The guinea pig as a model for Equine Amnionitis and Fetal Loss (EAFL)

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Abstract

A previously unrecognised form of abortion in mid- to late-gestation mares was reported in the Hunter Valley region of New South Wales in 2004. The term Equine Amnionitis and Fetal Loss (EAFL) was adopted to describe the syndrome. Some clinical and pathological findings of EAFL resembled those of Mare Reproductive Loss Syndrome (MRLS), a syndrome of abortions first recognised in the USA in 2001. The Eastern Tent Caterpillar (ETC), a caterpillar with barbed setae was shown to be the aetiological agent of MRLS. Research has shown that the Processionary caterpillar (Ochrogaster lunifer, PC) which also has barbed setae causes EAFL. However, the pathogenesis of EAFL has not yet been determined.

The use of pregnant mares to investigate the pathophysiology of EAFL is expensive both in the acquisition and maintenance of the mares and progress is slow due to their length of gestation. Therefore, an animal model with a short gestation is required for cost efficiency and to increase the rate of research. In the studies described here the guinea pig (GP) is investigated as a potential animal model as its gastrointestinal tract is anatomically and physiologically similar to that of the horse. The disadvantage of the GP is that it has a different type of placentation to the horse. The objectives of the present studies are to confirm that the GP is a suitable model for EAFL and to investigate the microbiological and histopathological outcomes of pregnancies over time following exposure to PC.

In the initial experiment, the objective was to determine if the oral administration of PC causes abortion in the guinea pig. Fifteen pregnant guinea pigs were allocated into a treatment group (n=9) and a control group (n=6). The treatment group received a capsule containing 0.2g/kg (n=6) daily for 3d from day 35 of gestation (GD35) or 1g/kg (n=3) macerated whole processionary caterpillar daily for 3d from GD26. The matching control guinea pigs were administered capsules containing commercial guinea pig pellets daily for 3d. All guinea pigs were monitored daily until 3d prior to term when they were moved into a separate pen for delivery. After delivery, the number of offspring and any gross abnormalities were recorded. One GP aborted. All other GPs delivered healthy offspring at term. The guinea pig that aborted had received the higher dose of PC (1g/kg).
and was treated for 3d from GD26. No fetal tissues were retrieved. The mother was euthanased and samples for microbiological examination were taken from the peritoneal cavity, uterus and heart blood. Bacteria were isolated from the uterus and were similar enteric and environmental organisms to those isolated from the EAFL experiments. Histopathology was performed on lung, liver, heart, gastrointestinal tract and uterus. The results showed that PC setae and setal fragments were found throughout all tissues.

Based on the findings of the first experiment, the objective of the second experiment was to confirm that abortion could be induced by administering PC to guinea pigs at GD25. Eight guinea pigs were used in the treatment group and four in the control group. The treatment group received PC exoskeleton and integument on 5 consecutive days from GD25. The control group did not receive any PC components. Pregnancies were monitored daily by trans-abdominal ultrasound. If fetal death was observed by ultrasound (n=2; GD31, 55) the guinea pigs were anaesthetised and sampled. If fetal death was not detected the guinea pig was anaesthetized and sampled at a set time point from the first day of treatment (n=6, GD26, 29, 40, 45, 50, 60). The control guinea pigs were also sampled (n=4, GD29, 40, 50, 60). Samples collected for microbiological examination were taken from the peritoneal cavity, uterus, maternal heart blood, junctional zone, placenta, amnionic fluid and fetal stomach contents. Samples were cultured on horse blood agar, McConkey's and chocolate agar plates at 37°C for 48h hours both aerobically and anaerobically. In addition, tissue and fluid samples, as well as swabs were placed in brain-heart infusion broth and further sub-cultured onto plates as described above. Bacteria were isolated from all the treated guinea pigs except one (GD26) which was sampled 24 hours after the first treatment. The bacteria isolated were environmental bacteria similar to those isolated from the field cases and experimental studies of EAFL. These findings supported the use of a pregnant guinea pig model in further investigation of EAFL.

In the final experiment, the objective was to investigate the microbiological and histopathological outcomes of pregnancies over time following exposure to PC. Twenty pregnant guinea pigs were used for this study, a treatment group (n=14) and a control group (n=6). Guinea pigs in the treatment group were administrated macerated PC exoskeleton and integument on 3 consecutive days starting on GD25. The control group did not receive any PC. Trans-abdominal ultrasound was
used to monitor the pregnancies. The guinea pigs were anaesthetized and sampled at a set time point from the first day of treatment (n=11, GD26, 27, 28, 29, 32, 33, 34, 35, 37, 39, 46) or if fetal death was observed by ultrasound (n=3; GD30, 31). The control guinea pigs were also sampled at set time points (n=6, GD26, 28, 30, 33, 37, 46). Samples for microbiological examination were collected from all GPs and all fetuses and processed as described above for Experiment 2. Tissue for histopathological examination was taken from maternal lung, heart, liver, kidney, spleen, gastrointestinal tract, both uterine horns and from each fetus and its placenta. Bacteria isolated from the treated guinea pigs and their fetuses were enteric and environmental organisms consistent with those isolated from equine fetuses aborted from EAFL.

The findings of these experiments suggest that the guinea pig can be used as a model for EAFL.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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No contributions by others.

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Keywords

guinea pig; horse; mare; Equine Amnionitis and Fetal Loss; EAFL; abortion; animal model; Processionary caterpillar

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List of Abbreviations

BHIB: Brain-heart infusion broth

CHOC: Chocolate agar

DART: Developmental and reproductive toxicology

EAFL: Equine amnionitis and fetal loss

EFL: Early fetal loss

ETC: Eastern tent caterpillars

FP: Fibrinous pericarditis

HBA: Horse blood agar

LFL: Late fetal loss

LM: Light microscopy

MAC: McConkey’s agar

MRLS: Mare reproductive loss syndrome

PC: Processionary caterpillar

TEM: Transmission electron microscopy

UU: Unilateral uveiti
CHAPTER 1

Introduction and Objectives

1.1 Introduction

During the winter of 2004, an abortion outbreak occurred in mares in the Hunter Valley region of New South Wales as well as Queensland and Victoria in Australia (Todhunter et al., 2009). This unusual form of abortion, which came to be known as equine amnionitis and fetal loss (EAFL), had not been previously recognised in Australia (Todhunter et al., 2009). The name represents the typical features of this form of abortion. EAFL has similar clinical and pathological features to those associated with mare reproductive loss syndrome (Perkins et al., 2007). MRLS was first recognised during the spring of 2001 when an outbreak of unusual early and late gestation abortions in mares occurred in central Kentucky in the USA (McDowell et al., 2010). At the same time, similar epidemics were also reported from the surrounding states of Ohio, West Virginia, Tennessee, Illinois and Indiana (Bernard et al., 2004, Sebastian et al., 2008b). This new abortigenic disease consisted of a reproductive component which included early fetal losses (EFL) and late fetal loss (LFL), and a non-reproductive component which included fibrinous pericarditis (FP) and unilateral uveitis (UU). Collectively, this syndrome was referred to as mare reproductive loss syndrome (MRLS) (Sebastian et al., 2008b). The EFL and LFL presented from late April, peaked in early May, declined rapidly thereafter and continued until June. The incidence of FP and UU occurred at the same time (Harrison, 2001, Kane and Kilby, 2001, Powell et al., 2002). The same pattern of abortion, with lesser intensity (one-third of 2001), occurred in 2002 (McDowell et al., 2010). Approximately $500 million of economic loss was caused by MRLS in 2001 and 2002, resulting in a severe impact on the thoroughbred industry (Sebastian et al., 2008b). In Australia, the horse industry contributes $5 billion to the country’s gross domestic product which is the fourth largest
industry in Australia. Approximately 30% of the fetuses presented for autopsy in 2004 (Ford, 2012) were attributed to EAFL. This constituted a significant economic loss to not only the Thoroughbred industry but also the other horse breeding industries.

A series of epidemiological investigations and pathological experiments were undertaken after the first abortion storm in the USA to determine the risk factors of MRLS. Eastern tent caterpillars (*Malacosoma americanum*, ETCs) were identified as the main causative agent (Webb et al., 2004). The similar clinical and pathological characteristics of MRLS and EAFL led to the suggestion that the causal factor could be similar for both syndromes (Perkins, 2005). The Processionary caterpillar (*Ochrogaster lunifer*, PC) which has external barbed setae similar to the ETC was identified as a potential agent for EAFL. Processionary caterpillars were investigated and demonstrated to induce abortion in mares (Cawdell-Smith and Bryden, 2009, Cawdell-Smith et al. 2012).

The clinical findings of both MRLS and EAFL suggest that these two syndromes share common pathology and may potentially have a similar pathogenesis (Perkins et al., 2007). However, the pathogenesis of these two conditions has not as yet been fully determined. Currently, there are three hypotheses that are commonly accepted: mechanical, toxicological or a combination of both (Cawdell-Smith et al., 2010). The use of pregnant mares to investigate the pathophysiology of MRLS and EAFL is expensive both in acquisition and maintenance of the mares. Therefore, laboratory animal models have been explored for investigation of the pathogenesis of these disease syndromes. Mice, rats and goats were trialled as animal models for MRLS. However, none of these were successful (Sebastian et al., 2008, Sebastian et al., 2002b, Sebastian, 2004). Pigs were also used and it was shown that ETC can induce abortion (McDowell et al., 2003) but pigs are an expensive model that provides little benefit over the use of the horse.

The guinea pig has been identified as a potential candidate as a bioassay for EAFL. It is a monogastric and a hind-gut fermenter (Crecelius and Rettger, 1943) similar to the horse but has different type of placentation. The use of guinea pigs is much cheaper and more time efficient than the use of horses, and would allow a wider range of experiments for investigation of these conditions to be undertaken.
1.2 Objectives

The objectives of the present study were to determine whether the GP model is suitable for studies of EAFL and to investigate the microbiological and histopathological outcomes of pregnancies over time following exposure to Processionary caterpillars. The hypothesis of current experiments is that the guinea pig is a suitable model to investigate the pathogenesis of EAFL.
CHAPTER 2

Literature Review

2.1 Scope of the review

Equine Amnionitis and Fetal Loss (EAFL) was first recognised in Australia in 2004 after an unusual abortion outbreak. The typical features which included inflammation of the amnion, umbilical cord and allantoic surface of the chorioallantois (Perkins et al., 2007) were similar to Mare Reproductive Loss Syndrome (MRLS) which occurred in central Kentucky in the USA in 2001 (McDowell et al., 2010).

A number of epidemiological studies were carried out to identify potential aetiological agents for MRLS (Dwyer et al., 2003). These were further investigated by a series of experiments with a number of possible factors considered for MRLS. Initially, an infectious cause was ruled out as the typical clinical features seen with infectious abortion were absent in aborting mares. Therefore, MRLS was identified as a non-infectious disease and environmental toxins were investigated (Sebastian et al., 2007). Fetal fluid samples from MRLS field cases were examined and a negative result for nitrate/nitrite ruled out its toxicity. Pasture samples were also collected and tested and phytoestrogens and other mycotoxins were ruled out (Sebastian et al., 2003). Researchers found that
mares bred in mid-February were more likely to have early term abortions compared to those bred in April (Cohen et al., 2005). This result led to a further epidemiological surveys to identified a relationship between MRLS and the presence of eastern tent caterpillars (ETC), wild black cherry trees, waterfowl and feeding hay off the ground (Dwyer et al., 2003). Finally, ETCs were determined to be the likely aetiologically agent (McDowell et al., 2003).

The investigation of EAFL was based on research of MRLS. The Processionary caterpillar (*Ochrogaster lunifer*, PC) was identified as the main cause of EAFL (Cawdell-Smith et al., 2012). However, using the mare for research of MRLS and EAFL is expensive and time consuming and an animal model for investigation of the pathogenesis of these disease syndromes is required.

This review will focus on the role of ETC in MRLS and PC in EAFL; animal models that have been trialled; animal models in general and more specifically the use the guinea pig as an animal model.

### 2.2 Overview of EAFL and MRLS

#### 2.2.1 Equine Amnionitis and Fetal Loss (EAFL)

A previously unrecognised form of abortion in mid- to late-gestation mares was reported in 2004 in the Hunter Valley region of New South Wales. The term Equine Amnionitis and Fetal Loss was adopted to describe the syndrome (Todhunter et al., 2009). Some clinical and pathological findings of EAFL resembled those of MRLS (Perkins et al., 2007). Several experiments demonstrated that the exposure of pregnant mares to Processionary Caterpillars or their exoskeleton can induce EAFL. The findings in both the experimental and field cases of EAFL have similar gross pathological and bacteriological findings. The PC was identified as the main risk factor of this disease (Cawdell-Smith et al., 2012).

**Gross pathological findings**

A range of pathological changes from acute to chronic were seen with EAFL. Acute cases were often associated with almost ‘no changes or haemorrhage on the pleura and amnion or sometimes
with mild interstitial oedema of the lungs, generalised vascular engorgement or some haemorrhage of the umbilical cord’ (Todhunter et al., 2009, p.36). Fetuses of younger gestational age were more likely to be associated with acute cases while fetuses of older gestational age more likely in subacute to chronic cases (Todhunter et al., 2009). The placental membranes showed an unusual pattern of inflammation. The umbilical cord of aborted fetuses was discoloured, thick and oedematous sometimes with focal diphtheritic membranes (Todhunter et al., 2009). Vascular engorgement, oedema, beading of the vasculature and haemorrhages were the typical features of the amnion in all of the abortions. The stomach contents of all aborted fetuses showed discoulouration and were turbid (Cawdell-Smith et al., 2010, Todhunter et al., 2009).

Microbiological findings

The majority of bacteria isolated from the aborted fetuses of both confirmed and suspect EAFL field cases were environmental coryneforms and gram-negative rods (Todhunter et al., 2009). Only 15% of identified cases had alpha haemolytic Streptococcus spp which is different from MRLS where the majority of isolated bacteria were Streptococcus spp. (Donahue et al., 2002). Bacteria were mainly found in the lung, amnion, chorioallantois, umbilical cord and stomach contents of aborted fetuses (Todhunter et al., 2009). In the experimental cases, the bacteria that were isolated from aborted fetuses included enteric or oral bacteria (Lactobacillus sp., Streptococcus spp., Enterobacter sp., Enterococcus spp., Cellulosimicrobium cellulans, Propionibacterium sp., E.coli, Actinobacillus sp., Pasteurella sp. and Proteus vulgaris) and environmental bacteria (Aeromonas spp., Staphylococcus spp., Aerococcus sp., Microbacterium spp., Dietzia maris, Corynebacterium spp., Bacillus spp., Aerosphearea sp., Bisgaard toxon 10, Pseudomonas spp., Stenotrophomonas maltophilia, Wautersiella sp. and Klebsiella sp.) (Cawdell-Smith et al., 2012).

Histopathological findings

In both confirmed and suspect cases of EAFL, the most common histopathological findings were microscopic pulmonary lesions in aborted fetuses. These were followed by the inflammation of chorioallantois and funisitis, amnionitis, umbilical cord oedema, haemorrhage and congestion
(Todhunter et al., 2009). In experimental cases, the setae or setal fragments of PCs were found throughout the gastrointestinal tracts especially in the caecum and large colon. The lesions of tissues caused by setae ranged from unapparent, superficial erosion to deep ulceration of the mucosa. The mares had varying degrees of typhlitis, colitis and endometritis. Focal hyperplastic serositis was mainly present in gastrointestinal tracts and uteri (Todhunter et al., 2013).

