats (n = 24), with the final 15 rats with foci of alveolar histiocytosis being equivocal for IIP. Additionally, rats with IIP tended to have more foci of alveolar histiocytosis and therefore high alveolar histiocytosis scores. As outlined previously, the majority of IIP-positive rats with alveolar histiocytosis had alveolar histiocytosis scores of 3 or 4, whilst in contrast; the majority of IIP-negative rats had few foci of alveolar histiocytosis, as evidenced by most having an alveolar histiocytosis score of 1. Therefore, whilst some of the alveolar macrophage aggregates seen in rats from this study probably represent background lesions, especially those foci noted in peripheral regions of the lung and those in the IIP-negative rats, the increased prevalence and alveolar histiocytosis scores seen in IIP-positive rats suggests that there is some association between alveolar histiocytosis and IIP. This, and the fact that the foci of alveolar histiocytosis were frequently present within areas of interstitial inflammation in IIP-positive rats, suggests that
this lesion probably represents a non-specific response to lung injury in these rats. The presence of karyorrhectic debris and phagocytised cells (cytophagia) within some macrophages in these foci further supports a role for these cells in the inflammatory response, even if it is simply cleaning up in response to prior injury to the surrounding lung. In one description of alveolar histiocytosis as a background lesion, it is noted that inflammatory cells, cholesterol clefts and pneumocyte hyperplasia are often seen in conjunction with the macrophage clusters and the author goes on to suggest that foci of alveolar histiocytosis may represent the remnants of resolved inflammatory foci (McInnes 2012). Indeed, it is thought that most forms of deep pulmonary injury cause at least some degree of alveolar histiocytosis in rats (Dungworth, Ernst et al. 1992).

3.5.3 Pigment-laden alveolar macrophages

Pigment-laden macrophages within foci of alveolar histiocytosis have been previously described in the literature. The pigment, usually variable shades of brown, may represent haemosiderin (Renne, Brix et al. 2009), as noted in the one case stained with Perl’s Prussian blue in this study, and/or other non-iron pigments such as lipofuscin or carbon (Dungworth, Ernst et al. 1992, Renne, Brix et al. 2009, McInnes 2012). Like the lesion of alveolar histiocytosis itself, the presence of pigment-laden macrophages within these foci has been associated with ageing (Dungworth, Ernst et al. 1992, Renne, Brix et al. 2009), however, in the current study, brown granular pigment morphologically consistent with haemosiderin was noted within alveolar macrophages in rats as young as 6 weeks. Although the pathogenesis of pigmented alveolar macrophages is uncertain, the haemosiderin component is presumed to be minor haemorrhage, whilst it has been
proposed that the probable lipofuscin component may be derived from lipid peroxidation of phagocytised surfactant lipid (Dungworth, Ernst et al. 1992).

3.5.4 Alveolar eosinophilic crystals

Another background lesion in this study was the presence of randomly scattered eosinophilic crystals in a small proportion of rats. Eosinophilic crystals, found free within the alveoli, have been previously described as a background lesion in the lungs of rats (Renne, Dungworth et al. 2003, McInnes 2012). One paper described these crystals as haemoglobin crystals (Renne, Dungworth et al. 2003), although a literature search failed to identify any published information regarding investigations into the histochemical or ultrastructural properties of these crystals. There is a single report (poster abstract) describing the presence of eosinophilic crystals in the lungs of three Sprague Dawley rats within control groups of a 6-month safety study (Schuh 2005). This study reportedly had problems with bacterial contamination (from chronic vascular implants) that lead to morbidity and mortality. The majority of survivors at 13 weeks and 6 months had multifocal chronic-active perivasculitis and vasculitis, which was restricted to the lungs and was accompanied by chronic thrombosis. The alveolar septa adjacent to affected regions were often hypercellular and alveoli contained activated alveolar macrophages. Additionally, in 3/13 affected rats there were rare (1-2) foci of small numbers of extracellular and intracellular (within alveolar macrophages) eosinophilic crystals. The author noted that these crystals are similar to those seen in acidophilic macrophage pneumonia (eosinophilic crystalline pneumonia) seen in various strains of mice associated with overexpression of chitinase 3-like 3 (Chi3l3) protein (formerly known as Ym1 protein)
(Elmore, Berridge et al. 2013). Additionally, the author also notes that as in mice, the crystals in rats were found in association with alveolar macrophages and secondary to lung injury, and concludes that although crystal formation is rare, that rats appear to have a protein present within the lung that is capable of autocrystalization during inflammation and is similar to the Ym1 (Chi3l3) protein in mice (Schuh 2005). Whilst, as the author acknowledges, thorough characterization of inducible pulmonary proteins in the rat would be required to determine the relationship, if any, to the Chi3l3 protein in mice, there is little evidence to suggest that the presence of eosinophilic crystals in the lungs of rats is pathogenic, in contrast to the disease in mice which may be associated with clinical disease and is a common cause of death in some strains of mice (Ward, Yoon et al. 2001, Hoenerhoff, Starost et al. 2006). Although the eosinophilic crystals in Schuh’s case series with perivasculitis/vasculitis and in the current study with infectious interstitial pneumonia (all affected rats were classified as either positive or equivocal for IIP), this is insufficient evidence for a definitive association with inflammatory disease, let alone a causal relationship. For example in acidophilic macrophage pneumonia of mice, the disease may be spontaneous or may be seen in conjunction with other pulmonary lesions such as neoplastic, fungal, allergic or parasitic disease (Hoenerhoff, Starost et al. 2006). Indeed, in one reference describing eosinophilic crystals as a background lesion in rats, the accompanying image also shows the presence of localized alveolar haemorrhage, few alveolar macrophages and possible septal thickening in the adjacent alveoli (McInnes 2012), just as described by Schuh (Schuh 2005) and documented in the current study (Figure 1.3.28).
3.5.5 Focal nodular interstitial inflammatory cell aggregates

