Introduction

Collectively, invasive animals, plants and microbes are a major threat to ecosystems worldwide, yet the compounding impact of invasive species is not widely recognised (van der Putten et al., 2007). Feral pigs (*Sus scrofa*) present an undefined threat to native ecosystems worldwide through their potential to spread secondary invasive species such as pathogens and weeds. While many animals are capable of acting as vectors for a number of disease agents including microbial pathogens, the non-fastidious diet, foraging habits and home range of feral pigs brings them in direct contact with invasive plant pathogens such as *Phytophthora cinnamomi* in many of the temperate regions of the world thus increasing the potential cumulative impact of both pigs and pathogens on native and horticultural plant communities (Feio *et al.*, 1999; Martin and Dale, 2001).

Feral pigs are a widespread pest species that are present throughout many regions and habitat types of the world (Tierney and Cushman, 2006), and they have been implicated in the decline of 257 and 8 plant and bird species, respectively, in the United States (Gurevitch and Padilla, 2004). They also impact on agricultural production and in Australia alone damage due to feral pigs is estimated to cost in excess of $107 million per annum (McLeod, 2004). The highly invasive plant pathogen *P. cinnamomi* infects over 3000 plant species worldwide including many agricultural, ornamental and forest species (Erwin and Ribeiro, 1996; Hardham, 2005). Shearer *et al.* (2004) estimated that 2284 (40%) of the 5710 plant species in the South-West Botanical Province of Western Australia are susceptible to *P. cinnamomi* infection. The pathogen has been estimated to cost the Australian economy $1.6 billion over the course of a decade (Carter, 2004). Despite the introduction of both feral pigs and *P. cinnamomi* in many temperate habitats worldwide, very little is understood of the compounding potential of these two highly destructive and invasive species. Anecdotally, feral pigs have long been implicated in the spread of *P. cinnamomi* although relatively little data exists to support these claims.

The non-fastidious diet of feral pigs typically incorporates a significant proportion of plant material including woody root material and underground structures (Masters, 1979; Choquenot *et al.*, 1996; Giménez-Anaya *et al.*, 2008), which are the primary sites for infection with *P. cinnamomi* in susceptible plants (Hardham, 2005; Hardy *et al.*, 2007). Such foraging behaviour presents a means of pathogen dispersal in addition to the transport of contaminated soil. This study investigated the potential for pigs to disseminate viable *P. cinnamomi* in their faeces following the ingestion of infected plant material. As such we demonstrate the ability of *P. cinnamomi* to survive passage through the pig digestive tract and the potential for long distance dispersal of the pathogen by feral pigs in native forest ecosystems.

Methods

Determining pig gastrointestinal passage rate; experimental and pig maintenance

Pig feed stained with Sudan III dye was prepared as described by Asplund and Harris (1970) to determine the intestinal passage rate. Briefly, 3 kg of pig feed was soaked in 4 L of distilled water for 10 min then drained and gently shaken to soften all particles and remove excess water. Dye was added by dissolving 900 mg of Sudan III (ProScitech, Thuringowa, QLD, Australia) in 750 ml of acetone and mixing with moist feed. Acetone and water were allowed to evaporate for 1 h at 25°C before rinsing with tap water twice and drying at 50°C for 72 h. Three female pigs (*Sus scrofa;* Large White) approximately 4 months of age (15-20 kg each) were individually housed in 2 m x 2 m pens in the pig handling unit at Murdoch University. Pigs were each fed approximately 1 kg of commercial "Baconers pellets" (Milne AgriGroup, Australia) daily during an acclimatisation period of 2 days. Water was provided *ad libitum*. The dried feed was fed to the pigs as per daily diet. Faecal samples were collected twice daily (am and pm) to record the time taken to passage all of the dyed feed. Pens were washed down twice daily, following faecal collection. Faeces were collected twice daily; once in the morning, prior to feeding, and again in the evening.

Culturing of Phytophthora cinnamomi *infected plant materials*

The three plant substrates used in this study were; millet (*Panicum miliaceum*) seed, pine (*Pinus radiata*) stems and slender-leaved Banksia (*Banksia leptophylla*) roots.