2.2.2 Mare Reproductive Loss Syndrome (MRLS)

The reproductive components (early fetal losses and late fetal loss) dominated the perception and impact of MRLS while the non-reproductive components (fibrinous pericarditis and unilateral uveitis) occurred in only a small number of horses and were considered on their own (Sebastian, 2004). Cohen (2005) noted that mares bred in mid-February were at greatest risk of early abortions. In addition, there was no long-term effect on fertility for mares which recovered from early or late-term abortions and those mares could reproduce normally in following years (Cohen et al., 2005).

MRLS related Early Fetal Loss (EFL)

Mares which suffered EFL showed no outward clinical signs. In most EFL cases, rectal palpation was normal but the typical ultrasonographic appearance of EFL was a dead fetus surrounded by echogenic allantoic fluid and amniotic fluid (Williams, 2002). In the cases where there was no fetal death, slow heart beats and slow fetal movements were detected by ultrasound with echogenic amniotic fluid. In some cases autolyzed fetuses were found in the vaginal canal (Williams 2002). The majority of EFL cases occurred between 40 and 80 days of gestation (Sebastian et al., 2007). The bacteria cultured from the allantoic fluid of three EFL mares were alpha-hemolytic Streptococcus spp. in two cases and Escherichia coli in one case. Uterine cultures were conducted on all aborting mares in 2001 and 2002 (Sebastian et al., 2007). Bacteria isolated were alpha- and beta-hemolytic Streptococcus spp., Actinobacillus spp., Escherichia coli and Enterobacter cloacae (Sebastian et al., 2007). Cytologic examination of the endometrium within 7 days of abortion in mares suffering EFL in 2001 showed moderate to severe inflammation (Riddle and LeBlanc, 2003). The pathological findings in fetoplacental units showed moderate neutrophilic infiltrates in the
placental membranes, allantoic and amniotic fluids. Bacterial placentitis was the major finding in EFL (Donahue et al., 2002; Williams 2002).

**MRLS related of Late Fetal Loss (LFL)**

The majority of LFL occurred in the last trimester and mares exhibited minimal signs of impending abortion. Clinical features included 'explosive parturition, dystocia, foaling while standing, premature placental separation, stillbirth, foals born weak, and agalactic mares' (Sebastian et al., 2008b, p.711). The most remarkable gross lesions included a thick, edematous and yellowish umbilical cord. Characteristic histopathologic changes associated with LFL included funisitis, inflammation of the amnion, perinatal pneumonia and occasionally placentitis (Williams 2002). The most frequently isolated bacteria were same as EFL, *Streptococcus* spp. and *Actinobacillus* spp.) (Sebastian et al., 2008b).

2.3 Caterpillars as a cause of fetal loss in the mare

2.3.1 Experimental studies of MRLS and EAFL

Initial research relating to the eastern tent caterpillar (ETC) investigated toxins associated with the caterpillar (Webb et al., 2004). The investigations were directed toward ETCs which were present around the affected farms and identified in the epidemiological studies (Webb et al., 2004). Cyanide, produced by the black cherry tree, was investigated. However, in a study where pregnant mares were exposed to different doses of cyanide this did not result in abortion (Harkins et al., 2002). Mandelonitrile, which yields cyanide and is also a defence chemical in ETC was also administered to late-term pregnant mares (Fitzgerald, 2002). No abortion or pathological changes were observed. Finally, cyanide and its precursors were discounted (Harkins et al., 2002).

From spring of 2002, experiments were mainly focused on the ETC itself. Pregnant mares were exposed to live ETC or their frass. There were abortions in the ETC and frass group, but no abortions in the frass only group. This experiment suggested that the exposure to ETC could induce MRLS abortions (Webb et al., 2004). An experiment conducted by Bernard and his colleagues
confirmed that exposure of early-term pregnant mares to ETC can result in fetal loss (Bernard et al., 2004). ETCs obtained from Michigan were also administered to late-term pregnant mares and caused abortions. This study indicated that ETC is abortigenic for late-term pregnant mares and ETCs from other regions can also cause MRLS (Sebastian et al., 2002).

To determine which body components of ETC is the abortifacient factor, early-term pregnant mares were exposed to exoskeleton, gut or internal contents. Only the exoskeleton induced abortions in mares which confirmed that ETC exoskeleton is the main causative agent in MRLS (Webb et al. 2004). Another experiment by Webb showed that the abortifacient agent in ETC is not lost when ETCs are frozen (Webb et al. 2004). Other experiments indicated that the causative agent is not a virus or bacteria associated with ETC (Sebastian et al., 2003).

The final hypothesis was that ETC may facilitate the invasion of potential pathogens. It was suggested that the sharp caterpillar setae may disrupt the gastrointestinal mucosal barrier and cause microgranulomatous lesions. It was also suggested that the bacteria invade the circulatory system of the mare through alimentary tract lesions and reduce immune surveillance of the mare. This allows haematogenous spread of bacteria resulting in infections in tissues such as the fetus and placenta, leading to fetal death and abortion (McDowell et al., 2003). After a series of epidemiological investigations and pathological experiments, the eastern tent caterpillar (Malacosoma americanum, ETCs) was identified as the main causative agent of MRLS (Webb et al. 2004).

The investigation of EAFL was based on research of MRLS, as both syndromes have similar clinical and pathological features. The Processionary caterpillar (Ochrogaster lunifer, PC) which has similar barbed setae to ETCs, was implicated as the cause of EAFL (Cawdell-Smith et al., 2012). Experiments were undertaken that involved administration of whole PC and PC exoskeleton to pregnant mares and showed that this exposure can induce abortion (Cawdell-Smith et al., 2012). Histopathological findings of fetuses aborted in these experiments have shown that caterpillar setae are found in the gastrointestinal tract of treated mares and setae and setal fragments can penetrate the gastrointestinal tract wall and migrate to the uterus. These findings suggest that the setae may facilitate movement of normal enteric or environmental bacteria across the gut wall and into the
uterus and placenta. Evidence strongly suggests that the mechanism is mechanical transfer of bacteria although the possibility that a toxin is involved has not been ruled out (Cawdell-Smith et al., 2012).

The ETC feeds mostly on the black cherry which is present on many farms in Kentucky, USA. When full-grown or when their food source is exhausted, the caterpillars drop from their habitat tree and travel long distances searching for sheltered sites (Fitzgerald, 2002). The epidemiological investigations found that when MRLS occurred, the caterpillar populations were at their peak in many of the affected areas (Sebastian et al., 2007). Many dispersing caterpillars were observed in the pastures, on rails of fences and water troughs (Fitzgerald 2002).

Processionary caterpillars are distributed in coastal and inland Australia ranging from temperate to tropical regions (Floater and Zalucki, 2000) and the larvae feed on Acacia spp. as well as eucalypts (Van Schagen et al., 1992). The nests of PC consist of silk, old skins, frass and other debris. The nests may be located at the base of the trees (Figure 2.1), attached to the trunk (Figure 2.2) or hanging from branches in the canopy (Figure 2.3). The exoskeleton found in the nest is very light and is easily blown about, spreading around the surrounding pasture.

![Figure 2.1: Processionary caterpillars. The nest located at the base of the tree.](image-url)
Figure 2.2: Processionary caterpillars. The nest attached to the trunk.

Figure 2.3: Processionary caterpillars. The nest was hanging from branches in the canopy.
ETC structure experiments

A series of experiments were conducted to understand mechanisms of the abortigenic agent and the structure of ETC. The first two experiments were conducted from May to July in 2002 to test whether exposure ETC to pregnant mares would induce fetal loss (Webb et al., 2002, Webb et al., 2004). The environmental exposure of mares to ETC, resulting in MRLS that occurred in 2001 was mimicked in these two experiments. The mares in treatment group were exposed to live ETC and nest materials or nest materials only. The results proved that ETC can induce fetal loss in pregnant mares (Webb et al., 2002, Webb et al., 2004).

Although the experiments showed that the ETC was the cause of MRLS, it was not determined whether this was a biological or toxicological mechanism. Another experiment showed that the abortigenic activity of ETC was stable to freezing and thawing but labile to autoclaving (Webb et al., 2004). To investigate which portion of the ETC has abortigenic activity, two additional experiments were performed between 2002 and 2003 (Webb et al., 2002, Webb et al., 2004). In the first experiment, ETC larvae were dissected into three different parts which were gut, internal tissues and cuticle. The results indicated that only the cuticle of the ETC was associated with abortigenic activity (Webb et al., 2004).

In the second experiment, insect cuticles, cuticle powder and lipids from ETC cuticle were used as the treatments. The results showed that the only treatment that caused fetal losses was insect cuticle and that when the cuticle of the ETC was disrupted, the abortigenic activity was reduced accordingly (McDowell et al., 2010).

2.3.2 Life tables of the Processionary Caterpillar (Ochrogaster lunifer) and the Eastern Tent Caterpillar (Malacosoma americanum) and the PC structure.

Processionary Caterpillar (Ochrogaster lunifer)

Processionary caterpillars (Ochrogaster lunifer) are widely distributed in coastal and inland Australia where they nest on the base of the trunk or in the canopy of wattle trees, but also eucalypts...
(Floater and Zalucki, 1999). Their nest consists of silk and forms a bag where large numbers of caterpillars rest together (Floater and Zalucki, 1999). The adult form of the caterpillar which is the Bag-shelter Moth emerges in late October. In early summer, the female moth lays a large number of eggs (around 150-500) at the bottom of the tree or in egg masses in the canopy. The eggs hatch into the first larval stage which does not feed. They shed their old skins and develop into second stage caterpillars which are bigger and feed on leaves (Floater, 1996). Before they metamorphose into pupa, the caterpillars need to go through eight developmental stages. In their early stages (mid-summer, December), the caterpillars feed on wattle leaves during the day and return to the nest at the base of the tree at night. The older caterpillars (late-summer, March) only feed at night and return to the nest before morning (Floater and Zalucki, 1999). The caterpillars that form nests in the canopy of trees and adherent to the trunk also follow this biological cycle.

As the caterpillars are developing, the nest grows bigger with more and more caterpillars shedding their skins (exoskeletons) (Floater, 1996). Sometimes, in the late summer and autumn (April), the caterpillars can defoliate their food tree when they are not fully grown. Therefore, they will leave the tree in a long, single procession, following each other head to tail and look for another suitable tree (Floater, 1996). In May, the caterpillars are fully grown and form into small groups (10 or less per group). At this time, the small group of caterpillars will travel out of the nest and burrow into the ground. Each caterpillar forms a cocoon chamber of soil and silk and stays in the chamber during the winter and pupates in spring. In September to October, the caterpillars hatch and the adult moths are present in the environment in late October (Floater and Zalucki, 1999). Figure 2.4 shows the seasonal life history of the Processionary Caterpillar.
PC structure

The Processionary caterpillar (*Ochrogaster lunifer*) is a member of the family Thaumetopoidea. Caterpillars in this family have mirrors along the dorsal surface holding true setae. The mirrors are cavities in the surface of the exoskeleton which contain many true setae with barbs along the shaft (Battisti et al, 2011). There are two distinct types of hairs on the Processionary caterpillar: true setae and body hairs. Both light and electron microscopy has shown that the setae have a spear-like appearance with many small barbs (Todhunter et al, 2013). These barbs are likely to encourage the forward movement of the setae through tissues as they prevent their backward motion (Figure 2.5a). This type of action is commonly seen with the progression of grass seeds through tissues in dogs (Johnston and Summers, 1971). Body hairs have also been identified. These have a thick shaft without barbs and it is these bristles that (Figure 2.5b black arrow) can be seen easily by eye and gives the caterpillar its hairy appearance. The hairs seen in the tissues of the mares and guinea pigs, and fetoplacental units of both are the much thinner, smaller and sharper setae, not the larger hairs (B.Cribb pers.comm).

**Figure 2.4:** Seasonal life history of the Processionary Caterpillar (*Ochrogaster lunifer*)
Eastern tent caterpillars (*Malacosoma americanum*)

The adult moths of the ETC lay eggs in late spring or early summer (Figure 2.5). A single egg mass contains approximately 200-300 eggs. Embryogenesis occurs rapidly after the eggs are laid and the fully formed caterpillars stay quiescent in the eggs until early spring (mid-February to mid-March) and then start to hatch at the same time as leaves and buds are emerging on the host plant (Fitzgerald, 1995). As soon as they emerge, the caterpillars construct a communal silk tent in the crotch of the host tree. As they grow, the tent expands daily to accommodate their size (Fitzgerald, 1995). Spring (April - May) is the feeding time for eastern tent caterpillars. The larva only feed for seven to eight weeks to complete their larval stages. When fully grown, the caterpillars leave their host plant and travel long distances on the ground for new sheltered sites. The caterpillars will spend two to three weeks spinning cocoons (late May to early June) and metamorphose before emerging as moths (Fitzgerald, 1995). The adult moths only live for a short period and do not feed. At this stage (mid- June to early July), female moths mate with males and oviposit in rapid succession, and die thereafter (Fitzgerald, 1995).
Figure 2.6: Seasonal life history of the ETC in Kentucky. The exact time of egg hatching is temperature dependent and varies from year to year (Fitzgerald 1995).

2.3.3 Temporal relationships between caterpillar exposure and mare abortion

The stage of gestation when abortion occurs in MRLS and EAFL is different. In MRLS, the abortions happened in both early and late gestation (April to May) (Cohen et al., 2003a, Cohen et al., 2003b), while in EAFL, the abortions were seen in mid- to late stages of pregnancy (April–July) (Cawdell-Smith et al., 2010). Early fetal loss has not been reported in field cases of EAFL. However, this may be related to management procedures (Cawdell-Smith et al., 2013) as the mares would not be tested until late gestation after the confirmation of pregnancy. Also, in the early stage of placentation abortions may not be detected (Cawdell-Smith et al., 2013). In addition, due to economic considerations, aborted fetuses may not be submitted for necropsy (Cawdell-Smith et al., 2013).

Experiments were undertaken to determine whether abortion could be induced by shed exoskeletons of processionary caterpillars in the pre-placentation (<35 days) or early placentation stages of pregnancy (45-60 days) (Cawdell-Smith et al., 2013). The results showed that the exoskeleton of Processionary caterpillars (PC) can induce early fetal losses according to the bacterial criteria in EAFL. Therefore, when unexplained early pregnancy loss occurs, the PC should be considered as a possible cause (Cawdell-Smith et al., 2013).
2.4 Laboratory animal models

2.4.1 Laboratory animal models of MRLS

As the high cost of using mares and their long gestation made the experiments complicated, laboratory animal models of MRLS and EAFL have been explored for investigation of the pathogenesis of these disease syndromes.

Experiments with ETC

Mice: The use of mice as an animal model of MRLS has been unsuccessful. Fresh and frozen ETCs, ETC homogenates, ETC frass and ETC setae fragments were given to pregnant mice orally between 5 to 12 days of gestation. No significant abortion was found. Several fetal resorptions were found, although the number was considered normal (Sebastian et al., August 2002,).

Rats: Finely chopped ETC was administered to pregnant rats from day 4 of gestation to the end of gestation. No statistically significant fetal resorption or abortion was observed (Sebastian, 2004).

These results indicate that it is not appropriate to use mice and rats as animal models for reproducing MRLS (Sebastian 2004).

Goats: Pregnant goats (>100 days gestation) were orally administrated 50g ETC per day for 10 days. None of the goats aborted (Sebastian et al., 2008a).