Nodular subpleural inflammatory cell aggregates were seen in over half of the rats in this study. The number of lesions ranged from 1-5 in affected rats. All ages were affected and the prevalence was very similar across strains and housing conditions. As discussed previously, the presence of this lesion was sometimes considered a confounding factor in the histopathological diagnosis of IIP, however ultimately there did not seem to be an association with this lesion and IIP, with approximately equal numbers of affected rats being positive and negative for IIP.

Additionally supporting a lack of association with IIP was the fact that this lesion was seen in 3 week-old rats, whereas no rats this young have ever been documented to have histopathological lesions consistent with IIP/RRV, both within this study and in the published literature. As such, this lesion is almost certainly an incidental background finding in rats, and likely reflects the foci of minimal, usually mononuclear cell subpleural inflammation described and illustrated as a background lesion in rats (McInnes 2012).

The origin and significance of the other nodular interstitial inflammatory foci, composed predominately of lymphocytes and macrophages as described in Chapter 3.2.3, is uncertain. As with the subpleural inflammatory aggregates described above, these lesions also needed to be distinguished from the lesions of IIP for classification purposes. Indeed, many of these foci may represent partial perivascular inflammatory cell cuffs or alternatively may represent BALT, the latter being common in rats and even observed in germ-free rats (Foo and Phipps 2010). If the latter is the case, then these could represent
aggregates of “classical” BALT or “inducible” BALT. Classical BALT is randomly distributed along the bronchial tree, but consistently present around bifurcations and always lies between an artery and a bronchus (Elmore 2006). Therefore, some of these interstitial foci likely represent classical BALT where the accompanying bronchus or bronchiole +/- artery does not lie within the plane of section. These foci lacked a lymphoid follicle, but this is not uncommon in rodent BALT, likely due to rapid transport of the immunological response to the draining lymph node(s) (Elmore 2006). Alternatively, it is possible that these interstitial inflammatory cell foci represent induced BALT, which can be triggered by a variety of underlying disease states including chronic inflammation, infection, and autoimmunity. Bronchus-associated lymphoid tissue induced by this mechanism is known as inducible BALT (iBALT), and it distinct from classical BALT in several aspects: these foci do not always have overlying epithelium, are not always found in association with an airway, and may be found at varying locations within the pulmonary parenchyma (Foo and Phipps 2010). Further evidence suggesting that these foci in the current study are BALT is provided by the high frequency of these nodular lesions, even in 3 week-old and/or IIP-negative rats. The presence of these foci in 3 week-old rats is particularly significant, as histological lesions of IIP/RRV in rats this young have never been described. Confirmation of the theory of at least some of these interstitial infiltrates being BALT would require further characterisation as to the exact cellular and non-cellular composition of these aggregates. Additionally, examination of a known Pneumocystis sp. negative colony for these lesions would be required for a definitive assessment of any association between Pneumocystis sp. and the presence and/or prevalence of these lesions. Given that there are known differences in the histopathology of lymphoid tissues in the athymic nude rat as compared to immunocompetent rats, additional information is also needed to characterise
the presence and nature of iBALT in athymic nude rats. In classical BALT, not only are interfollicular areas in athymic nude rats lymphodepleted (Schuurman, Hougen et al. 1992, Hanes 2006), but the formation of secondary follicles requires T-cell interactions, and as such secondary follicles tend to be absent in all lymphoid tissues in athymic nude rats (Hanes 2006). In regards to iBALT, however, the picture is less clear as information is emerging that many molecular and cellular cues critical for secondary lymphoid organ formation may not be necessary for the formation of iBALT (Foo and Phipps 2010).

3.5.6 Perivascular eosinophilic infiltrates

A lesion not recorded individually, but rather described as a variation of the loose perivascular cell infiltrates in the miscellaneous interstitial inflammatory cell infiltrates, were loose perivascular infiltrates of eosinophils. Similarly, although not recorded as a separate lesion category, variable numbers of eosinophils were also seen in some IIP-positive rats as a component of the dense perivascular inflammatory cell cuffs that are one of the hallmark features of this disease. Eosinophils have also been described as a component of the inflammatory cell infiltrate in some rats with RRV/IIP by other authors (Gore, Gower et al. 2004). A transient increase in perivascular eosinophils has also been described post-experimental RRV exposure, prior to the development of the characteristic mononuclear inflammatory cell infiltrates and subsequently declining with the onset of the more characteristic lesions of lymphohistiocytic interstitial pneumonia and dense perivascular inflammatory cell cuffs (Albers, Simon et al. 2009). That said, the non-specificity of this lesion was emphasised by the authors, with perivascular eosinophil infiltration having been described as a common background lesion in the lungs of both
young and old laboratory rats (McInnes 2012). Therefore, at least some of the loose perivascular inflammatory cell infiltrates noted in rats in this study, particularly those comprised of a large proportion of eosinophils, likely represent background lesions. The possibility, however, that some of these foci represented early lesions of IIP cannot be excluded and highlights another limitation of histopathology as a diagnostic tool in cases of early or subtle IIP.