Millet seeds (150 g) and 120 ml of water were placed into 500 ml Erlenmeyer flasks, stoppered with a non-absorbent cotton and a gauze bung and sealed with aluminium foil. Flasks were sterilised by autoclaving at 121^{o}C for 30 min once a day for three consecutive days. Following autoclaving flasks

were allowed to cool at room temperature before storing at 4°C overnight. Flasks were aseptically inoculated with *P. cinnamomi* isolate MP94.48 (Centre for Phytophthora Science and Management, Murdoch University) in a laminar flow cupboard using 4-5 agar plugs (100 mm^2) per 500 ml flask from three day-old cultures grown on NARPH agar, a *Phytophthora* selective medium plates as described by Hüberli *et al*. (2000). Inoculated flasks were placed inside zip lock bags and incubated for three weeks at 20°C with gentle shaking every three days to ensure uniform colonisation of *P. cinnamomi* through each substrate.

Lengths of pine branches of 10-15 mm diameter were harvested from 5-9 year-old trees, stripped of bark, cut into 2-3 cm lengths and soaked in deionised water for 24 h in a 2 L Erlenmeyer flask. Following soaking, water was drained and 50 ml of deionised water was added prior to autoclaving and inoculation as per the millet seeds. Flasks were kept at 20°C for 8 weeks and shaken once every three days to ensure uniform distribution and colonisation by *P. cinnamomi*.

Phytophthora cinnamomi infected plant roots were sourced from potted *Banksia leptophylla* plants grown in a container substrate inoculated with three *P. cinnamomi* colonised pine plugs. Briefly, the two year-old *B. leptophylla* seedlings were potted in 150 mm free-draining pots containing composted pine bark, coarse river sand and coco peat fibre (2:2:1 ratio, respectively; Richgro Garden Products, Canningvale, WA). Plants were fertilised with 15 g of a slow release low phosphate fertiliser (Osmocote Plus Native Garden, Scotts Australia, NSW) added to the surface of the potted soil. Three pine plugs colonised with *P. cinnamomi* isolate MP94.48 were placed in the soil to a depth of 4 cm. Plants were grown in a glasshouse at approximately 25°C degrees and watered daily via drip irrigation for 6 – 10 weeks until plants began to show symptoms of *P. cinnamomi* infection at which point the roots were harvested.

The viability of *P. cinnamomi* in each of the plant materials was confirmed by plating onto *Phytophthora* selective medium (NARPH agar plates).

Feeding of inoculated plant material

Each of the infected plant materials were fed separately to the individually housed pigs in conjunction with that days' pig feed by adding 150 g of infected material to 850 g "Baconers pellets" prior to feeding. Each plant substrate was fed separately and as a single bolus to each pig. Uneaten portions were removed 24 h after feeding. Following each feeding of inoculated material pigs were fed 1 kg of uninoculated "Baconer pellets" daily for a minimum of four days as determined by Sudan III dye experiment or until the last recognisable portion of plant inoculum was observed, whichever was longer. After the final passage of inoculated material was observed the next inoculated bolus was fed to the pigs after a 48 h clearing period was imposed.

Faecal sample collection and recovery of passaged inoculated plant materials

The total weight of each sample collected was recorded. The faecal material was sieved through 1 mm and 20 µm sieves sequentially using running water (2 L/min) to recover the inoculated plant material. The 1 mm sieve collected the undigested plant material and pig feed whilst the 20 µm sieve collected partially digested material and any chlamydospores and mycelium of *P. cinnamomi* that may have been present. Recovered faecal material from both the 1 mm and 20 µm sievings were stored at -20°C until further processing.

Analysis of recovered plant materials

Dry weight measurements of recovered infected plant material were used to determine the daily percentage of plant materials recovered. A representative portion of each type of material was collected for dry weight measurement; measurements were taken before and after drying in a 50°C oven overnight. The total passage time of each *P. cinnamomi* colonised plant material was also recorded.

Pooling of recovered plant materials

Due to the differential digestion of each plant material, samples collected from their respective individual pig replicates, were pooled to allow sufficient material for comparative isolation and PCR analyses (Table 1).

Testing viability of Phytophthora cinnamomi

(i) Baiting

Three day-old germinated *Lupinus angustifolius* cv. Mandelup cotyledons were used to detect the presence of *P. cinnamomi* zoospores via baiting from the recovered *P. cinnamomi* colonised plant material. Briefly, approximately 4.8, 6.6 and 5.4 g of recovered millet seed, pine plug and *B. leptophylla* roots, respectively was placed individually into 300 ml of deionised water held in 500 ml plastic containers. Cotyledons were placed on polyester floats with their tap root submerged in the inoculated water for five days. Cotyledons were examined for lesions and tap roots with lesions were then cut and plated onto NARPH plates aseptically. The plates were incubated at 20°C in the dark and observed daily under an Olympus CHS microscope (Olympus, Japan) to detect growth of *P. cinnamomi* mycelium. The *P. cinnamomi* colonised plant materials M_0, P_0 and R_0 were also baited as positive controls. Non-infected M_C, P_C and R_C were also baited as negative controls.