Pigs: The abortifacient capability of ETC was investigated in pregnant pigs (McDowell et al., 2003). Five mid-gestation gilts were fed ETCs daily for 10 days and another five gilts were used as controls. Two of the 5 gilts in the experimental group aborted while none of the controls aborted. *Streptococci* spp. was isolated from fetuses of all the gilts fed ETCs, whether or not they aborted. Numerous caterpillar setae were observed in mucosa and submucosa of the intestinal tracts of the sows in the experimental group. This experiment suggested that it is possible that other species can be affected by ETC and induce abortion (McDowell et al., 2003).
**Toxicological studies**

As described above, cyanide and its precursor, mandelonitrile were considered as an aetiological agent in MRLS. It is part of the ETC defence mechanism against predators. The intact black cherry leaf contains cyanide. After the caterpillar eats the leaf, cyanohydrin, glucose and mandelonitrile are produced as the result of enzyme conversion. Mandelonitrile is converted to benzaldehyde which is toxic to predators of ETC (Peterson et al., 1987) and decays to cyanide under appropriate conditions (Sebastian 2004). A number of laboratory animal models have been used to investigate the effect of cyanide on pregnancy but no evidence has been found that suggests that this is related to MRLS (Harlins et al., 2002).

Sheep: Previous research has investigated the effects of cyanide in sheep. Pregnant sheep receiving cyanide sodium nitroprusside (SNP) had a significantly increased rate of fetal death but no significant changes were observed in uterine blood flow. The results suggest that pregnant sheep can tolerate higher levels of cyanide than the ovine fetus (Naulty et al., 1981).

Hamsters: In research by Doherty et al. (1982) it was found that 83% of fetuses were resorbed following continuous administration of cyanide to pregnant hamsters (Doherty et al., 1982).

Goats: The pregnant goats received cyanide orally with 1 to 3 mg/kg body weight from 24 gestation day. No abortions were observed indicating that in some species abortion may not be caused by low doses of cyanide (Soto-Blancob and Gorniak, 2004).

**2.4.2 Characteristics of a good animal model**

Animal models can be classified into three types: homologous, isomorphic and predictive. Commonly, animals are used for scientific purposes in nine distinct ways: as predictive models for human disease; as predictive models to evaluate human exposure safety in the context of pharmacology and toxicology; as sources of spare parts; as bioreactors; as sources of tissue in order to study basic physiological principles; for dissection and study in education and medical training; as heuristic devices to prompt new biological/biomedical hypotheses; for the benefit of other
nonhuman animals; and for the pursuit of scientific knowledge in and of itself (Shanks and Greek, 2009).

The following criteria are required for a suitable animal model:

1. The animal model should closely reproduce the disease or condition under study.
2. The animal model should be easily available to many researchers, that is, not a rare or exclusive animal. This allows validation and stimulates further investigations.
3. The animal model, in the case of a vertebrate model should be large enough for multiple biological sampling (tissue, blood, etc).
4. The animal model should fit into available animal facilities of the average institution.
5. The animal model should be easily handled by most investigators.
6. The animal model should be available in multiple sub-species.
7. The animal model should survive long enough for results to be meaningful.
8. The animal model should be sufficiently robust for the purpose of the study. (Chow et al., 2008)

The established models should be used if they are available or possible. When selecting an appropriate animal model, consideration needs to be given to the relevance of the model to the aims of the study. The model should be a relevant species to the target species (Chow et al., 2008).

Festing (2012) describes other aspects of the laboratory animal that are important when selecting the appropriate model. When choosing an animal model for specific study, the model should be appropriate for the condition of the study and respond in the same way as the target species. The 'belief' should be established on specific evidence or it may be assumed from the biological similarity between the model and target animals. However, it is acceptable if the ‘belief’ is incorrect. More importantly, the experiments that use animal models should be well designed in order to ensure the accuracy of the results (Festing, 2012).

Festing (2012) developed the following criteria for selecting an animal model:

1. The model and the target can only have one similarity, but also have any number of differences. Sometimes, the useful models are highly abstract. In other words, substantial asymmetry in the
number of similarities between the model and target is allowed as long as they have a single feature in common.

2. The differences between the model and the target are as important as similarities. This allows manipulation of the model which would not be possible with the target species.

3. Particular studies should use specific models.

4. Models need to be validated.

5. Models can be improved by further research. Some studies are aimed at investigating the possibility of using an animal model for particular conditions. The model needs to be developed and refined.

2.4.3 The guinea pig as a laboratory animal model (general)

The guinea pig (Cavia porcellus) has been used as an animal model for biomedical research for a long time as they are tame, small and easy to handle. The most frequently utilized species is the albino form which is the short-haired English variety (Gad and Peckham, 2007). The guinea pig has been used to study immunology, audiology, nutrition, genetics, pharmacology, allergies, radiology, reproduction and infectious diseases. Approximately 2% of the laboratory animals used annually are guinea pigs which make them the third or fourth most popular species in toxicology and safety assessment in 2007 in USA (Gad and Peckham, 2007).

Models of disease

Hearing

The anatomy of the guinea pig ear and associated structures make them an important animal model for inner ear or ototoxicity studies. Permanent sensorineural hearing loss in humans is caused by cochlear hair cell loss. The guinea pig was used to model this condition by chemical deafening. Aminoglycoside compounds were administrated to guinea pigs to achieve this condition (Taylor and Lee, 2012).
Toxicology

Traditional laboratory animals such as rats, mice and rabbits are usually used for developmental and reproductive toxicology (DART) studies (Rocca and Wehner, 2009). Nonetheless, for certain studies traditional animal models are not appropriate and alternative models need to be investigated (Rocca and Wehner, 2009). The guinea pig is applicable for their high sensitivity to teratogenic effects of environmental and chemical agents (Smith et al., 1992). It has a relatively long gestation period (63–68 days) when compared to other laboratory animal species and produces more than one offspring, usually 3-4 (Percy and Barthold, 2007). These characteristics have an advantage over other laboratory rodents. The birth of offspring with a mature central nervous system is important for toxicology and teratology studies (Taylor and Lee, 2012). Guinea pigs are also used in the General Safety Test for toxic contaminants in biological products (Taylor and Lee, 2012).

Allergic diseases

Allergic conjunctivitis is a common condition affecting humans. Guinea pigs were the first species selected to study allergic conjunctivitis and still remain popular for these kinds of studies. Guinea pigs are also used in other allergic diseases studies, such as IgE-mediated disease, allergic rhinitis and food allergies (Taylor and Lee, 2012).

Non-infectious respiratory disease

The anatomical feature of the airway in the guinea pig is similar to that of the human. This makes the guinea pig well-suited to studies of respiratory disease. Guinea pigs are commonly used for studies of asthma and chronic obstructive pulmonary disease (COPD) (Taylor and Lee, 2012). In addition, the guinea pig has a sensitive respiratory system which allows them to be used in studies of inhalation pneumonia and environmental pollution (Taylor and Lee, 2012).

Reproduction and pregnancy-related diseases

Guinea pigs are suitable models for studies of reproductive physiology, anatomy and endocrinology as they have similar reproductive features to humans (Lee and DeMayo, 2004). Their long gestation and rapid brain development in utero make them the best rodent model for studies of chronic
maternal stress, development of the neonate and many other conditions relating to pregnancy (Taylor and Lee, 2012).

**Infectious disease**

Guinea pigs are extensively employed in studies on research and diagnosis of infectious diseases such as brucellosis, cholera, diphtheria, foot-and-mouth disease, glanders, Q fever and various strains of typhus (Reid, 1959). In addition, guinea pigs are commonly used in the diagnosis and isolation of tuberculosis (Jolly and Heywood, 1979) as they are highly susceptible to both human and bovine-type tuberculosis bacteria. Studies on a wide variety of therapeutics such as antibiotic, antifungal and antiviral compounds have also been carried out in the guinea pig model (Hanes, 2003).

**Other diseases**

Scurvy is another disease that the guinea pig shares with humans. Since the guinea pig cannot synthesize vitamin C it must be obtained from the diet which makes them an ideal model for researching scurvy (Gad and Peckham, 2007). Moreover, the requirement of vitamin C makes the guinea pig a favourable model for nutritional and metabolic studies (Navia and Hunt, 1976). Complement was first isolated from the blood of guinea pigs and guinea pig serum has higher levels of activity in haemolytic complement than other laboratory animals. Therefore, they are widely used in serology studies (Taylor and Lee, 2012). The guinea pig with its unique characteristics has become a popular animal model in biomedical research. There is scope to investigate the guinea pig as an animal model for new studies.

**2.4.4 The guinea pig as a model for EAFL**

Generally, there are two benefits of using animal models. These are that there is a short time to the development of the lesion being investigated, and the ability to study pathology in a large number of individual animals (Cullen et al., 2003).

As previously mentioned, the guinea pig is a good model for reproductive studies because it can produce more than one offspring (usually 3-4) which allows a large number of individuals for experiments, and it has a relatively long gestation when compared to other laboratory species. This
provides more opportunity to investigate the influence on the fetus by the causative agent. Guinea pigs are commonly used in DART studies due to their high sensitivity to teratogenic effects of environmental and chemical agents (Smith et al., 1992). These factors make it a good model for investigation of abortion. Most importantly, the guinea pig is a monogastric and a hind-gut fermenter (Crecelius and Rettger, 1943) similar to the horse. From the economic point of view, it is much cheaper and time-saving to use guinea pigs than the equine model. The pathogenesis of EAFL involves developmental and reproductive toxicology studies. Therefore, for these reasons the guinea pig is suitable as a model for EAFL. However, there are some ways in which the guinea pig and horse differ that are a disadvantage for its use as a model for EAFL. The most important of these relates to the difference in placentation between the two species.

**Placentation**

All eutherian mammals share common placentas in structure and function (Bowen, 2011). There are four basic functions of the placenta which include nutrient synthesis, nutrient transfer, as an immunological barrier and as a temporary endocrine organ (Morresey, 2011). However, the gross and microscopic structure of the placenta differs between species (Bowen, 2011). Based on placental shape and contact points, there are 4 types of placenta which are diffuse, cotyledonary, zonary and discoid. Based on layers between fetal and maternal blood, the placentas are classified into epitheliochorial, endotheliochorial and hemochorial placenta. Diffuse, epitheliochorial placenta is present in horses and discoid, hemochorial placenta can be seen in rodents (Bowen, 2011).

The equine placenta is classified as adeciduate, diffuse, microcotyledonary and epitheliochorial. Different from the discoid placenta of guinea pig, the diffuse placenta involves almost the entire surface of chorioallantois except a small area beside the cervix (Morresey, 2011). The placenta of the guinea pig is discoidal, labyrinthine hemochorial placenta with a fetal/maternal transport barrier (Figure 2.6). It also has separate subplacenta and yolk sac placenta. The chorioallantoic placenta is formed and vascularised by allantois (Kaufmann, 2004). The guinea pig has two uterine horns and one to five implantation sites can be contained in each horn (Hargaden and Singer, 2012). Each fetus is covered by three membranes from inner to outer which are the inner fetal membrane, the
amnion and the outer fetal membrane. The connection between the placenta and uterine wall is placental stalk, a stem-like connection. The role of the chorioallantoic main placenta is a fetal/maternal exchange organ. It is consisted by labyrinth which is used by both maternal and fetal blood channels. The majority of fetal/maternal exchange tissue is provided by the labyrinth. The outer mantle of the main placental disc is the interlobium. It is the area where the labyrinthine lobes are embedded into syncytiotrophoblast. It divided the labyrinthine lobes in several sectors. The central excavation is the centre of the main placenta and is the continuation of umbilical cord. It crosses the placenta and connects to the subplacenta (Kaufmann, 2004).

Figure 2.7: Diagram of the guinea pig placenta (Kaufmann, 2004).

Gastrointestinal tract anatomy

Equine

The gastrointestinal (GI) tract of equine is composed of mouth, esophagus, stomach, small intestine, caecum, large and small colons and rectum. The foregut consists of the stomach and the small intestine, and the large intestine and rectum make up the hindgut of the equine. The equine stomach is monogastric and relatively small when compared to the other segments of GI tract and only comprises about 10% of the entire tract (Frape, 2004). It mainly contains digestive enzymes and hydrochloric acid and differs from cattle which have billions of microorganisms that can digest and
utilize some feed component and potentially toxic substances that the enzymes cannot (Young, 2009). With the digestion of food, the stomach has variations in degree of acidity. It is neutral at the entrance, slightly acidic in the main part of the stomach and very acid at the exit which stimulates protein digestion (Cuddeford, 2003).

The small intestine is composed of three parts: the duodenum, the jejunum and the ileum. Most enzymes are produced in the small intestine and nutrients are degraded into components which can be absorbed into the bloodstream (Young, 2009). Then the digesta empties into the caecum and microbial fermentation and further nutrient absorption occurs in the large intestine (Young, 2009). The large intestine consists of caecum and colon. In horses, the caecum is situated primarily on the right side of the body (Fritz, 2012). The second section of the large intestine is the colon. Unlike human beings, the colon in horses can be up to 10 meters long. In order to fit such a large colon in the limited abdominal cavity, it lies in a horseshoe shape on the ventral aspect of the abdominal cavity from the right inguen to the left. It then turns up towards the back and forms another horseshoe shape on the dorsal aspect in the abdominal cavity (Fritz, 2012).

**Guinea pig**

Compared to equine GI tract, the guinea pig has a similar GIT structure. The monogastric stomach includes cardia, fundus, body and pylorus. The small intestine has three parts: duodenum, jejunum and ileum and the large intestine consists of the cecum and the colon (Cooper and Schiller, 1975). Moreover, the caecum of the guinea pig is the largest dilatation of the alimentary tract which produces large amounts of vitamins and it is located on the left side of the abdominal cavity and occupies most of the ventral abdominal cavity (Manning et al., 1984). According to the location in the abdominal cavity, the colon can be identified into three sections: ascending, transverse and descending (Cooper and Schiller, 1975). Similar to the horse, the guinea pig is a hind-gut fermenter with nutrient absorption mainly happening in the large intestine (Suckow et al., 2012).
**Gastrointestinal tract flora**

**Equine**

As mention above, fermentation usually occurs in the fundus of the stomach where the pH is about 5.4. The microorganisms in this region can tolerate moderate acidity. The species include lactobacilli, streptococci and *Veillonella gazogenes* (Frape, 2004). The exact composition of the equine microflora is unclear, although it has been known that the ratio between anaerobes and aerobes was 1:1 (Garrett et al., 2002). The predominate lactic acid producing bacteria (LAB) in equine intestine are *Streptococcus bovis, Streptococcus equinus, Lactobacillus salivarius* and *L. mucosae* (Milinovich et al., 2006). Two phyla, low % guanine cytosine (GC) Gram-positive cocci and the cytophagia/flexibacter/bacteroides (CFB) group constitute about 92% of bacterial species in equine GI tract (Daly et al., 2001). The entire GI tract is colonized by lactobacilli, streptococci and lactate-utilizing bacteria (Frape, 2004).