### 3.5.7 Alveolar neutrophils

Another feature seen in some rats in this study were variable numbers of neutrophils within the alveoli of IIP-affected rats. This finding has also been previously, but inconsistently, documented in reports of RRV/IIP (Elwell, Mahler et al. 1997, Farrar and LaRegina 1997, Riley, Simmons et al. 1999). Interestingly, a more recent report specifically noted the absence of this finding (Albers, Simon et al. 2009) and concluded that given the presence of bacteria on electron microscopic evaluation in the Elwell et al report, that the affected rats likely had a secondary bacterial infection as well as infection with RRV. Whilst it is plausible that the presence of bacteria noted in the Elwell et al paper may have been a contributing factor for the recruitment of neutrophils seen in those rats, it should be noted that neutrophils were also noted in another study (Riley, Simmons et al. 1999), in which extensive ancillary testing was undertaken to specifically exclude a bacterial agent. All tests undertaken in this latter study were negative and included numerous histochemical stains (H&E, Gram, acid-fast and modified Steiner’s silver stain), microbial culture, PCR and serology for CAR bacillus and Mycoplasma pulmonis and lastly, a PCR using conserved 16S rRNA gene primers capable of detecting all known
bacterial species. Less extensive, but similar work-ups were undertaken by other researchers (Farrar and LaRegina 1997) and the results included negative culture, histochemical stains, *Mycoplasma* serology and as in the Elwell *et al* study, electron microscopic evaluation of affected lungs. Taken together, the negative results for bacteria in these latter studies suggest that although secondary bacterial infection cannot be ruled out as a contributor in the rats described by Elwell *et al*, neutrophils can be seen within the spectrum of IIP-induced lesions in the absence of a concurrent bacterial infection.

In this study, neutrophils were a relatively common finding, being present in 31.5% of ARC rats and 50% of rats from each of the externally housed groups. Almost of all of which had concurrent lesions of interstitial pneumonia and perivascular inflammatory cell cuffing consistent with RRV. Routine aerobic and anaerobic cultures of all lungs with alveolar neutrophilic infiltrates were negative for bacterial growth. Indeed, bacterial culture of the lungs yielded positive results in only 3/210 rats tested and neutrophils were not identified on histopathological examination of the lungs from any of these rats, as would be expected in a true bacterial infection. Additionally, no gross or microscopic lesions were noted on examination of the lungs of 2/3 cases (3 week SD isolator rats), from which coagulase negative *Staphylococcus* sp. was isolated on lung culture. This finding, in conjunction with the knowledge that coagulase negative *Staphylococcus* sp. are normal flora of the human skin (Roth and James 1988), suggests that the positive lung culture result is likely due to inadvertent microbial contamination during necropsy. The likelihood of inadvertent contamination is further increased by the fact that these rats were part of the first group to be necropsied, during a time when procedural techniques and skills were still being optimised. Lastly, an Enterobacteriaceae species (possible *Pseudomonas* sp.) was
isolated from the lung of the third culture-positive rat (12 week-old SD isolator housed). This rat had no gross lesions and a single lymphohistiocytic perivascular cuff was identified on histopathological examination, accompanied by mild alveolar histiocytosis and low numbers of loose lymphohistiocytic perivascular inflammatory cell infiltrates. As mentioned previously, no neutrophils were seen and as the lesions seen are not consistent with those described for *Pseudomonas* sp.-associated pneumonia (Percy and Barthold 2007), the positive lung culture result in this animal is most consistent with an environmental contaminant.

Therefore, whilst the results of this and more than one prior study do not support a concurrent bacterial infection as the cause of the alveolar neutrophils seen in some rats with IIP, the exact mechanism(s) contributing to their presence is uncertain. Similarly, the reasons for the discrepancy between reports of RRV and the presence/absence of neutrophils is also uncertain, although given the presence of neutrophils in several studies, in the absence of demonstrable bacteria, it is likely that the presence of neutrophils in these cases is rather a non-specific indicator of acute or ongoing lung injury.