(ii) Direct plating of inoculated plant materials

Individual fragments of each recovered substrate were surface sterilised with 70% ethanol for 30 sec, rinsed in sterile distilled water and placed onto NARPH agar plates to determine if *P. cinnamomi* survived passage through the pig guts. The *P. cinnamomi* colonised plant materials M_0, P_0 and R_0 were also plated as positive controls and non-infected M_C, P_C and R_C were plated as negative controls. The NARPH agar plates were incubated at 20°C in the dark and observed daily for hyphal growth typical of *P. cinnamomi* under an Olympus CHS microscope (Olympus, Japan). When observed, hyphae were sub-cultured onto fresh NARPH agar plates and confirmed as *P. cinnamomi* based on hyphal morphology identification. Representative isolates were stored on corn meal agar plugs (100 mm^2) in sterile water at room temperature for future reference.

(iii) Plant infection bioassay trial

Recovered pine plugs were placed into container substrate containing two year-old *B. baxteri* seedlings in 150 mm free-draining pots. The container substrate consisted of composted pine bark, coarse river sand and coco peat fibre (2:2:1 ratio, respectively; Richgro Garden Products, Canning Vale, Western Australia). Each pot was top-dressed with 15 g of slow release low phosphate fertiliser (Osmocote Plus Native Gardens, Scotts Australia, New South Wales). Recovered pine plugs P_1, P_3, P_5 and P_7 were placed into the container substrate at a depth of 4 cm. Positive and negative control plants were also inoculated with P_0 and P_C pine plugs, respectively. There were 6 replicate pots per treatment giving a total of 36 pots. Plants were grown in a glasshouse at 25°C and watered via drip irrigation daily to container capacity. Plants were observed daily for visible signs of infection (chlorosis of leaves and wilting). The onset of visible symptoms was noted. The roots of the plants were harvested after 10 weeks of inoculation and plated directly onto NARPH agar to determine if *Phytophthora* was present in the roots. In addition, roots and the container medium were baited as previously described.

DNA extraction

DNA was extracted from the *P. cinnamomi* colonised plant materials recovered from faecal samples using a BioSprint 15 (Qiagen GmbH, Hilden Germany) and Qiagen DNA Plant Kit (Qiagen GmbH, Hilden Germany Cat. No 941517) with the following minor alterations to the manufacturer's protocols: A TissueLyser (Qiagen GmbH, Hilden Germany) was used to macerate 1 g of each sample with two stainless steel balls 3 mm in diameter and 5 g of black silica in 5 ml of DNA Extraction buffer (2% CTAB, 100 mM Tris-HCl pH8.0, 1.4 M NaCl, 2% PVP-40) (Graham et al., 1994). These were stored on ice after shaking with TissueLyser at 30 hz for 1.5 min. The homogenized samples were centrifuged at 600 rpm for 5 min in an Allegra® X-15R benchtop centrifuge (Beckman Coulter, Germany). Five milliliters of supernatant was added to 20 µl Qiagen Magattract and 5 ml Isopropanol (Fisher Biotech, Australia) for 5 min on ice. The Magattract beads were then harvested and incorporated into the Qiagen DNA Plant Kit and used together with a Biosprint 15 machine to extract DNA from the supernatant according to the manufacturer's instructions. The DNA isolates were stored at -20°C.

PCR and DNA sequencing

Isolates subcultured twice from NARPH plates were grown on half-strength Potato Dextose Agar, PDA (Becton Dickinson Co., 19.5 g Potato Dextrose Agar, 7.5 g Difco Agar mixed with 1 L deionised water) plates at 20°C for 2 weeks. The mycelium was harvested by scraping it from the agar surface with a sterile blade and placing it in a 1.5 ml sterile Eppendorf tube. Harvested mycelium was frozen in liquid nitrogen, ground to a fine powder and genomic DNA extracted according to Andjic *et al.* (2007). The region spanning the internal transcribed spacer (ITS) 1-5.8S-ITS2 region of rDNA was amplified using primers ITS6 (5' GAA GGT GAA GTC GTA ACA AGG 3') (Cooke *et al*., 2000) and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White *et al*., 1990). The PCR reaction mixture,

amplification conditions, clean up of products and sequencing were as described by Andjic *et al*. (2007).