**Guinea pig**

The GI flora of the guinea pig is similar to the rabbit which contains Gram-positive organisms (Navia and Hunt, 1976b). Coliforms, yeasts and clostridia are present in small numbers (Harkness et al., 2010). There are few coliform bacteria and enterococci in the stomach and small intestine. Gram-positive non-spore forming rods are the predominating flora in the upper section of the small intestine. In the stomach and duodenum-jejunum, the gram-positive rods which belong to grain types of lactobacilli can be seen (Crecelius and Rettger, 1943). The population of bacteria in the caecum is distinctly different from the bacteria in the small intestine. The spiral-shaped bacterium is the one not present in the small intestine. There are also abundant fusiform-type organisms as well as several types of rods and cocci (Knehans and O'dell, 1980). Anaerobic lactobacilli are the most common bacteria in the large intestine (Cheeke, 1987).
CHAPTER 3

Materials and Methods

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Three experiments were undertaken as part of this study. All experimentation was approved by The University of Queensland Production and Companion Animal Ethics Committee and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

3.1 Animals and their husbandry

Female IMVS (Institute of Medical and Veterinary Science) coloured guinea pigs, aged 4-7 months, were obtained from the Guinea Pig Unit at the Gatton Campus of The University of Queensland for use in this study. The guinea pigs were maintained under their normal management system until treatment was commenced. Prior to the commencement of treatment they were housed in the breeding facility in floor pens with ten female guinea pigs and 1 male guinea pig per pen. The floor pens were 140cm x 200cm in size with wood shaving bedding. A commercial pelleted diet formulated for guinea pigs was fed *ad libitum* with daily supplementation of fresh vegetables. In addition, vitamin C was added to the drinking water which was also available *ad libitum*. The room in which they were housed was well-ventilated and maintained at a temperature between 18°C and 21°C with artificial light for 12 hours daily. The animals were identified photographically and assigned a number. Large boxes were provided in each pen for the guinea pigs to use as a hide. The boxes were cleaned daily and the shavings in the pens were replaced weekly.
The guinea pigs were checked each day for oestrus as evidenced by the opening of the vaginal seal. Each female was left in the pen with the breeding male for 24 hours after oestrus was detected and then removed from the pen for 3 days. The day that the guinea pig was taken out of the pen was recorded as the day of ovulation, gestational day (GD) 0. Daily oestrus detection was continued after mating to ensure that pregnancy had occurred. Guinea pigs that returned to oestrus were re-mated. Those that did not return to oestrus were maintained in the original pen until the experiment was completed for Experiment 1; for Experiments 2 and 3 the guinea pigs remained in Guinea Pig Unit until GD21 and were then transferred to the Laboratory Animal Research Facility, approximately 500 metres from the Breeding Facility. The animals were transferred to the Laboratory Animal Research Facility in a ventilated guinea pig transfer box with wood shaving bedding. Each guinea pig was then maintained in a separate cage. Each cage was 85cm x 85cm and the cages were arranged in tiers of 2 cages. These cages had stainless steel mesh walls which allowed the guinea pigs to interact with the other guinea pigs in the unit. The flooring of the cages consisted of a solid plastic sheet with drainage holes to allow the ready passage of urine into the stainless steel trays beneath each pen. The pens were cleaned daily. In each pen a large diameter plastic cylinder was provided as a hide. As with the floor pens, water and guinea pig pellets were provided ad libitum with daily supplement of fresh vegetables and Vitamin C in the water.

3.2 Collection of Processionary Caterpillars

Processionary caterpillar (PC) nests were collected from trees on properties on the Darling Downs in Queensland during April 2011. At this time, the PCs were in their 8th larval stage, the last larval stage prior to leaving the nests to enter pre-pupal diapause in the ground (Floater and Zalucki, 1999). The nests were stored at 5°C overnight and the following morning they were opened and caterpillars, caterpillar frass (excrement) and exoskeleton that had been shed during the growth stages of the caterpillars were recovered. Caterpillars were separated, weighed and stored in 100 g aliquots at -20°C until required.
3.3 Assessment of pregnancy

In Experiment 1 following mating, the guinea pigs were checked daily for return to oestrus. Any guinea pigs that did not return to oestrus were examined at 21 days post-mating by transabdominal ultrasound (Esaote, Model 7300®) for pregnancy using a 5-10 MHz linear probe. The ventral abdomen was clipped and scanned on the left and the right side. Pregnancy was confirmed by detection of placentas and fetal heartbeats. The number of the fetuses and sites was determined. Once pregnancy was confirmed the females were matched according to gestational stage and allocated into either a treatment or control group. Thereafter the guinea pigs were examined daily from the day prior to the first treatment until completion of the experiment.

In Experiment 2 and Experiment 3, following transfer to the Laboratory Animal Research Facility the guinea pigs were allowed 2 days to habituate to the new conditions and were then examined for pregnancy via transabdominal ultrasound examination (Esaote, Model 7300®) on GD21 (Figure 3.1) and GD23, respectively. Once the guinea pigs were confirmed pregnant they were matched according to gestational age and allocated to either the treatment group or the control group (Appendix 1).

![Image](image.png)

**Figure 3.1: Experiment 2.** Guinea pigs were examined for pregnancy via transabdominal ultrasound examination (Esaote, Model 7300®) on GD21. Two fetuses were detected by the ultrasound (red arrows).
The guinea pigs were then had ultrasound examinations each morning either until the set time point for euthanasia or until fetal death was detected at which point the guinea pig was euthanased and samples collected.

3.4 Preparation and administration of Processionary caterpillars

Preliminary EAFL experiments on mares showed that 100g PC administrated to a 500kg mare for 5 days could induce abortion (Cawdell-Smith et al., 2010). Based on this dose rate, in Experiment 1, the guinea pigs were administered a whole PC dose of 0.2g/kg. This dose was increased to 1g/kg for the last 3 guinea pigs. Results from this study indicated that the higher dose of PC was required to induce abortion in the guinea pig. Therefore, in Experiment 2, 0.3g/kg of PC exoskeleton and integument without head and tail were used for the treatment group. The caterpillar was cut open and the internal organs, head and tail removed. The remaining exoskeleton and integument was cut into pieces and placed into gelatin capsules (size 5, 0.13ml). The capsules were prepared daily and used within 2 hours. The weight of the PC components in each capsule was determined by subtracting the weight of the empty capsule from that of the loaded capsule. In Experiment 3, 0.3g/kg of PC exoskeleton and integument with head and tail were used. The guinea pigs in the treatment groups were administered capsules containing PC exoskeleton and integument and the control guinea pigs were administered capsules containing the commercial guinea pig pellets fed in their normal diet. The capsules were prepared using the same procedure as Experiment 2, the only difference was whether the head and tail of PC remained or not. Prior to administration, the capsules were lubricated with vegetable oil to facilitate passage of the capsule in the oesophagus. The capsules were placed into the guinea pig's oropharynx using tweezers and the guinea pig’s throat was massaged to stimulate swallowing. This was followed by administration of 2-3ml of water into the oral cavity via syringe to ensure the guinea pig swallowed the capsules.
3.5 Experimental design and protocol

Experiment 1

In the initial experiment, the objective was to determine if the oral administration of Processionary Caterpillars *(Ochrogaster lunifer)* causes abortion in the guinea pig. Sixteen pregnant guinea pigs were matched according to gestational age and randomly allocated into two groups, the treatment group (n=9) and control group (n=6). The treatment regimen is outlined in Table 3.1. The initial treatments were administered in mid-gestation (GD35 to GD41) to correspond with the stage of gestation when EAFL occurs in mares. The first six GPs in the treatment group (GD35 - GD41) received 0.2g/kg of macerated processionary caterpillars in a single capsule, daily for 5 days. Their corresponding controls (n=4) received a single capsule containing commercial guinea pig feed. The remaining three guinea pigs in the treatment group (GD26-GD36) received two capsules containing a total dose of 1g/kg of macerated processionary caterpillar, daily for 5 days. The remaining control guinea pigs (n=2) were administered two capsules containing commercial guinea pig pellets daily for 5 days. All guinea pigs were monitored daily by clinical examination and fetal viability was monitored by trans-abdominal ultrasound until 3 days prior to term when they were moved into a separate pen for delivery. After delivery, the number of offspring and any gross abnormalities were recorded. One GP aborted and this GP was placed into a separate pen and the number of aborted fetuses recorded.
<table>
<thead>
<tr>
<th>Guinea Pig No.</th>
<th>Dose (g whole caterpillar/kg bodyweight)</th>
<th>Number of days of treatment (days)</th>
<th>Gestational age at 1st treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0 (1 capsule)</td>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td>14</td>
<td>0 (1 capsule)</td>
<td>5</td>
<td>41</td>
</tr>
<tr>
<td>15</td>
<td>0 (1 capsule)</td>
<td>5</td>
<td>35</td>
</tr>
<tr>
<td>19</td>
<td>0 (1 capsule)</td>
<td>5</td>
<td>38</td>
</tr>
<tr>
<td>9</td>
<td>0 (2 capsules)</td>
<td>5</td>
<td>31</td>
</tr>
<tr>
<td>27</td>
<td>0 (2 capsules)</td>
<td>5</td>
<td>36</td>
</tr>
<tr>
<td><strong>Treated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>0.2g/kg (1 capsule)</td>
<td>5</td>
<td>39</td>
</tr>
<tr>
<td>32</td>
<td>0.2g/kg (1 capsule)</td>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>0.2g/kg (1 capsule)</td>
<td>5</td>
<td>39</td>
</tr>
<tr>
<td>12</td>
<td>0.2g/kg (1 capsule)</td>
<td>5</td>
<td>41</td>
</tr>
<tr>
<td>20</td>
<td>0.2g/kg (1 capsule)</td>
<td>5</td>
<td>35</td>
</tr>
<tr>
<td>28</td>
<td>0.2g/kg (1 capsule)</td>
<td>5</td>
<td>38</td>
</tr>
<tr>
<td>5</td>
<td>1g/kg (2 capsules)</td>
<td>5</td>
<td>31</td>
</tr>
<tr>
<td>10</td>
<td>1g/kg (2 capsules)</td>
<td>5</td>
<td>36</td>
</tr>
<tr>
<td>7</td>
<td>1g/kg (2 capsules)</td>
<td>5</td>
<td>26</td>
</tr>
</tbody>
</table>
Experiment 2

Based on the findings of the first experiment, the objective of the second experiment was to confirm that abortion could be induced by administering PC to guinea pigs at GD25.

Twelve pregnant guinea pigs were used for this study. They were matched according to gestational stage and randomly allocated to a treatment group (n=8) or a control group (n=4). Guinea pigs in the treatment group were administrated 0.3g/kg PC exoskeleton and integument without head and tail in gelatine capsules daily from GD25 for 5 days (n=6) except for two guinea pigs that underwent scheduled euthanasia prior to the completion of the 5 days, one at GD 26 (1 treatment) and one at GD28 (3 treatments). The final doses had a mean of $0.39 \pm 0.01$g/kg BW of PC components. The control group did not receive PC exoskeleton. Each guinea pig underwent daily transabdominal ultrasound examination to monitor the pregnancies. If fetal death was observed on ultrasound examination (n=2; GD31, 55) the guinea pig was anaesthetized and sampled as described below. If fetal death was not detected the guinea pigs were anaesthetised and sampled at a set time point from the first day of treatment (n=6; GD26, 28, 40, 45, 50, 60) (Table 3.2). The control guinea pigs were sampled at set time points (n=4; GD29, 40, 50, 60).

<table>
<thead>
<tr>
<th>Guinea pig number</th>
<th>Number of days of treatment (days)</th>
<th>Sampling day (gestation day)</th>
<th>Days from the 1st treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>1</td>
<td>26</td>
<td>1</td>
</tr>
<tr>
<td>93</td>
<td>3</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td>56</td>
<td>5</td>
<td>31 (Fetal death)</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>40</td>
<td>15</td>
</tr>
<tr>
<td>78</td>
<td>5</td>
<td>45</td>
<td>20</td>
</tr>
<tr>
<td>21</td>
<td>5</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>29</td>
<td>5</td>
<td>55 (Fetal death)</td>
<td>30</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>60</td>
<td>35</td>
</tr>
</tbody>
</table>
**Experiment 3**

The objective of experiment 3 was to investigate the histopathological and microbiological outcomes of pregnancies over a greater range of set time points following exposure to PC from GD25.

Twenty pregnant guinea pigs were used for this study and they were allocated to a treatment group (n=14) or a control group (n=6). Based on the results from Experiment 2, some adjustments were made to the methodology for this experiment. Guinea pigs in the treatment group were administrated 0.3g/kg BW PC exoskeleton and integument with head and tail in gelatine capsules daily on 3 consecutive days starting on GD25 (average 0.31 ± 0.01g/kg BW). The control group did not receive any PC exoskeleton. Pregnancy was monitored by transabdominal ultrasound as previously described. The guinea pigs were anaesthetized and sampled at a set time point from the first day of treatment (n=11; GD26, 27, 28, 29, 32, 33, 34, 35, 37, 39, 46) or if fetal death was observed by ultrasound (n=3; GD30, 31, 31) (Table 3.3). The control guinea pigs were also sampled at set time points (n=6; GD26, 28, 30, 33, 37, 46). The samples for microbiology and histopathology were collected as described below.
Table 3.3: Experiment 3. Administration days of PC exoskeleton and sampling days

<table>
<thead>
<tr>
<th>Guinea pig number</th>
<th>Number of days of treatment (days)</th>
<th>Sampling day (gestation days)</th>
<th>Days from the 1st treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>119</td>
<td>1</td>
<td>26</td>
<td>1</td>
</tr>
<tr>
<td>114</td>
<td>2</td>
<td>27</td>
<td>2</td>
</tr>
<tr>
<td>118</td>
<td>3</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>29</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>30 (Fetal death)*</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>31 (Fetal death)*</td>
<td>6</td>
</tr>
<tr>
<td>107</td>
<td>3</td>
<td>31 (Fetal death)*</td>
<td>6</td>
</tr>
<tr>
<td>109</td>
<td>3</td>
<td>32</td>
<td>7</td>
</tr>
<tr>
<td>106</td>
<td>3</td>
<td>33</td>
<td>8</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>34</td>
<td>9</td>
</tr>
<tr>
<td>102</td>
<td>3</td>
<td>35</td>
<td>10</td>
</tr>
<tr>
<td>104</td>
<td>3</td>
<td>37</td>
<td>12</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>39</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>46</td>
<td>21</td>
</tr>
</tbody>
</table>

*: abortion or fetal death was detected on ultrasound examination. Therefore, the sampling day was different with planned sampling day.

3.6 Monitoring

The guinea pigs were monitored twice daily (8:00 and 16:00) by evaluating appearance, clinical signs (body temperature, appetite and faeces) and behaviour. The number and viability of the fetuses (movement and fetal heart beat) were confirmed via transabdominal ultrasound (Esaote, Model 7300®) as described above. The guinea pigs in the treatment group were examined by ultrasound daily once the treatment commenced and those in the control group every second day until sampling.
If fetal death, abortion or other unusual signs were observed, the guinea pig was anaesthetized and sampled. If no fetal death, abortion or other unusual signs were observed, the guinea pig was anaesthetized and sampled at a set time point from the first day of treatment in experiments 2 and 3. In experiment 1 the guinea pigs were allowed to carry their pregnancies to term.

3.7 Collection of samples

In Experiment 1, one guinea pig aborted. Uterine swabs were taken at necropsy following euthanasia of this guinea pig. Samples for microbiology and histopathology were taken as described in following. The remaining guinea pigs delivered normal guinea pigs at term and no samples were taken.