As with the overall prevalence of IIP, there was also increased prevalence of neutrophils within the alveoli in older rats, reaching peak prevalence of 67.5% at 24 weeks of age (n = 27). This high prevalence was contributed to greatly by the 100% prevalence of this lesion in both groups of nude rats at this age. Nude rats were also greatly overrepresented overall, with this lesion being seen in over twice as many nude rats as in SD rats. The reasons for this are uncertain, however it can be postulated that the neutrophils may be increased in the altered immune response to pulmonary *Pneumocystis carinii*
infection in athymic animals versus immunocompetent individuals. Given that nude rats had an increased prevalence of IIP and alveolar neutrophils compared to Sprague Dawley rats, as well as a tendency for increased IIP severity scores, another contributing, yet non-specific influence on the presence of alveolar neutrophils could be the severity of IIP. Indeed, rats with IIP and alveolar neutrophils had a median severity score of 6, while rats with IIP and no alveolar neutrophils had a median severity score of 3. There was no association between the presence of this lesion and housing conditions, with 29 barrier-housed and 34-isolator rats affected in the ARC-housed groups and equal numbers (5 each) affected in each interstate facility group.

3.5.8 Peribronchial and peribronchiolar inflammation

The cause of the submucosal lymphohistiocytic infiltrate seen in bronchi and bronchioles in some rats is unknown. The nature of this infiltrate was not consistent with descriptions of BALT (Elmore 2006) and is not considered a criterion for the histopathologic diagnosis of IIP (Albers, Simon et al. 2009). Notably, almost all affected rats (28/31) were athymic nude rats, with the lesions in the three SD rats being rare and mild. Approximately half of the affected rats had concurrent increases in the amount of BALT. Given the preponderance of nude rats affected, however, and the increased severity of the lesion in this strain, an effect of strain is presumed. Given the immunodeficiency of this strain, this peribronchial/peribronchiolar nonsuppurative inflammatory infiltrate is considered most likely the result of an altered immune response compared to immunocompetent rat strains. That said, no mention of this lesion was found in a published study of IIP that utilised athymic nude rats (Henderson, Dole et al. 2012).
3.5.9 BALT hyperplasia

BALT hyperplasia was described as a histological feature in some of the early reports of RRV (Elwell, Mahler et al. 1997, Slaoui, Dreef et al. 1998, Raffaella, Sara et al. 2003). However, other reports have specifically reported the absence of BALT hyperplasia/increased peribronchiolar lymphoid tissue in affected rats. It seems likely that the presence of BALT hyperplasia is an inconsistent response that occurs in a small proportion of affected rats. Accurate recognition and assessment of BALT hyperplasia is also difficult in studies lacking clean, known *Pneumocystis*-free, age-matched control animals and without detailed morphometric analysis.

3.6 Infectious interstitial pneumonia classification

Application of the proposed diagnostic criteria for the histopathological diagnosis of RRV (IIP) (Albers, Simon et al. 2009) was, for the most part, relatively straightforward, with many of the rats having frequent perivascular inflammatory cell cuffs and/or foci of lymphohistiocytic interstitial pneumonia required for positive classification and a diagnosis of IIP. That said, for some rats, classification was more difficult, with at least some degree of subjective interpretation of the guidelines being required for classification. This prompted some minor modifications to the grading scheme for ease of classification (as outlined in Part 2: Chapter 2.1). Given the subsequent revelation that *Pneumocystis carinii* was the cause of the lesions of RRV/IIP and that molecular and serological techniques are now the preferred techniques for diagnosing the *P. carinii* infection within a group of
rats (Henderson, Dole et al. 2012), difficulties with histopathological classification now seem less critical. That said, as discussed previously, there does seem to be variation in sensitivity with PCR assays. Additionally, it is worth noting the time frame of this study, and the fact that during much of the study, the aetiology of RRV/IIP was still unknown. As such, at the time, the importance of the interpretation of the lesions outlined below seemed much more important than they are presently. Given, however, that histopathology will likely still be used, as least in some capacity, by some institutions for classification and screening of this disease, some commentary on the nature and interpretation of these lesions seems relevant.

One difficulty encountered with the histopathological classification of RRV/IIP was how to classify rats that lacked typical foci of interstitial pneumonia and dense perivascular inflammatory cell cuffs, but which had one or more, sometimes numerous dense aggregates of inflammatory cells (predominately lymphocytes and plasma cells) adjacent to/within the perivascular connective tissue, but not actually surrounding a vessel as specified in the guidelines. Similarly, in some rats, similar inflammatory cell aggregates were also seen subpleurally, expanding the alveolar septa or within the connective tissue surrounding terminal bronchioles. These foci were not considered sufficient for RRV/IIP-positive or equivocal classification. Rather, in many cases, particularly when faced with perivascular inflammatory aggregates, these foci were considered most likely to represent classical BALT in which the associated bronchus/bronchiole was out of the plane of section, or possibly iBALT as described previously in section 3.5.5. Additional information supporting the BALT origin hypothesis for at least some of these foci include that they were identified in 3 week age group of rats and the histopathological lesions of IIP have not been identified
in any rats this young, in both this study and in prior reports. Several of these foci were also present in a lung section deemed IIP-negative by the consulting pathologist at Charles River Research Animal Diagnostic Services. This increased our level of confidence in the non-specificity of these lesions for the diagnosis of RRV/IIP and led to the inclusion of two additional negative criteria: i) dense nodular perivascular lymphohistiocytic infiltrates (presumptive BALT) and ii) dense nodular interstitial or subpleural lymphohistiocytic infiltrates to the list of suggested criteria for the histopathological diagnosis of RRV infection that was originally proposed by Albers et al (See Part 2: Chapter 2.1).