Polymerase Chain Reaction (PCR) products were sequenced with the Big-Dye terminator cycle sequencing kit (PE Applied Biosystems, California, USA) using the same primers that were used in the initial amplification. The sequencing products were cleaned using Sephadex G-50 columns (Sigma Aldrich, Sweden) according to the manufacturer's instructions and were separated by using an ABI3730 48 capillary sequencer (Applied Biosystems, California, USA). Isolate identification was determined by sequence similarity comparison with Blast searches found in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Q-PCR

Polymerase Chain Reaction (PCR) was set up with 1 x PCR buffer (20 mM Tris-HCl, 50 mM KCl) (Fisher BioTech), 2.0 mM $MgCl_2$ (Fisher Biotech), 0.25 mM dNTP's (Fisher Biotech), 1 U Taq Ti Polymerase (Fisher Biotech), 0.4 mg/ml Bovine Serum Albumin (Fisher Biotech), 0.4 mM PcinF3 (5' ATT AGT TGG GGG CCT GCT C 3', IDT Technologies), 0.4 mM PcinR7 (5' CAG TAT TAC AGA ATG GGT TTA AAA GAG AGG 3', IDT Technologies), 0.4 mM Probe (5' ATC ACT GGC GAG CGT TTG GGT C 3', IDT Technologies) and 1 µl DNA template, DNA-free water (Fisher Biotech, Australia) was added to make a final volume of 25 µl. Negative controls contained 1 µl of PCR-grade water instead of target DNA.

Amplification of products was performed over 50 cycles with denaturing temperature at 95°C for 3 min, followed by annealing at 95°C for 10 s and final temperature at 60°C for 30 s, on a Biorad MyiQ PCR (Biorad) with DNA concentrations analysed using Biorad IQ (Biorad). Data were subjected to One Way ANOVA analysis and means compared to determine differences between substrate type and passage days using SPSS 19.0 (IBM).

Results

Passage rates of dye and plant materials

Sudan III dye feeding showed that the initial passage time of pig feed through a pig's digestive tract is approximately 6 h, with total passage completed within 4 days (80-96 h). Passage rates differed for each plant substrate, with the smaller sized *B. leptophylla* roots passaging over 3 days and the larger pine plugs taking up to 7 days to passage (Figure 1). Millet seeds passaged over 5 days.

Consumption and digestion rates differed for each substrate based on palatability, maceration and digestion. Approximately one third (53.4 g ± 1.2) of the infected pine plugs were consumed whilst all of the millet seeds and *Banksia* roots provided were eaten. Of the bolus eaten, the highest recovery of infected plant material was from the pine plugs (94.9%) followed by millet seeds (33.2%) and *Banksia* roots (10.4%) demonstrating that the millet seeds and *Banksia* roots were readily digested by the pigs.

Viability of inoculated plant materials

Viable *P. cinnamomi* was recovered from all three pigs and all plant substrate type. Plating of the recovered plant materials onto the *Phytophthora* selective medium (NARPH plates) and baiting with lupin cotyledons showed that all of the colonised materials were 100% viable prior to feeding. No mycelium production or infection was observed in the negative controls M_C, P_C and R_C. Viability of *P. cinnamomi* was highest in the first three days following feeding pine plugs (98.3%), followed by roots (41.3%) and millet seeds (25.5%); see Table 2. Viability of *P. cinnamomi* within the recovered pine plugs was observed to reduce over time with only 43.9% of pine pieces recovered between 4 and 7 days after feeding found to contain the pathogen. Baiting demonstrated that *P. cinnamomi* within the recovered plant material could produce zoospores and infect the lupin baits under laboratory conditions (Table 2). No mycelium or zoospore production was observed in the lupin baits inoculated with negative controls M_C, P_C and R_C.

Plant infection bioassay trial

Potted *Banksia baxteri* plants inoculated with *P. cinnamomi* within passaged pine plugs recovered from pig faecal material showed disease symptoms consistent with infection by the pathogen 6 weeks post inoculation. Disease symptom development within the host plants was most severe in those plants inoculated with 80 % of the roots infected in those plants inoculated with the unfed (P_0) pine plugs. Similarly, 74% of those roots inoculated with passaged materials recovered within 24 hours of feeding

(P_1) were infected with *P. cinnamomi*. The percentage of root infection resulting from exposure to passaged materials decreased inversely in relation with the time of passage, decreasing to 35% of roots infected in plants inoculated with materials recovered 7 days post feeding. Lupin baiting demonstrated that *B. baxteri* roots grown in container substrate inoculated with the recovered pine plugs could produce zoospores and infect available plant material under laboratory conditions. No *P. cinnamomi* was isolated from the plants inoculated with P_C (negative control).