In Experiment 2 and 3, the guinea pigs were anaesthetised for sample collection. Prior to anaesthesia, the abdominal area was clipped and excess hair removed. The guinea pigs were anaesthetized with gaseous isoflurane using an Ohmeda Isotec anaesthetic machine. When the guinea pig achieved a surgical plane of anaesthesia, heart blood was collected by cardiac puncture and the guinea pig was then euthanized using intra-cardiac administration of 2 ml of Lethabarb (Virbac (Australia) Pty Ltd) (29.7%, sodium pentobarbital). The guinea pig was placed on its back and the abdomen was prepared for necropsy. The abdomen skin was wiped with 70% alcohol, followed by povidone iodine applied twice in order to minimise contamination. The abdominal cavity was opened using sterile instruments and the peritoneal cavity was swabbed using a sterile transport swab (FL medical Amies swabs). The uterus was visualized, removed and placed in a sterile petri dish. The number of fetuses in each horn was then determined. Fetuses were identified by a defined numbering system relating to position in each of the uterine horns. Fetuses in the left uterine horn were designated the letter ‘L’ and a number with the numbers starting from 1 for the fetus closest to the ovary. The fetus in the left uterine horn and closest to the ovary was numbered ‘L1’ with the other fetuses in that horn being numbered sequentially such that the fetus closest to the cervix had the highest number. Thus, if there were 3 fetuses in the left horn the fetuses were numbered L1, L2 and L3 with the fetus closest to the cervix being numbered ‘L3’. The same numbering system was applied to fetuses in the right uterine horn with the prefix letter being ‘R’.
The two uterine horns were incised separately at a site where there was no placental attachment and samples were taken for bacteriology. The uterine horns were then opened and each placenta was removed from the placental site and the junctional zone was sampled. The fetuses, within their amnionic sacs and still attached to the placenta by the umbilical cord were then removed and placed into separate sterile Petri-dishes. Amnionic fluid was collected aseptically from each fetoplacental unit using a 1ml syringe and 25G needle. The fetus was then necropsied as described in Appendix 2 and stomach contents were collected for bacteriology using a 1ml syringe and 25G needle.

Tissue was collected aseptically from each of the uterine horns, and all placentas, fetal lungs and liver were placed into brain heart infusion broth (BHIB). The fluid samples collected were heart blood, amnionic fluid and fetal stomach contents. Following collection of samples for microbiology, the entire gastrointestinal tract, spleen, liver, lung and heart from the mother and the remainder of the fetuses with the attached placenta were placed in 10% formal saline for histopathological examination (Table 3.4).

In addition, in experiment 3, one small piece of colon, caecum, placenta and implantation site were fixed in 3% glutaraldehyde 0.1M sodium cacodylate buffer for transmission electron microscope (TEM) and light microscope (LM) examination (Table 3.5).

### Table 3.4 Sites for samples taken from dam and fetus for bacteriology

<table>
<thead>
<tr>
<th>Swab</th>
<th>Fluid</th>
<th>BHIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peritoneum</td>
<td>Heart blood</td>
<td>Placenta</td>
</tr>
<tr>
<td>Left uterine horn</td>
<td>Heart blood</td>
<td>Uterine horn</td>
</tr>
<tr>
<td>Right uterine horn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal Samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Junctional zone</td>
<td>Amnionic fluid</td>
<td>Lung</td>
</tr>
<tr>
<td>Placenta</td>
<td>Stomach contents</td>
<td>Liver</td>
</tr>
</tbody>
</table>
Table 3.5 Sites for samples taken from dam and fetus for TEM and histopathological examination

<table>
<thead>
<tr>
<th>Maternal Samples</th>
<th>TEM</th>
<th>Histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Implantation site</td>
<td>Uterine horns</td>
<td></td>
</tr>
<tr>
<td>Placenta</td>
<td>Placenta</td>
<td></td>
</tr>
<tr>
<td>Caecum</td>
<td>Lung</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>Gastrointestinal tract</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td></td>
</tr>
<tr>
<td>Fetal Samples</td>
<td>Lung</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Remainder of fetus</td>
<td></td>
</tr>
</tbody>
</table>

3.8 Sample processing for microbiology

In Experiment 1, uterine swabs were streaked onto horse blood agar (HBA) and McConkey’s agar (MAC). In Experiment 2, fluid samples and swabs collected (Table 3.3) from the dam and each fetus were streaked onto 2 plates each of horse blood agar (HBA), McConkey’s agar (MAC) and chocolate agar (CHOC). In Experiment 3, the same fluid samples and swabs were collected and streaked onto horse blood agar (HBA) and McConkey’s agar (MAC) in the same manner. After being streaked onto the culture plates, the swabs and remaining fluid were incubated in brain-heart infusion broth (BHIB). One of each of the different culture plates was incubated aerobically and one anaerobically at 37°C for 24 h. The samples in BHIB bottles were incubated aerobically at 37°C. After 24 h, all the plates and BHIB bottles were checked for evidence of bacterial growth and the details recorded. The plates were returned to the same environmental conditions (aerobic or anaerobic) and incubated for a further 24 hours. If the BHIB bottles were cloudy, a sample was taken using a sterile loop and streaked onto HBA, MAC and CHOC culture plates in experiment 2 and HBA and MAC plates in experiment 3. The plates were then incubated aerobically and anaerobically at 37°C for 24 hours and the BHIB samples were returned to the incubator for another 24 hours. After 48 hours incubation, all the plates with evidence of bacterial growth and any BHIB
bottles with evidence of bacterial growth were sealed, refrigerated and sent to the laboratory for identification of bacteria.

Isolates were identified using standard bacterial identification techniques. Gram-negative oxidase-negative and some oxidase-positive bacteria were further identified using API 20NE or 20E kits (BiomerieuxAustralia Pty Ltd, Baulkham Hills, New South Wales, Australia). *Streptococcus* spp. were classified according to haemolytic patterns, Lancefield groupings and/or API rapid *Streptococcus* kit (Biomerieux). Any bacteria isolates identified as Gram-positive coryneform-like rods were identified using API Coryne kits (Biomerieux). One isolate was further speciated using partial 16SrRNA sequencing and compared to the GenBank® database using BLASTN® (National Centre for Biotechnology Information, Bethesda, MD, USA) for identification to species (≥99% sequence homology) or genus level (≥97% sequence homology).

3.9 Transmission Electron Microscope (TEM) and Light Microscope (LM)

Samples from the dam and the fetus were fixed in 3% glutaraldehyde in 0.1M cacodylate buffer PH7.2 at the time of collection. The samples were rinsed in a buffer then fixed in osmium tetroxide, rinsed with water, dehydrated, infiltrated with resin followed by resin polymerization, and then fixed in epon. The samples were then cut using an ultracut ultramicrotome. The samples were then transferred to the Centre for Microscopy and Microanalysis (CMM) laboratory at St. Lucia campus. The samples were processed as detailed in Appendix 3.

3.10 Statistical analysis

Statistical analysis was performed on the results from all 3 experiments. Differences between the treated and control groups were analysed using a Fisher’s exact chi-square test using Minitab software (Minitab® 16). The P value for judging significance is 0.05.
CHAPTER 4

Results

4.1 Clinical findings and ultrasonography

Abnormal clinical signs were seen in a few guinea pigs in each of the three experiments. These included weight loss, respiratory dyspnea and inappetance.

Experiment 1

In Experiment 1, only one guinea pig (GP7) aborted. None of the guinea pigs treated from GD31 or later aborted, irrespective of the dose of PC. The guinea pig that did abort was one of the three guinea pigs treated with 1g/kg BW PC for 5 days. Treatment of this guinea pig commenced on GD26. While this guinea pig was compliant with treatment for the first 2 doses, she resisted treatment on the last 3 days and was agitated following treatment. On the last day of treatment this guinea pig became inappetant and began to lose weight. On the morning of GD32, 6 days after the first treatment she was noted to have a haemorrhagic vaginal discharge. Two placentas were retrieved from the cage but no fetuses were obtained. The other two guinea pigs (GP5 & GP10) which were treated with the higher dose treatment also resented taking the capsules after the initial treatment, showing some agitation such as scratching and subsequently lost weight. Neither of these GPs aborted.
Experiment 2

In Experiment 2, the treatment group received PC exoskeleton and integument without head and tail at dose 0.3g/kg BW for 1-5 days. Fetal death occurred in 2 (GP56 & GP29) of the 8 treated guinea pigs at 31 and 55 days of gestation respectively. Both guinea pigs had completed the 5 days treatments prior to the detection of fetal death. Fetal death was detected on ultrasound examination in GP56 (Figure 4.1); however fetal death was determined in GP29 at the time of scheduled euthanasia on GD55. Sampling of GP56 was carried out immediately after fetal death was detected on ultrasound.

![Figure 4.1](image)

**Figure 4.1: Experiment 2.** Fetal death was detected on ultrasound examination on GD31 in GP56 which was treated with 0.3g/kg PC for 1-5 days. Red square: fetus. Yellow arrow: no heartbeat detected.

GP56 showed obvious clinical signs on GD31 after five-day treatments. The mother showed signs of clinical illness with dyspnoea and anorexia. There was evidence of fetal death on ultrasound. GP29 was scheduled to be sampled at GD55 and one dead fetus was found on sample day. Before the dissection, ultrasound was failed to detect this dead fetus. There were no abnormal clinical signs seen in this guinea pig. None of the other treated or control guinea pigs showed any obvious clinical signs prior to sampling. Thus, 2 out of 8 (25.0%) treated guinea pigs had abnormal clinical outcomes (Table 4.1) but only one of these guinea pigs showed overt clinical signs of disease.
Table 4.1: **Experiment 2.** Outcome of pregnancies in guinea pigs treated with PC exoskeleton and integument without head and tail at dose 0.3g/kg daily for 1 to 5 days from GD25.

<table>
<thead>
<tr>
<th>Guinea pig No.</th>
<th>Administration (days)</th>
<th>1st treatment (days)</th>
<th>Euthanasia (days)</th>
<th>1st treatment to fetal death</th>
<th>1st treatment to abortion</th>
<th>Outcome of Pregnancies</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP12</td>
<td>1</td>
<td>25</td>
<td>26</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>GP93</td>
<td>3</td>
<td>25</td>
<td>29</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>GP56</td>
<td>5</td>
<td>25</td>
<td>31</td>
<td>6</td>
<td>-</td>
<td>Dead fetuses</td>
</tr>
<tr>
<td>GP6</td>
<td>5</td>
<td>25</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>GP78</td>
<td>5</td>
<td>25</td>
<td>45</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>GP21</td>
<td>5</td>
<td>25</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>GP29</td>
<td>5</td>
<td>25</td>
<td>55</td>
<td>#</td>
<td>-</td>
<td>Dead fetus</td>
</tr>
<tr>
<td>GP1</td>
<td>5</td>
<td>25</td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
</tbody>
</table>

*Normal fetuses

#Unknown
Experiment 3

In Experiment 3, the treatment group received 0.3g/kg BW of PC exoskeleton and integument with head and tail for 1-3 days. Seven out of 14 (50.0%) treated guinea pigs with abnormal outcomes (Table 4.2). GP5 and GP107 aborted on GD30 and GD31 respectively. Fetal death was not picked up before abortion. Ultrasound examination of GP1 on GD31 could not detect any heartbeats and all fetuses were found to be dead at necropsy. GP15 showed obvious respiratory dyspnea on GD34, 9 days after the initial treatment. Ultrasound examination picked up three heartbeats but did not detect the lack of heartbeat in the fetus that was dead at necropsy.

Table 4.2: Experiment 3: Outcome of pregnancies in guinea pigs treated with Processionary caterpillar exoskeleton daily for up to 3 days from GD25.

<table>
<thead>
<tr>
<th>Guinea pig No.</th>
<th>Administration time (days)</th>
<th>Gestational Age</th>
<th>Days from 1st treatment</th>
<th>Outcome of Pregnancies</th>
</tr>
</thead>
<tbody>
<tr>
<td>119</td>
<td>1</td>
<td>25</td>
<td>26</td>
<td>*</td>
</tr>
<tr>
<td>114</td>
<td>2</td>
<td>25</td>
<td>27</td>
<td>*</td>
</tr>
<tr>
<td>118</td>
<td>3</td>
<td>25</td>
<td>28</td>
<td>Abnormal placenta</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>25</td>
<td>29</td>
<td>*</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>25</td>
<td>30</td>
<td>Unknown 5 Aborted</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>25</td>
<td>31</td>
<td>6 Dead fetuses</td>
</tr>
<tr>
<td>107</td>
<td>3</td>
<td>25</td>
<td>31</td>
<td>Unknown 6 Aborted</td>
</tr>
<tr>
<td>109</td>
<td>3</td>
<td>25</td>
<td>32</td>
<td>*</td>
</tr>
<tr>
<td>106</td>
<td>3</td>
<td>25</td>
<td>33</td>
<td>8 Dead fetuses</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>25</td>
<td>34</td>
<td>Unknown Dead fetus</td>
</tr>
<tr>
<td>102</td>
<td>3</td>
<td>25</td>
<td>35</td>
<td>*</td>
</tr>
<tr>
<td>104</td>
<td>3</td>
<td>25</td>
<td>37</td>
<td>*</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>25</td>
<td>39</td>
<td>Unknown 1 Dead fetus</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>25</td>
<td>46</td>
<td>*</td>
</tr>
</tbody>
</table>

* Normal fetuses
4.2 Gross pathology

In Experiment 1, GP7 was euthanased and necropsied after abortion. When the abdomen was cut open, distended small intestine was observed. The caecum was very dark in colour and there was a red, paste-like ingesta.

In Experiment 2, a necropsy was performed to GP56 immediately after fetal death was detected by ultrasound. The uterus was congested (Figure 4.2a) and a dark red discharge was observed in the uterine cavity. The surface of gastrointestinal tract was hyperaemic (Figure 4.2b). All three fetuses were dead.
Figure 4.2: Experiment 2. GP56, treated with 0.3g/kg PC components for 5 days from GD25 and euthanased 7 days (GD31) after the first day of treatment.
a) Exteriorised uterus with evidence of congestion.
b) The surface of GI tract was hyperaemic.
After the abdomen was opened, the surface of GI tract of GP21 was bright red and hyperaemic (Figure 4.3ab). It was euthanased on GD50 and had two normal fetuses. The peritoneal cavity swab was taken for microbiological examination and bacteria were then isolated from this site.

Figure 4.3: Experiment 2. GP21 was euthanased on GD50 following 5 days of treatment (0.3g/kg) from GD25. Bacteria were isolated from the peritoneal cavity.

a) The surface of GI tract was bright red and hyperaemic
b) Enlarged view of the GI tract.

Guinea pig 29 had one dead fetus (of 3) in the left uterine horn adjacent to the cervix. (Figure 4.4) This fetus had a thickened umbilical cord and amnion. There was also a greenish mucoid discharge in the left uterus where the dead fetus was found. There were 2 normal fetuses in the left horn. No fetuses were found in the right uterine horn.

Figure 4.4: Experiment 2. GP29, euthanased on GD55 following 5 days of treatment from GD25. One dead fetus (L3) with haemorrhagic amnionic fluid was found. The size of this dead fetus was much smaller than the other two normal fetuses.
In Experiment 3, GP1 was necropsied immediately after ultrasound examination failed to pick up any heart beats. Three dead fetuses were found. In GP15, after the uterus was opened, a haemorrhagic discharge was observed in both uterine horns. There were 3 normal fetuses and one small, dead fetus (Figure 4.5a) with a small dense placenta. The placenta of this dead fetus was dark red in colour and differed from the other placentae. No fetuses were observed in the left uterine horn. GP8 had similar fetal outcomes to GP15 with three normal fetuses and one dead small fetus near the cervix (Figure 4.5b and Figure 4.5c). A comparison of the size of these two dead fetuses with the normal fetus sampled at 26 days of gestation (Figure 4.5d), indicates that these fetuses died at 26 days of gestation or soon after, 1 day after the first treatment with caterpillars.