The diagnostic criteria proposed by Albers et al. lists lymphohistiocytic interstitial pneumonia under the criteria for RRV (IIP) positive. Whilst multiple dense perivascular inflammatory cell cuffs are also included in the positive category, a single dense perivascular inflammatory cell cuff was considered only equivocal. In three of the ARC-housed rats in this study, small foci of alveolar septal thickening, interpreted as mild interstitial pneumonia, were found exclusively in association with small eosinophilic intra-alveolar crystals and in the absence of any other lesion considered consistent with a positive or even equivocal diagnosis of RRV. As discussed previously, eosinophilic crystals have been previously described as a background or idiopathic lesion in rats, sometimes in association with changes such as alveolar septal thickening and alveolar histiocytosis (Schuh 2005; McInnes 2012). Given the small, focal nature of the lesion in these three rats and the prior documentation of similar lesions as a background lesion in this species, a decision was made to classify these three rats as equivocal for IIP under the criterion of “unexplained (e.g. no foreign body visible, inflammatory lesions that in any way resemble RRV (IIP) lesions, rather than IIP-positive under the criterion of lymphohistiocytic
Lastly, as per the Albers et al. guidelines, perivascular eosinophil infiltration that was not dense, usually in one to two areas and sometimes accompanied by lymphocytes was considered RRV (IIP)-negative. Similarly, perivascular lymphoid infiltrates loosely arranged around vessels, incompletely surrounding the vessel and in low numbers was also considered RRV (IIP)-negative (Albers, Simon et al. 2009). Despite the latter stipulation, IIP classification was sometimes complicated in rats which lacked typical foci of interstitial pneumonia and dense perivascular inflammatory cell cuffs, but had i) dense partial/incomplete perivascular inflammatory cell cuffs and/or b) frequent (often variably) loose perivascular inflammatory cell infiltrates, usually comprised of lymphocytes, eosinophils or a combination of both with occasional plasma cells and macrophages in some rats. Although on strict interpretation of the guidelines, it seems that the latter should be considered RRV-negative, it was noted that these dense, incomplete or frequent, loose, mixed, inflammatory cell cuffs were relatively common concurrent lesions in rats classified IIP-positive due to the presence of dense complete inflammatory cell cuffs or foci of interstitial pneumonia. Like definitive lesions of IIP, these dense incomplete or frequent loose perivascular inflammatory cell cuffs were not seen in rats in the 3 week-old age group. These findings seemed to suggest that the presence of these lesions, especially if frequent or prominent, could potentially be associated with IIP. In particular, it is proposed that these lesions could potentially indicate early or resolving lesions of IIP and therefore may be better included in the equivocal rather than the negative category. Additionally, the presence of these “harder to interpret” lesions further highlight the limitations of histopathology as a singular diagnostic method for the diagnosis of IIP. That said; it must
be recognised that screening larger numbers of rats within a colony would also increase the
diagnostic sensitivity of histopathology as screening tool for IIP. Diagnostic sensitivity can
be improved further by using histopathology as only part of a diagnostic regimen for the
detection of IIP, e.g. in combination with more recent developments such as commercially
available PCR and serological tests for *P. carinii* infection in rats.
Summary, Conclusions & Areas for Further Research

The results of part one of this investigation, which characterised and reported the baseline occurrence and progression of background lung lesions in a colony of SPF rats, showed there was a high prevalence of microscopic lung lesions within the colony. A smaller proportion of rats also had macroscopic lung lesions, which were first noted at the time of necropsy or became evident following formalin-fixation of the tissues. Additionally, similar lesions were also identified in two smaller groups of rats that were sourced from the SPF facility, but were later transported and maintained within one of two external facilities.

The predominating lesions were inflammatory in nature and affected the pulmonary interstitium and the alveoli. Interstitial lesions included lymphohistiocytic interstitial pneumonia and dense perivascular inflammatory cell cuffing, while foci of alveolar histiocytosis and neutrophil accumulation predominated within the alveoli. Rats with alveolar inflammation almost always had concurrent interstitial inflammation. A variety of lesions were also noted in some rats, including other patterns of interstitial inflammatory cell infiltration, changes in BALT, and several miscellaneous lesions. Some of these lesions were considered likely artefacts of euthanasia, sample collection and/or tissue processing, while others were compatible with lesions described within the literature as common background findings in the laboratory rat.

Despite the variety of different lesions observed within the study population, the
predominant and most important lesions identified were the lymphohistiocytic interstitial pneumonia and dense perivascular inflammatory cell cuffing. As for all identified lesions, the number and extent of the foci of lymphohistiocytic interstitial pneumonia and dense perivascular inflammatory cell cuffing were highly variable between rats and in many cases, there was also marked variability within lung lobes within the same rat. In athymic nude rats, lesion prevalence and severity tended to increase with age, while the prevalence and severity trends in Sprague Dawley rats tended to be more variable, and in some cases, decreased with advancing age. There were no consistent trends with lesion prevalence and severity between rats of different housing conditions, particularly between ARC-housed rats from the isolator and barrier groups. In 12 week old rats, however, rats from the externally housed groups (Murdoch University Animal House and the interstate facility) often had increased lesion prevalence and/or severity compared to rats of the same age housed within the ARC isolator or barrier.