Figure 4.5: Experiment 3. GP15 and GP8 were euthanased on GD34 and GD39 respectively following 3 days of treatment with PC commencing GD25.

a) GP15: one small dead fetus with a small dense placenta
b) GP 8: dead small fetus (black arrow) compared with normal fetus in the same uterine horn (blue arrow)
c) GP 8: one dead small fetus near cervix
d) Normal fetus from control group euthanased on GD26
GP 119 which underwent scheduled euthanasia on GD26 had caecal distension (Figure 4.6a and Figure 4.6b) and is compared with normal GIT of a guinea pig from the control group (Figure 4.6c). This guinea pig had three normal fetuses (two on the right uterine horn, one on the left side). The left fetus had an abnormal implantation site near the cervix which had extensive mucoid discharge (Figure 4.7). All the fetuses were normal.

**Figure 4.6: Experiment 3.** GP119 showed caecal distension after the abdominal cavity was opened on GD26 (1 day after 1st treatment).

a) Caecal distension  
b) Caecal distension as above  
c) Normal GIT from control group
Figure 4.7: Experiment 3. GP119 had an abnormal implantation site (black arrow) which had extensive mucoid discharge on GD26 (one day after 1st treatment). This guinea pig had three normal fetuses (two on the right uterine horn and one on the left).

GP106 had two normal fetuses in the left uterine horn and one implantation site with no placenta or fetus near the cervix (Figure 4.8).

Figure 4.8: Experiment 3 GP106 had two normal fetuses on the left uterine horn and one implantation site with no placenta or fetus (black arrow) on GD33 after 3-day treatment with PC exoskeleton. Adjacent to this abnormal implantation site, a normal implantation site which was evident after the placenta was removed (blue arrow).
GP 118 had one abnormal placenta with a normal fetus. It was half grey and half dark red and hemorrhagic (Figure 4.9a). A normal placenta from a control guinea pig of the same gestation stage is shown in Figure 4.9b.

Figure 4.9: Experiment 3. Placentas from two guinea pigs (GP118 and GP112) both euthanased at GD28. Guinea pig 118 was treated with PC for 3 days from GD25 and GP112 was from the control group.

a) Abnormal placenta from GP118. Note that one half of the placenta was grey and the other was dark red with distended vasculature.

b) Normal placenta from GP112 (control group) euthanased on GD28.

4.3 Microbiology

Bacteria isolated from treated guinea pigs in these three experiments were predominantly enteric or environmental bacteria similar to those isolated in the horses.

In Experiment 1, uterine swabs from the one guinea pig that aborted grew bacteria on culture. The bacteria isolated were identified as Corynebacterium spp..

In Experiment 2, bacteria were isolated from all treated guinea pigs except one (GP12) which was euthanased 24 hours after receiving a single dose of PC exoskeleton. A range of environmental bacteria were isolated from both the dams and fetuses. The majority of isolated bacteria belonged to three species: Staphylococcus spp., Corynebacterium spp. and anaerobes (Table 4.3). The uterus was the most frequently affected site followed by the placenta, peritoneal cavity, amnionic fluid and junctional zone (Table 4.4). No bacteria were isolated from maternal heart blood or fetal stomach.
contents of any of the animals (Table 4.5). However, some bacteria were identified as contaminants. Bacteria were also isolated from one control guinea pig (GP2), which were identified as contaminants.

**Table 4.3: Experiment 2.** Site and category of bacteria isolated from treated GPs

<table>
<thead>
<tr>
<th>GP No.</th>
<th>Site</th>
<th>Bacteria</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Uterine horn</td>
<td><em>Staphylococcus aureus</em></td>
<td>Environmental/ Human/Animal</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Corynebacterium glucuolyticum</em></td>
<td>Environmental</td>
</tr>
<tr>
<td>R1</td>
<td>Junctional zone</td>
<td><em>Corynebacterium glucuolyticum</em></td>
<td>Environmental</td>
</tr>
<tr>
<td>93</td>
<td>R3 Placenta</td>
<td><em>Flavimonas oryzhabitans</em></td>
<td>Environmental</td>
</tr>
<tr>
<td>56</td>
<td>L2 Placenta</td>
<td><em>Staphylococcus aureus</em></td>
<td>Environmental/ Human/Animal</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Corynebacterium glucuolyticum</em></td>
<td>Environmental</td>
</tr>
<tr>
<td>R1</td>
<td>Placenta</td>
<td><em>Staphylococcus aureus</em></td>
<td>Environmental/ Human/Animal</td>
</tr>
<tr>
<td></td>
<td>Uterine discharge</td>
<td><em>Staphylococcus aureus</em></td>
<td>Human/Animal</td>
</tr>
<tr>
<td>R2</td>
<td>Amnionic fluid</td>
<td>*Corynebacterium renale group/</td>
<td>Environmental</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Corynebacterium accolens</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peritoneal cavity</td>
<td><em>Corynebacterium Group G</em></td>
<td>Environmental</td>
</tr>
<tr>
<td>21</td>
<td>Peritoneal cavity</td>
<td><em>Corynebacterium Group G</em></td>
<td>Environmental</td>
</tr>
<tr>
<td>29</td>
<td>L3 Amnionic fluid</td>
<td>Anaerobe</td>
<td>Enteric</td>
</tr>
<tr>
<td></td>
<td>L3 Placenta</td>
<td>Anaerobe</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Uterine discharge</td>
<td>Anaerobe</td>
<td></td>
</tr>
</tbody>
</table>

Environmental/Human/Animal: Bacteria that commonly found in the environment and on human and animal skin.
Environmental: Bacteria that commonly found in nature such as soil, water and plant.
Enteric: Bacteria that commonly found in the guinea pig’s gastrointestinal tract.
Human/Animal: Bacteria found only on human and animal skin – considered to be a contaminant in these studies.
Table 4.4: Experiment 2. Sites at which bacteria were isolated from the treated guinea pigs. The value represents the frequency of isolation of each type of bacteria as a percentage of the total number of sites from which bacteria were isolated.

<table>
<thead>
<tr>
<th>Bacteria Isolates</th>
<th>Uterus</th>
<th>Amnionic fluid</th>
<th>Placenta</th>
<th>Junctional zone</th>
<th>Peritoneum</th>
<th>Heart blood</th>
<th>Stomach contents</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus</em> spp.</td>
<td>25.0%</td>
<td>0</td>
<td>9.5%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Corynebacterium</em> spp.</td>
<td>12.5%</td>
<td>4.8%</td>
<td>4.8%</td>
<td>4.8%</td>
<td>12.5%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anaerobes</td>
<td>12.5%</td>
<td>4.8%</td>
<td>4.8%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Other bacteria</td>
<td>0</td>
<td>0</td>
<td>4.8%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.5: Experiment 2. The sites from which bacteria were isolated as a percentage of the total number of sites from which bacteria were isolated.

<table>
<thead>
<tr>
<th>Uterus</th>
<th>Placenta</th>
<th>Peritoneal cavity</th>
<th>Amnionic fluid</th>
<th>Junctional zone</th>
<th>Heart Blood</th>
<th>Stomach Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>50.0%</td>
<td>23.8%</td>
<td>12.5%</td>
<td>9.5%</td>
<td>4.8%</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

In Experiment 3, bacteria were isolated from 6 of 14 treated guinea pigs. A range of environmental and enteric bacteria were isolated from both the dams and fetuses (Table 4.6 and Table 4.7). The majority of isolated bacteria belonged to four species: *Staphylococcus* spp., *Corynebacterium* spp., *Streptococcus* spp. and Gram-Negative Bacteria (Table 4.8). Some bacteria were identified as contaminates. The placenta was the most frequently affected site followed by uterus, amnionic fluid, peritoneum and heart blood (Table 4.9).

Bacteria were also isolated from some of the control group. All of the isolated bacteria from these animals are environmental or normal flora from human or animal skin (Table 4.10).
### Table 4.6: Experiment 3. Category of the isolated bacteria from treated guinea pigs

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>Erysipelothrix rusiopathiae</td>
</tr>
<tr>
<td><em>Enterococcus faecium</em></td>
<td>Cellulomonas</td>
</tr>
<tr>
<td>(Actinobacteria)</td>
<td><em>Staphylococcus</em></td>
</tr>
<tr>
<td><em>Staph sciuri</em></td>
<td><em>Staphylococcus</em></td>
</tr>
<tr>
<td><em>Staph intermedius poss.</em></td>
<td><em>Staphylococcus</em></td>
</tr>
<tr>
<td><em>Staph cohnii poss.</em></td>
<td><em>Staphylococcus</em></td>
</tr>
<tr>
<td><em>Corynebacterium</em></td>
<td><em>Pasteurella pneumotropica</em></td>
</tr>
<tr>
<td><em>Cellulomonas</em></td>
<td><em>Brevibacterium</em></td>
</tr>
</tbody>
</table>

### Table 4.7: Experiment 3. Site and category of which bacteria isolated from treated GPs

<table>
<thead>
<tr>
<th>GP No.</th>
<th>Site</th>
<th>Bacteria</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>109</td>
<td>L1 Placenta</td>
<td><em>Enterococcus faecium</em></td>
<td>Enteric</td>
</tr>
<tr>
<td></td>
<td>L2 Placenta</td>
<td><em>Enterococcus faecium</em></td>
<td>Enteric</td>
</tr>
<tr>
<td></td>
<td>L2 Placenta</td>
<td><em>Enterococcus faecium</em></td>
<td>Enteric</td>
</tr>
<tr>
<td></td>
<td>R2 Placenta</td>
<td><em>Erysipelothrix rusiopathiae</em></td>
<td>Environmental/</td>
</tr>
<tr>
<td></td>
<td>Left uterus</td>
<td><em>Erysipelothrix rusiopathiae</em></td>
<td>Human/Animal</td>
</tr>
<tr>
<td></td>
<td>Right uterus</td>
<td><em>Erysipelothrix rusiopathiae</em></td>
<td>Human/Animal</td>
</tr>
<tr>
<td>5</td>
<td>Placenta 1#</td>
<td><em>Escherichia coli</em></td>
<td>Enteric</td>
</tr>
<tr>
<td></td>
<td>Placenta 2#</td>
<td><em>Staphylococcus</em> sp. poss.</td>
<td>Human/Animal</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>intermedius</em></td>
<td></td>
</tr>
<tr>
<td>102</td>
<td>Heart blood</td>
<td><em>Staphylococcus</em> sp. poss.</td>
<td>Human/Animal</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>hominis</em></td>
<td></td>
</tr>
<tr>
<td>106</td>
<td>L2 Placenta</td>
<td><em>Cellulomonas</em></td>
<td>Environmental/Human/Animal</td>
</tr>
<tr>
<td>15</td>
<td>R2 Stomach Content</td>
<td><em>Staphylococcus</em> sp. poss.</td>
<td>Human/Animal</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>cohnii</em></td>
<td></td>
</tr>
<tr>
<td>119</td>
<td>Peritoneum</td>
<td><em>Pasteurella pneumotropica</em></td>
<td>Human/Animal</td>
</tr>
<tr>
<td></td>
<td>R1 placenta</td>
<td><em>Brevibacterium</em> sp.</td>
<td>Human/Animal</td>
</tr>
</tbody>
</table>

#GP5 aborted and the placentae were recovered from the cage so their implantation site could not be determined.

Environmental/Human/Animal: Bacteria that commonly found in the environment and on human and animal skin.

Environmental: Bacteria that commonly found in nature such as soil, water and plant.

Enteric: Bacteria that commonly found in the guinea pig’s gastrointestinal tract.

Human/Animal: Bacteria found only on human and animal skin – considered to be a contaminant in these studies.
Table 4.8: **Experiment 3.** Sites from which bacteria were isolated from the treated guinea pigs. The value represents the frequency of isolation of each type of bacteria from the given site as a percentage of the total number of sites tested across all treated guinea pigs.

<table>
<thead>
<tr>
<th>Bacteria Isolates</th>
<th>Uterus</th>
<th>Amnionic fluid</th>
<th>Placenta</th>
<th>Junctional zone</th>
<th>Peritoneum</th>
<th>Heart blood</th>
<th>Stomach contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus spp.</td>
<td>0</td>
<td>0</td>
<td>6.3%</td>
<td>0</td>
<td>0</td>
<td>7.1%</td>
<td>0</td>
</tr>
<tr>
<td>Corynebacterium spp.</td>
<td>7.1%</td>
<td>0</td>
<td>4.2%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus spp.</td>
<td>0</td>
<td>0</td>
<td>8.5%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gram-Negative Bacteria</td>
<td>0</td>
<td>0</td>
<td>2.1%</td>
<td>0</td>
<td>7.1%</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.9: **Experiment 3.** The sites from which bacteria were isolated as a percentage of the number of sites from total number of sites tested across all treated guinea pigs.

<table>
<thead>
<tr>
<th>Uterus</th>
<th>Placenta</th>
<th>Peritoneal cavity</th>
<th>Amnionic fluid</th>
<th>Junctional zone</th>
<th>Heart Blood</th>
<th>Stomach Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.3%</td>
<td>14.9%</td>
<td>7.1%</td>
<td>0</td>
<td>0</td>
<td>7.1%</td>
<td>2.1%</td>
</tr>
</tbody>
</table>

Table 4.10: **Experiment 3.** Bacteria isolated from control group

<table>
<thead>
<tr>
<th>GP No.</th>
<th>Site</th>
<th>Bacteria</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>176</td>
<td>Heart blood</td>
<td><em>Leifsonia aquatica</em></td>
<td>Human/Animal</td>
</tr>
<tr>
<td></td>
<td>L2 Stomach Contents</td>
<td><em>Staphylococcus saprophyticus</em></td>
<td>Human/Animal</td>
</tr>
<tr>
<td>116</td>
<td>R2 Placenta</td>
<td><em>Staphylococcus intermedius</em> or <em>cohnii</em></td>
<td>Human/Animal</td>
</tr>
<tr>
<td>112</td>
<td>L2 Stomach Contents</td>
<td><em>Staphylococcus warneri</em> or <em>intermedius</em></td>
<td>Human/Animal</td>
</tr>
<tr>
<td></td>
<td>R1 Stomach Contents</td>
<td><em>Staphylococcus capitis</em> (42%)</td>
<td>Human/Animal</td>
</tr>
<tr>
<td>113</td>
<td>R3 Junctional zone</td>
<td><em>Micrococcus sp.</em></td>
<td>Human/Animal</td>
</tr>
</tbody>
</table>
4.4 Histopathology

Samples from maternal lung, heart, liver, kidney, spleen, gastrointestinal tract and each uterine horn and each fetus and placenta were taken for histopathological examination. Setal fragments have been identified throughout the GIT, uterus, fetoplacental unit and other organs in horses treated experimentally with PC. They have also been identified in the placenta of experimental studies of EAFL (Todhunter et al., 2013).

The histopathological results of these experiments are the subject of another thesis and are covered only briefly here.

In Experiment 1, several setal fragments were observed throughout the GIT, lung and uterus of GP7 which aborted 7 days (GD32) after the first treatment (Figure 4.10). A photomicrograph of the setae situated in the mirror of the exoskeleton of a PC (Figure 4.10a) is used here to demonstrate their appearance for reference to their presence in tissues.
Figure 4.10: Experiment 1. GP7 that aborted on GD32 following treatment with 1g/kg PC daily for 5 days from GD26.

a) Photomicrograph of a section of PC exoskeleton with setae barbs in situ.
b) Lungs with setal shaft (arrow)
c) Uterus with setal fragment in cross section (arrow)
d) Endometrial gland with setae in cross section (arrow)

In Experiment 3, setae fragments were observed from the colon of GP109. Based on the clinic results, samples of caecum, colon, placenta and implantation site) from GP119, GP106 and GP15 were examined by light microscopy (LM) and transmission electron microscopy (TEM) at the Centre for Microscopy and Microanalysis at The University of Queensland. No setal fragments were identified in any of the samples. However, the LM results from the placenta of the malformed fetus (R4) in GP15 (Figure 4.11a and Figure 4.11b) showed a significant difference between this placenta and that of the placenta of a normal fetus from the same guinea pig (Figure 4.11c and Figure 4.11d). This guinea pig had four fetuses in the right uterine horn: three normal fetuses and one small malformed dead fetus in the same horn adjacent to the cervix.
Figure 4.11: Experiment 3. GP15 euthanased on GD34 following 3 days treatments. There was one dead small fetus R4 and three normal fetuses (R1, R2, R3). R4 placenta showed a significant difference compare with R3 placenta.

a) GP15 R4 placenta. Toluidine blue staining. (Bar: 200μm)
b) GP15 R4 placenta. Toluidine blue staining. (Bar: 50.0μm)
c) GP15 R3 placenta. Toluidine blue staining. (Bar: 200μm)
d) GP15 R3 placenta. Toluidine blue staining. (Bar: 50.0μm)

Table 4.11: Abnormal outcomes (abortion, fetal death, abnormal placentae and positive bacterial isolation) of pregnancies between treatment and control guinea pigs in each experiment.

<table>
<thead>
<tr>
<th></th>
<th>Treatment Group</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>1 / 9</td>
<td>0 / 7</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>5 / 8</td>
<td>0 / 4</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>8 / 14</td>
<td>0 / 6*</td>
</tr>
</tbody>
</table>

* bacteria isolated from control group in Experiment 3 were not counted as they were identified as contamination.
4.5 Statistical analysis

Abnormal results compared with the total number of animals in each group for the three experiments are listed in Table 4.11. In the first experiment, the abnormal findings (abortion, dead fetuses, bacterial growth) in the treated group were not significantly different (P>0.05) to the control group. The findings were the same for the second experiment (P>0.05) While in the third experiment it was shown that there was a significant difference between treatment group and control group (P<0.05). Growth of environmental and enteric bacteria has been included in the statistical analysis as the termination of pregnancies at earlier time points may have preempted fetal death or abortion. Cases in which the species of bacteria were of human/animal origin were excluded from the analysis in both the treated and control guinea pigs.

In these three experiments, different treatments were used. A comparison between the different treatments showed that the outcomes of each experiment were not statistically different to each other (P>0.05). However, these results should be considered with caution due to the small sample sizes.
CHAPTER 5
Discussion and Implications of the Research

5.1 Why the guinea pig as a model?

The use of pregnant mares to investigate the pathophysiology of MRLS and EAFL is expensive both in acquisition and maintenance of the mares. Therefore, laboratory animal models have been explored for investigation of the pathogenesis of these disease syndromes. However, the attempts to develop a suitable alternative have been largely unsuccessful. Mice, rats and goats were used but did not abort following exposure to ETC (Sebastian et al., 2008a, Sebastian et al., 2002, Sebastian, 2004). Although abortions were observed in the pigs after exposure to ETC (McDowell et al., 2003), no additional work has been published using this species as a model for MRLS. The pig is an expensive and time consuming bioassay compared to small laboratory animal models. The gestation length of the sow is an average of 114 days which is much shorter than that of the mare but the cost of purchase of pregnant sows, their maintenance and feed consumption do not make them an economically viable option for extensive experimentation.

In the initial experiment in the present study, the guinea pig was chosen as a potential animal model for EAFL due to its ready availability, cost of purchase and care. When compared to other rodents, the guinea pig model has advantages for the study of EAFL. They are easy to handle, have a longer gestation period (about 65-67 days) compared to rats and mice, but not so long that experiments are excessively time consuming. It also allows easier detection of changes during pregnancy as they produce more than one offspring, usually 3-4 (Percy and Barthold, 2007), which allows more
opportunities to investigate fetal infection. Most importantly, the guinea pig is a monogastric and a hind-gut fermenter (Crecelius and Rettger, 1943) similar to the horse. The use of guinea pigs is much cheaper and more time-saving than using horses. These features make the guinea pig more representative than other animal models for EAFL studies.

5.2 Anatomical relationships

‘The large intestine of the mare lies in close proximity to the non-gravid uterus with the uterine horns lying on either side of the small (descending) colon and its mesocolon and the uterine body and cervix lying ventral to the terminal part of the descending colon and rectum’ (Kainer, 2011, p.1588). As the fetus grows, the mare’s intestines are displaced by the increasing size of the uterus (Fritz, 2012). As the horse has a large caecum and colon, the gravid uterus is in apposition to the large intestine in the mid- to late-gestational stages and is closely associated with the small colon in early pregnancy. In the EAFL experimental cases, the setae or setal fragments of PCs were found throughout the gastrointestinal tract with greater numbers found in the caecum and large colon (Todhunter et al., 2010). Focal hyperplastic serositis was found to mainly be present in the gastrointestinal tract and uterus (Todhunter et al., 2010) suggesting the same inciting agent may be responsible for both organs.

The guinea pig has a bicornate uterus (Hargaden and Singer, 2012) as does the horse but the uterine horns are longer, allowing for the accommodation of more than one fetus. In the horse, the body of the uterus (18-20cm) is similar in length to the uterine horns (20-25cm) (McKinnon, 2011) whereas in the guinea pig the body of the uterus is less than half the length of the uterine horns (Hargaden and Singer, 2012). The caecum of the guinea pig lies on the left side in the abdominal cavity and occupies most of the ventral abdominal cavity (Manning et al., 1984) is likely to be in close association with the uterus. The colon in the guinea pig differs from the horse and is more like that of the human. It is short and consists of three sections: ascending, transverse and descending (Cooper and Schiller, 1975).
In the initial experiment, one guinea pig aborted on GD 32, seven days after the first administration of PC components and setae fragments were found throughout the gastrointestinal tract. This guinea pig had a severe typhlitis. In the mares that were necropsied as part of the EAFL experiments (Todhunter et al., 2013) all had evidence of focal necrosis of the caecum and other lesions ranged from inapparent to granulomas. Importantly, the caecum and large colon were the site of higher concentrations of caterpillar setae in the mares (Todhunter et al., 2013). The guinea pig has a relatively short colon and it lies at the top of the uterus. This anatomical relationship of the uterus to the colon may facilitate the penetration of setae from the colon into the uterus. The size and weight of the gravid uterus in the mare results in the uterus predominantly lying in the midline, ensuring that it remains in close contact with the caecum and large colon with the small colon lying on the dorsal surface of the uterus as it passes between the uterine horns and over the uterine body. The large size of the cecum in the guinea pig means that it is likely to be in apposition to the gravid uterus but the relatively long uterine horns allows them to be pushed laterally in the abdomen. This may reduce the opportunity for continuous contact to be maintained with the caecum. Close to the cervix there is less mobility of the reproductive tract and it is held in closer contact with the caecum and descending colon. In the guinea pig experiments, the fetuses near the cervix were more likely to be infected. The relative mobility of the uterine horns may afford them and their fetuses some protection from penetration by PC setae but the cervix has less mobility and is fixed in relation to the caecum and descending colon which may allow more ready penetration of setae at this site. In experiments 2 and 3, fetal infection and death were more likely to occur at this site (66.7%). Due to the small sample size in these experiments the statistical power was reduced and this did not reach statistical significance. However, the overall outcomes closely reflected the situation of the mare experiments and field cases.

5.3 Immunology and placentation

The timing of exposure of the pregnant guinea pig is an important consideration with regard to its use as a biological model for infective conditions of the fetus in other species, in this case the mare.
The transfer of antibodies from mother to fetus is determined by the structure of the placenta (Tizard, 2013). The mare has a diffuse, epitheliochorial placenta with the intact uterine epithelium in contact with the fetal chorionic epithelium. This type of placentation has the most layers separating the maternal and fetal blood. This prevents trans-placental passage of the large immunoglobulins from the mare to the fetus. Hence the need for antibodies to be acquired from the colostrum by the newborn foal (Tizard, 2013). As a result, the equine fetus has little immediate protection from bacterial invasion.

In humans and other primates, IgG can be transferred directly to the fetus during pregnancy, as the trophoblast is in direct contact with the maternal blood in their hemochorial placenta (Tizard, 2013). The guinea pig has the same type of placenta and a similar process of trophoblast invasion as the human (Carter et al., 2006). During pregnancy, the embryo and its membranes do not break out of the decidua capsularis until 26 days of gestation (Barnes, 1959). With the progression of pregnancy, the decidua capsularis gradually becomes thinner until it is completely broken down by the 30th day of gestation. At this stage, the yolk-sac endoderm is exposed to the uterine lumen which allows the transfer of antibodies from the maternal circulation to the fetus (Barnes, 1959). Thus, the timing of exposure of the guinea pig fetus to infectious agents such as those seen in EAFL is likely to affect the fetal outcomes as in the later stages the guinea pig fetus will have protection from maternal IgG. This is reflected in the ability to induce fetal death and abortion in the guinea pig with exposure of the dam to PC components from GD25-27 (3-day treatments) or GD25-29 (5-day treatments) whereas exposure at GD33-40 in the first experiment did not cause fetal death or abortion, but exposure of one guinea pig at GD26 did.

The structure of the placenta has other relevance to EAFL abortion. It has been reported that in sub-Saharan Africa and Niger, abortion in camels is associated with proliferation of caterpillars in Acacias. In these cases the reported species is *Lasiocampidae* sp. caterpillars and Lepidopteran caterpillars (Volpato et al., 2013). Interestingly the ETC is also a member of the Lasiocampidae family of moths. Experimental studies of MRLS have shown that ETC can induce abortion in pigs (McDowell et al., 2003). The pig, camel and horse all have a diffuse epitheliochorial placenta (Abd-Elnaeim et al., 1999, Enders and Blankenship, 1999). Ruminants also have an epitheliochorial
placenta but it is cotyledonary rather than diffuse. Exposure of pregnant goats to ETC failed to result in abortion (Sebastian et al., 2008a). There are no documented reports of abortion in sheep or cattle associated with caterpillars. This may indicate that the diffuse structure of placenta (horse, camel and pig) is more susceptible to the effects of caterpillar setae than the cotyledonary, epitheliochorial placenta. It is possible that the greater surface area of the diffuse placenta that is in contact with the endometrium permits more ready penetration by the setae than that of the cotyledonary placenta found in ruminants, where the chorionic villi are concentrated in the cotyledons. The guinea pig has the same discoid placenta as do other rodents and exposure of the guinea pig to PC components has induced abortion in the current studies. The guinea pig and the horse are both monogastrics and are hind-gut fermenters with a large caecum that is in close association with the uterus. This may provide more chance for the setae to pass from the caecum into the uterus and placenta. Preliminary histology on guinea pigs that have aborted from PC administration show that PC setae do pass from the guinea pig gut into the uterus (Figure 4.10). However, a much higher dose of PC components is required to cause abortion in the guinea pig than in the horse and this may be a result of the separate, discoid placentas of the guinea pig fetus, which provides relatively less surface area for setal penetration into the fetomaternal unit than does the diffuse, epitheliochorial placenta of the horse.

5.4 Clinical features

In the case definition for EAFL, fetal death and abortions were associated with distinctive changes in the amnion and umbilical cord. One of the changes was oedema of the umbilical cord (Todhunter et al., 2009). These findings were replicated in the EAFL experiments (Cawdell-Smith et al., 2012). In the second of the experiments described here, fetal death occurred on 26 days of gestation (1st day of treatment) with one guinea pig, and on day thirty with another two guinea pigs (GP 29 and GP 1). The dead fetus of GP 29 had a thickened umbilical cord with oedema which was similar to those associated with EAFL field and experimental cases.

In the EAFL experiments, the mares which were euthanized showed gastric ulceration, enteritis and colitis (Cawdell-Smith et al., 2012). These pathological findings could be a response of these organs
to mechanical irritation or an allergic response to the caterpillar setae (Hossler, 2010). Similar findings such as hyperaemia of the gastrointestinal tract were seen in some of the guinea pigs that suffered fetal death or abortion in current studies. Caecal distension was also observed in a number of the guinea pigs described in these experiments. The uterus of one of these guinea pigs was congested and a serosanguinous discharge was observed in the uterine cavity. These pathological signs may also be a result of mechanical irritation or allergic reactions to the presence of setae. However, whether this, or bacterial infection is the cause cannot be determined until the histopathology has been completed. This is the subject of a further thesis.

In the EAFL experimental studies, mares showed few clinical signs of illness prior to abortion when treated with 5g shed exoskeleton, but did when treated with whole caterpillars (Cawdell-Smith et al., 2010). In these three current experiments, some of the guinea pigs treated with higher doses of PC that had abnormal outcomes to pregnancy showed obvious clinical signs such as dehydration, weight loss, dyspnoea and anorexia. This suggests that clinical disease in both the maternal guinea pig and the horse is related to higher doses of caterpillar components. Geldings that were treated with whole PC as part of the EAFL experiments also showed signs of colic similar to that in mares (A.J. Cawdell-Smith, pers comm).

In Experiment 2, 2 of 8 treated guinea pigs had abnormal clinical outcomes and one guinea pig (out of 2, 50%) had a dead fetus near the cervix. In Experiment 3, 4 out of 7 (57.1%) guinea pigs had abnormal fetuses or implantation sites near the cervix. Therefore, in these two experiments, 5 out of 9 (66.7%) infected guinea pigs had abnormal outcome at the site near the cervix. This finding may reflect the relative lack of mobility of the uterus at the cervix which could hold the uterus more closely to the caecum and colon at this location.

The necropsies on sequential days were to allow the histopathological investigation of setal migration in tissue. However, this may have preempted fetal death in guinea pigs euthanased prior to GD33 thereby affecting the statistical significance of the results. To account for this the isolation of bacteria known to be of environmental or enteric origin were included in the statistical analysis.
The other bacteria that are known to be related only to human or animal skin were not included as positive results in either the treated or control groups.

5.5 Microbiology and Histopathology

Based on the findings of the mare experiments of EAFL, it is suggested that the caterpillar setae carry environmental and enteric bacteria from the gastrointestinal tract into the adjacent uterine horns (Cawdell-Smith et al., 2012). Therefore, the bacteria isolated in treated GPs should have been similar to those found in mares. This is confirmed by the results: a range of enteric and environmental bacteria similar to those isolated from mares, both experimentally and in field cases of EAFL were isolated from treated guinea pigs. These included *Staphylococcus* spp., *Corynebacterium* spp. and Anaerobes. Enteric bacteria such as *Escherichia coli* and *Enterococcus faecium* were also frequently isolated.

*Staphylococcus aureus* was isolated from the uterine discharge and placentas of one guinea pig in the second experiment. It has been shown that *S. aureus* can cause pyaemia in lambs (Webster and Mitchell, 1989), pigs (King-Tiong et al., 1991) and humans (Hay, 1960). The septicaemia resulting from infection by *Staphylococcus aureus* causes widespread abscesses of a metastatic nature. It can cause whole body inflammation with associated changes in the urine, blood, lungs, skin and other tissues (Liu et al., 2005, Clauditz et al., 2006). This pathology is consistent with those observed in this guinea pig. However, bacteria were not isolated from maternal heart blood of this guinea pig suggesting that the clinical signs resulted from the uterine infection rather than a bacteraemia.

*Corynebacterium* spp. group G were isolated from the peritoneal cavity of GP21 and at necropsy the GIT of this guinea pig was bright red and hyperaemic. This bacterium can be found commonly in the environment. This suggests that the setae of the PC carried the bacteria through the GIT and infected the peritoneal cavity.