Part one of this study fulfilled the aim of surveying the ARC population for evidence of lung disease, with a variety of lesions being noted within the study population, including the lymphohistiocytic interstitial pneumonia and dense perivascular inflammatory cell cuffing that predominated. Similar lesions were also identified at a high prevalence in both groups of rats that were raised at the ARC but then maintained under routine experimental husbandry conditions at one of two external research facilities. These results allowed rejection of the null hypothesis that there would be no identifiable gross or microscopic lung lesions detected within the rats from the ARC colony or those housed at external facilities. Review of the prevalence and severity scoring data of the most prevalent lesions (lymphohistiocytic interstitial pneumonia and dense perivascular inflammatory cell
cuffing as well as alveolar histiocytosis and alveolar neutrophils) showed some trends in lesion prevalence and severity between rats of different ages, strains and housing conditions, including a trend for increasing prevalence and severity with age, especially in athymic nude rats. These trends, however, were variable between groups. Given this and the relatively small group sizes, additional investigation, including statistical analysis with larger group numbers, would be required in order to be able to definitively reject the original hypotheses that rat age, strain and housing conditions have no effect on the occurrence of gross and microscopic lung lesions within the ARC rat colony.

Upon review of the historical and recent literature relevant to inflammatory lung lesions in rats, it became clear that the combination and nature of the lesions of lymphohistiocytic interstitial pneumonitis and dense perivascular inflammatory cell cuffing that predominated in the study were not consistent with any of the historical and well-recognised lung diseases of rats. Rather, these lesions were consistent with an emerging and at the time, poorly characterised disease of rats known as rat respiratory virus (and later, infectious interstitial pneumonia) (Albers, Simon et al. 2009). This was considered an important finding, as the combination of lesions compatible with this disease had not, to the author’s knowledge been previously recognised in Australia. Given the possible confounding effects of this disease on research, particularly on experiments focused on the lower respiratory tract, it is essential that this disease be recognised and taken into consideration in both the planning stages of an experiment and when interpreting experimental data.

Therefore, in Part two of this study, the original lung slides were re-examined and
each rat was classified as being positive, negative or equivocal for IIP, based on a modified version of the original guidelines published for the histopathological diagnosis of IIP (RRV). In order to help evaluate lesion severity and progression, a lesion severity score for IIP was also developed. These results were then used to assess for trends in lesion prevalence and severity between rats of different strain, age and housing conditions. As expected, the prevalence of IIP was high within the study population, with just under 50% of ARC rats being either positive or equivocal. In externally housed groups, this prevalence was even higher. Definitive lesions of IIP were first noted in all groups at 6 weeks and the overall prevalence increased with age. This effect was consistent across the athymic nude rats, but inconsistent in the Sprague Dawley rats, with the prevalence across the isolator housed groups increasing consistently with age as for the athymic nudes, but with a dramatic drop in prevalence noted at 24 weeks within the barrier housed animals. Infectious interstitial pneumonia severity scores also tended to increase with age (compatible with lesion development and progression over time), and the proportion of rats with higher severity scores was higher in athymic nude rats.

Lastly, given the emergence of two papers demonstrating *Pneumocystis carinii* to be the cause of the lesions of IIP, efforts were made to identify *P. carinii* within the study population. Flocculent eosinophilic material compatible with *P. carinii* infection had already been identified in two athymic nude rats within the study population and efforts were not being made to monitor or exclude this organism from the ARC colony, so infection within the colony was already strongly suspected. Fungal cysts were subsequently demonstrated using a modified silver histochemical stain in just under half of the IIP-positive rats and in almost 100% of the IIP-positive athymic nude rats. Subsequently, a
commercially available PCR assay for *P. carinii* was used to look for the organisms in a subgroup of the 3, 6, and 12 week rats. The limitations of not performing silver stain histochemistry and *P. carinii* PCR testing on all rats are acknowledged, however despite this limitation, the results of these tests did provide some useful information, including confirming the presence of the organism within rats of all ages, strain and housing types. PCR testing also detected *P. carinii* DNA in two 3 week-old rats, which was earlier than when IIP lesions had been detected via histopathology. Another significant finding was imperfect agreement between the PCR results and presence of histopathological lesions of IIP, including negative PCR results in two rats in which scattered fungal organisms were identified within silver-stained sections of lung. These results differ to the literature in which a significant association has been identified between the presence of IIP lung lesions and *P. carinii* DNA, suggesting that the PCR testing in the current study may have lacked sensitivity. There are a variety of reasons that this could have occurred, including issues related to sample collection, processing and storage unrelated to the actual laboratory assay itself, however further investigation would be required to assess this theory. The possibility of more widespread issues with the sensitivity of PCR for the detection of *P. carinii* in lungs with lesions of IIP is also supported by early publications of RRV in which *P. carinii* PCR was either negative or inconsistently positive. The reasons behind this are unclear, but despite the strong association between IIP lesions and the presence of *P. carinii* DNA that have been demonstrated in the literature, the imperfect agreement seen in this study suggests caution is warranted if using PCR alone as a diagnostic tool for *P. carinii* infection, as it seems that for whatever reason, that false negatives do occur. Combining PCR with other techniques such as histopathology and serology, and ensuring an adequate sample size in all testing, is important for maximising diagnostic sensitivity.
While the high prevalence of *P. carinii* and lack of evidence for other infectious agents within the study population supports the recent literature documenting *P. carinii* as the cause of IIP, many questions remain. Firstly, although *Pneumocystis* sp. infection has been documented within rats for over 100 years and with a relatively high prevalence since the 1950s and 1960s, interestingly the distinctive lesions of IIP were not documented until the late 1990s. Similarly, *P. carinii* has always been considered a disease of immunosuppressed and immunocompetent animals, and so the occurrence of striking lesions that have the ability to interfere with research in immunocompetent rats is also not well understood. Additionally, despite the fact that there are differences documented in the serological response and subsequent time course of lesion progression and resolution, it is interesting that the lesions of IIP are qualitatively similar on histopathology between athymic nude and immunocompetent rat strains. As such, further investigations into IIP in rats must include efforts to characterise the nature of the immune response and its contribution to lesion development, progression and resolution. Additional investigations are also required to assess the role that *P. carinii* infection, manifesting as IIP, may have in altering physiological and immunological responses to a variety of stimuli that could impact adversely on a range of different types of experiments utilising rats. In the meantime, researchers concerned as to the possible impacts of this disease on experimental results should seek to source and maintain animals known to be free from *P. carinii* infection. To this end, commercial rodent suppliers with infected colonies should consider the use of caesarean rederivation and subsequent management in order to eradicate and maintain rat populations that are negative for *P. carinii*, particularly now that lesions associated with *P. carinii* have now been documented in both immunocompetent and immunodeficient rats.
Appendix A:

<table>
<thead>
<tr>
<th>ORGANISMS MONITORED AND EXCLUDED</th>
<th>TEST CODE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Viruses</strong></td>
<td></td>
</tr>
<tr>
<td>Pneumonia Virus of Mice</td>
<td>E12</td>
</tr>
<tr>
<td>Theiler Encephalomyelitis Virus (GD VII)</td>
<td>E12</td>
</tr>
<tr>
<td>Sendai Virus</td>
<td>E12</td>
</tr>
<tr>
<td>Adenovirus Type 1 &amp; 2</td>
<td>E12</td>
</tr>
<tr>
<td>Sialodacryoadenitis/Rat Corona Virus</td>
<td>E12</td>
</tr>
<tr>
<td>Rat Parvoviruses (KPV, RPV, Toolan’s H1)</td>
<td>E12</td>
</tr>
<tr>
<td>Lymphocytic Choriomeningitis Virus</td>
<td>E12</td>
</tr>
<tr>
<td>Hantaan (Korean Haemorrhagic Fever)</td>
<td>E12</td>
</tr>
<tr>
<td>Reovirus -3</td>
<td>E12</td>
</tr>
<tr>
<td>Rat minute virus</td>
<td>E</td>
</tr>
<tr>
<td>Rat Theilovirus</td>
<td>E12</td>
</tr>
<tr>
<td><strong>Bacteria, Mycoplasma and Fungi</strong></td>
<td></td>
</tr>
<tr>
<td>CAR bacillus</td>
<td>AE12</td>
</tr>
<tr>
<td>Clostridium piliforme</td>
<td>AE12</td>
</tr>
<tr>
<td>Mycoplasma pulmonis</td>
<td>AE12</td>
</tr>
<tr>
<td>Helicobacter species</td>
<td>H12</td>
</tr>
<tr>
<td>Salmonella enteriditis</td>
<td>C12</td>
</tr>
<tr>
<td>Corynebacterium kutscheri</td>
<td>D12</td>
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<tr>
<td>Bordetella bronchiseptica</td>
<td>D12</td>
</tr>
<tr>
<td>Pasteurella pneumotropica</td>
<td>D12</td>
</tr>
<tr>
<td>Pasteurella multocida</td>
<td>D12</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>D12</td>
</tr>
<tr>
<td>Streptobacillus moniliformis</td>
<td>D12</td>
</tr>
<tr>
<td><strong>Parasites and Protozoa</strong></td>
<td></td>
</tr>
<tr>
<td>Encephalitozoon cuniculi</td>
<td>E12</td>
</tr>
<tr>
<td>Arthropods (Fur mites, lice, fleas)</td>
<td>B12</td>
</tr>
<tr>
<td>Nematodes (roundworms, pinworms)</td>
<td>FG12</td>
</tr>
<tr>
<td>Cestodes (sentinels)</td>
<td>FG12</td>
</tr>
<tr>
<td>Protozoa: Giardia + Spironucleus sp.</td>
<td>F12</td>
</tr>
</tbody>
</table>
ORGANISMS MONITORED BUT NOT EXCLUDED

*Staphylococcus aureus*  
D12

**Code for tests performed**


**Minimum Frequency testing:** 4= monthly. 12= quarterly. 52= annually. 26= tested bi-annually.

For additional details, see http://www.arc.wa.gov.au.
Appendix B:

Euthanasia and necropsy protocol

1. Euthanasia via intraperitoneal pentobarbitone injection.

2. Collect blood sample via cardiac puncture – allow sample to clot, then spin down serum, collect and freeze at -20 °C.

3. Position the rat in dorsal recumbency.

4. Wet the fur down with 70% ethanol.

5. Remove and reflect skin (mandibular symphysis to pubis) as shown in image below.

6. Spray the subcutaneous tissues with 70% ethanol again prior to opening the body cavities (allow to dry).

7. Open the abdominal cavity and reflect the abdominal wall musculature.

8. Open the thoracic cavity. Cut the diaphragm as close as possible to its junction with the ribcage (avoid touching lungs). Extend the incision up along both sides of the rib cage. Reflect and remove (cut) the thoracic wall to expose the thoracic viscera (see image below).
9. **Collect lung samples** for culture and storage in RNA later:
   a) Use a separate set of instruments that have been soaking in 70% ethanol – keep aside for lung sampling.
   b) Collect approximately half to one-third (depending on rat size) of the right cranial lung lobe for aerobic/anaerobic culture – place in sterile container.
   c) Collect approximately one-third of the right middle lung lobe for RNAlater®/freezing.

10. Remove and examine the thoracic viscera in its entirety:
   a) Make incision through ventricles of heart (to allow formalin penetration).
   b) **Infuse lungs to maximum physiological size with formalin.**

11. Collect remaining organs in formalin for archival purposes:
   a) Remove, examine and collect the abdominal viscera:
      i. Remove spleen and pancreas together and then remove the liver.
ii. Make multiple linear incisions into liver/spleen to assess for lesions and aid formalin penetration.

iii. Remove and “unzip” the tubular gastrointestinal tract, starting at the rectum.

iv. Open the stomach along its greater curvature (with scissors), remove and discard contents.

v. Infuse the intestines with formalin fixative (as for lungs) – care, only do if adequate facilities and personal protective equipment.

vi. Collect the kidneys and adrenals together, longitudinally bisect kidneys

vii. Collect all components of the reproductive tract and the urinary bladder

b) Remove the head at the atlanto-occipital joint and split the head sagittally, exposing the brain and nasal cavity.

Examine all organ systems for abnormalities and photograph anything abnormal – especially if any gross changes evident in the lungs
Appendix C:

Llewellyn’s Sirius red (modified) for eosinophil granules

(Llewellyn 1970)

Reagents:

Alkaline Sirius red solution
1. Sirius red F3B* 0.5 g
2. Distilled water 45 mL
3. Ethanol, absolute 50 mL

* Note: This method uses Sirius red F3B. The dye Sirius red 4B is not suitable.

Tissue:
- 5µm paraffin sections of neutral buffered formalin fixed tissue are suitable. Other fixatives are likely to be satisfactory.

Method:
1. Bring sections to water via xylene and ethanol.
2. Stain nuclei with a progressive alum haematoxylin for a few minutes.
3. Rinse with tap water.
4. Rinse with ethanol.
5. Dehydrate with absolute ethanol.
6. Clear with xylene and mount with a resinous medium.

Expected results:
- Eosinophil and Paneth cell granules – red.
- Nuclei – blue.
- Background – colourless.
Appendix D:

Modified PAAS (Periodic Acid Ammoniacal Silver) method for the detection of fungi
(Spoelstra, 2011, personal communication)

Reagents:
1. Ammoniacal Silver Solution*
   Using a 100ml conical flask, add around 5-10ml of distilled water to 0.2g of Silver Nitrate. Dissolve and slowly add fresh ammonium hydroxide drop by drop until the precipitate first formed has nearly disappeared. Top up to 50ml with distilled water.
2. 1% Periodic Acid
3. 0.2% Gold Chloride –Aqueous
4. 2% Sodium Thiosulphate - Aqueous
5. Harris’s Haematoxylin
   *Note: Always make up fresh.

Method:
1. Take sections to water.
2. Treat with periodic acid for 20 min.
3. Wash sections well with distilled water.
4. Preheat Ammoniacal Silver Solution to 60 degrees centigrade in a Coplin jar in MW oven using temperature probe or Microwave Calibration lide.
5. Working quickly place sections in the Coplin jar and heat in MW oven for 1 min on low setting. (Times based on 700W microwave oven)
6. Remove from microwave oven and let sit for 5 min.
7. Wash sections well with distilled water.
8. Treat with 0.2% Gold Chloride solution until colour changes ~ at least 30 seconds.
9. Wash sections well with distilled water.
10. Treat with 2% Sodium Thiosulphate 30 seconds.
11. Wash sections well with distilled water.
12. Counterstain with Harris’s Haematoxylin, 2 dips then blue.
13. Wash well, dehydrate, clear and mount

**Expected results:**

- Pneumocystis spp. – shades of black.
- Nuclei – blue
- Melanin, argentaffin substances black (Elastin and some other structures may also stain) – colourless
References


Foo, S. Y. and S. Phipps (2010). "Regulation of inducible BALT formation and
contribution to immunity and pathology." Mucosal Immunology 3(6): 537-544.


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XXI Convegno Nazionale, Grugliasco.