In the guinea pig, *Enterococci* spp. are normal flora in the stomach and small intestine (Crecelius and Rettger, 1943). In the final experiment, *Enterococcus faecium* was isolated from the placentae.
of 3 of the 4 fetuses in GP109. This was a scheduled autopsy at GD32, and at the time of sampling all of the fetuses were alive. This supports the findings in mares that normal enteric bacteria may be carried from the intestine directly into the uterus and the fetoplacental unit.

In the MRLS experiments, freshly dissected exoskeletons of ETC were shown to induce abortions in mares (McDowell et al., 2010). In experimental studies of EAFL, whole PC or shed PC exoskeleton administrated via a nasogastric tube to pregnant mares induced abortions. The experiments relating to both MRLS and EAFL showed that whole caterpillars, dissected caterpillar exoskeleton, and shed caterpillar exoskeleton have the ability to be an abortifacient. Based on this evidence, the guinea pigs in this experiment were orally administrated capsules filled with exoskeleton dissected from frozen PC. The capsules allowed easy dosing of the guinea pigs. Generally the guinea pigs did not chew the capsules so that the oral bacteria were not exposed to the caterpillar setae. As a consequence, oral bacteria were not isolated during these studies. Similar differences in the incidence of oral bacteria isolated from aborted fetuses in the MRLS studies were seen when mares were fed caterpillars mixed in feed rather than being administered to them by gastric lavage (Webb et al., 2004, Bernard et al., 2004).

Contamination occurred in a number of the controls in these studies, while in the EAFL horse experiments contamination did not occur. The tissues and fluids available for sampling in the horse are more easily accessible due to the large size of both the mare and the fetus which reduces the risk of contamination. However, in the guinea pig, the tissues and organs that are sampled are small. Due to the difficulty in isolating bacteria using standard culture techniques, enhancement media (brain heart infusion broth, BHIB) was used for the guinea pig studies. The advantage of using this media is the highly nutritious environment for microorganisms (CHEMUNEX) enabling the isolation of bacteria from both small samples and samples with few bacteria. The use of BHIB has enhanced the results for these experiments. However, it has the disadvantage of aiding the growth of contaminants from the sampling procedures, thereby complicating the findings. It is therefore essential when using the guinea pig model that meticulous asepsis is practiced during the sampling process. In these experiments the small size of the sampling sites, lack of experience and changes in the personnel assisting during the sampling procedures did result in contamination in some samples.
A laminar flow cabinet, separate to that where plating occurs should be used for the autopsy procedure and caps, masks and sterile gloves must be worn. Scalpel blades used for incising the abdomen must not be used for incisions of the uterus and different sterile equipment must be used for the autopsy of each fetus and fetoplacental unit.

Preliminary light microscopy studies of the placenta of one malformed fetus (of 4 fetuses) retrieved at scheduled euthanasia of the dam on GD34 was histologically very different to the placenta of one of the grossly normal fetuses. Based on the size and conformation of the malformed fetus, it was estimated that fetal death had occurred at approximately GD26-27. The apparent loss of density of the placenta reflects degeneration of the placenta following fetal death. The presence or absence of microscopic evidence of inflammation in the tissues will be further elucidated in the histological studies that are the focus of another thesis.

Preliminary histology from one guinea pig that aborted following treatment with PC showed the presence of setae in a uterine endometrial gland (Figure 5.1a). This finding replicates that seen in mares from the EAFL studies (Figure 5.1b). This indicates that the way setae penetrate the uterus is the same in both mare and guinea pig.
Figure 5.1: Setal fragments present in endometrial glands of both guinea pig and mare (from K.H. Todhunter).

a) **Experiment 1**: GP7 had been treated with PC (1g/kg) for 3 days from GD26 and aborted on GD32 (100x).

b) The mare euthanatized 2 days after the first treatment (50g whole PC) (Bar: 30μm)
5.6 Conclusion

Based on the criteria of a suitable animal model (Chow et al., 2008), the guinea pig met the requirements in these experiments. Firstly, the results of microbiology and clinical signs showed that this model reproduces the disease. Secondly, the guinea pigs are readily available and can be easily accessed by researchers. The guinea pig can produce multiple offspring providing a large number of individuals for experiments, they are well suited for use in the available animal facilities and can be easily handled. Finally, the guinea pigs survive long enough and are sufficiently robust for the purpose of this study.

The disadvantage of the guinea pig as a model for pregnancy in the horse is its different placental structure. However, the results of this experiment indicate that although abortion may be more reliably achieved in the horse, the differences between the model and the target allows manipulation of the model which would not be possible with the target species.

The hypothesis that the guinea pig is a suitable model to investigate the pathogenesis of EAFL has been met. However, a number of factors must be considered for the success of this model: care must be taken to ensure that the time of exposure relative to the stage of gestation avoids complications of the different fetal immunity of the 2 species; dose of caterpillar components administered per kg BW will need to be higher in the guinea pig; enhancement media will be needed for successful isolation of bacteria and as a consequence, meticulous aseptic technique when collecting samples for bacteriology is essential.

Details of the histopathology of the specimens from these experiments will be reported elsewhere but the results that are currently available further support this model (K.H. Todhunter, pers comm). This animal model for EAFL will be improved and refined by further research.
5.7 Further study

The mechanism of action of PC setae still needs to be further elucidated. As described from previous EAFL cases (Todhunter et al., 2009, Todhunter et al., 2010), EAFL experiments (Cawdell-Smith et al., 2010, Cawdell-Smith et al., 2012) and this paper, caterpillar setae can migrate through tissues and organs in both mares and guinea pigs. Bacteriological evidence supports the belief that the setae can facilitate the transfer of bacteria across the gut wall into the fetoplacental unit resulting in fetal infection (Todhunter et al., 2010). Further studies in the guinea pig using trans-generically modified bacteria and bioluminescence imaging technology could identify the mechanism of bacterial progression in real time through the gut into the uterus and fetoplacental unit. Ryan (2011) has shown that this novel approach provides a better understanding of pathogen progression in ascending uterine infections on pony mares but euthanasia of the mare is required. The small size of the guinea pig will permit monitoring of bacterial progression over time without euthanasia. This technique may provide a novel means of studying the pathogenesis of EAFL in the guinea pig model.

The cost effectiveness of the guinea pig as a model will allow experiments to investigate the association between the structure of caterpillar setae and bacteria and elucidate the role of toxins, if any, associated with the PC. The development of the guinea pig as a model for EAFL will also allow investigation of the role of other hirsute caterpillars in EAFL. Discovering the mechanism by which caterpillar setae cause animal abortions will allow development of timely diagnosis, targeted intervention and novel management approaches.


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Table A.1.2 Experiment 3 guinea pig allocation and sampling date

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APPENDIX 2

Procedure for sampling the fetuses

The fetus was placed into a sterile Petri-dish to autopsy. Using a new scalpel blade the left limbs of the fetus were removed and an incision made through the left abdominal wall to allow exposure of the fetal stomach. Stomach contents were collected by 1ml syringe with a 22 G needle. Then, the liver was taken out and put into a BIHB bottle. Next, the ribs were cut by using scissors in order to exposure the lung. The fetal lung was then sampled in the same manner.
APPENDIX 3

Procedure for Electron Microscope (TEM) and Light Microscope (LM)

BioWave Microwave
Initially, the samples were chopped into several small pieces (1mm x 1mm) and the Pelco BioWave Microwave (PELCO 34700 BioWave®) (Figure 3.1 & Figure 3.2) was used for fixed samples.

Buffer Rinse
In a fume hood, the sample was put into a 10ml plastic container and added fresh buffer to rinse for about 1 minute and changed to new buffer. Then the sample was put into the microwave and set as follows:

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<th>Time sequence</th>
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Osmium Tetroxide Fixation
After the buffer rinse, changed the buffer and added 1% osmium for osmium tetroxide fixation. Then the sample was put back to the microwave and, the following procedure was used.

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<td>2 min on, 2 min on off, 2 min on Cool to 20°C and repeat time sequence</td>
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Water Rinse
The samples were then taken out of the microwave and water was used to rinse the osmium in the fume hood. New water was then added and put back into the microwave.

<table>
<thead>
<tr>
<th>Step</th>
<th>Solvent</th>
<th>Wattage</th>
<th>Time</th>
<th>Vacuum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>water</td>
<td>80</td>
<td>40 sec</td>
<td>on</td>
</tr>
</tbody>
</table>
Dehydration

The samples were taken out after the water rinse and the following procedure was used for dehydration.

<table>
<thead>
<tr>
<th>Step</th>
<th>Solvent</th>
<th>Wattage</th>
<th>Time</th>
<th>Vacuum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acetone 50%</td>
<td>150</td>
<td>40 sec</td>
<td>no</td>
</tr>
<tr>
<td>2</td>
<td>Acetone 70%</td>
<td>150</td>
<td>40 sec</td>
<td>no</td>
</tr>
<tr>
<td>3</td>
<td>Acetone 90%</td>
<td>150</td>
<td>40 sec</td>
<td>no</td>
</tr>
<tr>
<td>4</td>
<td>Acetone 100%</td>
<td>150</td>
<td>40 sec</td>
<td>no</td>
</tr>
<tr>
<td>5</td>
<td>Acetone 100%</td>
<td>150</td>
<td>40 sec</td>
<td>no</td>
</tr>
</tbody>
</table>

Rinse Infiltration

Resin (epon) was used for rinse infiltration. In the first step, the sample was add into the resin mix which resin and solvent (100% acetone) were mixed at the ratio of 1:2. The sample was then put into the microwave for 3 minutes with wattage 250w and vacuum on. The following procedures were in the same manner except for the resin mix ratio.

<table>
<thead>
<tr>
<th>Step</th>
<th>Resin mix</th>
<th>Wattage</th>
<th>Time</th>
<th>Vacuum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Resin : solvent 1:2</td>
<td>250</td>
<td>3 min</td>
<td>on</td>
</tr>
<tr>
<td>2</td>
<td>Resin : solvent 1:1</td>
<td>250</td>
<td>3 min</td>
<td>on</td>
</tr>
<tr>
<td>3</td>
<td>Resin : solvent 2:1</td>
<td>250</td>
<td>3 min</td>
<td>on</td>
</tr>
<tr>
<td>4</td>
<td>100% Resin</td>
<td>250</td>
<td>3 min</td>
<td>on</td>
</tr>
<tr>
<td>5</td>
<td>100% Resin</td>
<td>250</td>
<td>3 min</td>
<td>on</td>
</tr>
</tbody>
</table>

Resin Polymerization

After the rinse infiltration, all the samples were put into a block tray and 100% resin was added (Figure 3.3) and then left in an oven overnight at 60°C. Then, the samples in the resin block were ready to use for ultramicrotome.
Figure A.3.1: Pelco BioWave Microwave (PELCO 34700 BioWave®)

Figure A.3.2: Pelco BioWave Microwave setting panel
**Ultracut ultramicrotome**

**Preparation of glass knives**

Glass knives were prepared by using a knife maker (Leica® EM KMR3). Once the good quality knife was made, the truf was carefully attached onto the knife with melted wax. Then, the truf edges were sealed with molten wax.

**Ultramicrotome sectioning**

**Trimming the block**

The sample block was inserted into the chuck holder and tightened. Then, adjusted the microscope was adjusted (Leica® EM UC6) until a clear vision of the block could be seen. Next, the block was trimmed by using a blade. Cut the excess resin and left the desire tissue sample into a trapezoid body. Then, the sample block was ready for sectioning.
Thick sectioning
The glass knife and sample block were placed into right position. Aligned the block with the glass roughly and smoothed the surface of the block. Then, filled the turf with water and started to cut until whole section was obtained. Cut the block slowly until required numbers of sections were gained. Picked up the sections in a loop and placed the loop on a glass slide. After the slide dried completely, stained the slide with toluidine blue for a few seconds and rinsed with water. The slide was examined under the light microscope to determine if thin sections were required. Finally, a smaller area of interest was selected and retrimmed it under the microscope (approximately 0.5mm x 0.5mm).

Thin sectioning
After retrimmed the sample block, the chuck holder was put back to the arm and a diamond knife (Drukker International®) was used instead of glass knife for thin sectioning. The procedure was in the same manner of the thick sectioning. The turf of the knife was filled with water. Then, the knife edge and the block surface were orientated until the knife and the whole surface of the block were vertical. Next, the 60nm feed at speed of 1.5 was selected. After about 40 sections were cut, a heat pen was used to straighten the sections before collecting them (Figure 3.4).

Collecting sections
Good quality forceps were used to hold a clean grid and the sections were maneuvered with the dog-hair stick to a suitable position. Then the grid was brought up from underneath and lifted the section. A filter paper was used to dry the grids.
Figure A.3.4: Thin sectioning by using diamond knife

**Transmission Electron Microscope (TEM)**

Before the TEM examination, the grids were stained. On a glass plate, placed few drops of water and adhered a large piece of parafilm (left the cover on until ready to stain). Put four beakers filled with distilled water aside. Dispensed equal amounts of uranyl acetate and lead citrate into eppendorfs (2.0 ml) and spined the eppendorfs in centrifuge at full speed for 5 minutes. Cleaned the forceps with ethanol and removed the cover from parafilm. Placed one drop of uranyl acetate and used forceps to place the grid (section side down) onto the uranyl acetate drop for 2 minutes. Used the forceps clamping the grid and rinsed the grid in the first beaker for few seconds and then rinsed in second beaker for 10 to 15 seconds. After rinsing, removed the excess water with torn filter paper and cleaned the forceps with ethanol again. Placed one drop of lead citrate on parafilm and placed
the grid (section side down) onto it for 1 minute. Rinsed the grid in the third beaker for few seconds and rinsed in fourth beaker for 10 to 15 seconds. The grid was dried with filter paper.

A TEM (JEM-1010) (Figure 3.5) was used for taking micrographs of all the samples. First of all, pulled the sample loaded arm out gently, loaded two sample grids once at a time and returned the arm to the original position. Turned the TEM on, the chamber screen was filled with green light. To find the sample on the grid, pressed LOW MAG button on right hand side (RHS) console and moved out objective aperture by turning silver lever to the right. The full grid was observed at this point and used the two direction wheels to find the sample. Moved the objective aperture in and pressed MAG 1 button and spread the beam by turning the brightness knob on left hand side (LHS) console. To get clear image, adjusted magnification button and coarse and fine focus knobs on RHS console. After the sample could be seen clearly in the chamber screen, turned the iTem software on in the computer. Selected camera in icon and the sample picture could be seen on the computer screen. RTFFT (RealTime Fast Fourier Transformation) was used for focussing the TEM. The micrographs of the samples were taken and saved into the computer.

Figure A.3.5: Transmission Electron Microscope - JEM-1010 at Centre for Microscopy and Microanalysis (CMM)
Light Microscope (LM)

The slides (toluidine blue staining) made after thick sectioning were covered with a glass cover and a LM (OPTICAL OLYMPUS BX61 BIOL) was used for taking LM pictures (Figure 3.6). Turned on the top box (1), loaded the slide and ensured that the stage was lowered (2) and at x4 objective (a). Opened the DP controller and DP manager program on the computer. To get the bright field appropriate, all filters were out and mirror unit (3) read 6, if not change it with MU (b). Selector (4) read BF or changes it with CDT (c). Adjusted brightness with (5) and checked transmitted light lens (6) was in upright position, change with TL (d). After all the steps were completed, a clear image could be seen on the computer.

**Figure A.3.6:** OPTICAL OLYMPUS BX61 BIOL