DNA Sequencing
ITS1-5.8S-ITS2 sequences of isolates grown on NARPH plates from the recovered P_{1-3} and P_{4-7} pine plugs were identified as *P. cinnamomi* with 100% homology to those isolated from P_0 pine plugs and *P. cinnamomi* isolate MP94.48 (GenBank Accession no.: JX113294).

DNA analysis with Q-PCR
Phytophthora cinnamomi DNA was detected at decreasing concentrations from the recovered colonised plant substrates (millet seed, pine plugs and roots) following passage through pig digestive tract as shown in Figure 2. DNA concentrations of *P. cinnamomi* in the ingested plant materials were seen to decline after three days of passage (Figure 2). The *B. baxteri* root isolates from the plant infection bioassay trial were also confirmed to be infected with *P. cinnamomi*. No significant difference were noted when One Way ANOVA analysis was performed using substrate type ($F(12,245)= 0.942$; $p= 0.506$) and time ($F(6,39)= 1.599$; $p= 0.174$) as independent factors; and means comparison (source $Eta^2= 0.044$; time $Eta^2= 0.197$).

Discussion
This feeding trial demonstrated for the first time to our knowledge the potential for pigs to spread *P. cinnamomi* through the ingestion of *P. cinnamomi* infected plant material. Although *P. cinnamomi* has been previously shown to survive digestion in birds (Keast and Walsh, 1979), the present study is the first to demonstrate a potential vector pathway of the pathogen via ingested plant material with a likely animal vector. Furthermore, this study demonstrates the potential compounding impact of these two invasive species within the ecosystems where they have been introduced.

Although Kliejunas and Ko (1976) found that feral pigs could transport *P. cinnamomi* in soil lodged on their hooves, this has proven to be difficult to reproduce under study conditions in the south western Australia due to the majority of soil being dislodged prior to sampling. Trapping of feral pigs in this region is most successful in summer months when pigs concentrate within water catchments. Due to the high sand and gravel contents of soils lodged on the bodies/ hooves of feral pigs, material is readily dislodged when dry or via tree-rubbing activities. The present study has established an alternate mode by which pigs can transport plant pathogens such as *P. cinnamomi* as demonstrated by its survival through the gastrointestinal tract. This poses a greater risk for dispersal as the plant pathogen can be kept viable and transported across greater distances over a period of up to 7 days following the consumption of a single infested bolus. Feral pigs were found to travel at least 2.4 km daily (Peter Adams, *pers. comms*) with huge implications for the movement of *P. cinnamomi* from infested into disease-free native vegetation over large areas.

This is the first demonstration of the survival of *P. cinnamomi* within infected plant material following pig ingestion. While the previous study had used axenic cultures of *P. cinnamomi* spores in attempting to demonstrate survival, such inocula are less likely to be encountered in the pig's natural diet than infected root materials given that pigs often forage for soil-borne root, tuber and fungal bodies. Individual spores are also labile to the hostile environment of the stomach making individual spores a poor demonstration of the potential risks of pathogen spread through ingestion of colonised plant materials. This is supported by the findings of the current study as conditions in the pig digestive tract decreased the viability of *P. cinnamomi* within the infected material over time. Re-isolation of *P. cinnamomi* from ingested plant material was significantly reduced after three days of exposure to digestion conditions. Detection of *P. cinnamomi* via PCR also showed a reduction in *P. cinnamomi* DNA concentrations as digestion time was increased.

Dietary studies had previously demonstrated that the pig digestive tract has poor utilisation and digestion of both b-glucan and cellulose (Teague and Hanson, 1954; Knudsen *et al.*, 1993) which are components of *P. cinnamomi* cell walls (Zevenhuizen and Bartnicki-Garcia, 1969). This could lead to survival of the pathogen through digestion. However, the reduction in viability observed is likely due

to the temperature, chemical or enzymatic activity within the pig digestive tract as suggested by Masters (1979). The temperature of pig stomachs is typically around 39^{o}C (McCauley *et al.*, 2005), a temperature which is sustained throughout digestion and that is inhibitive to *P. cinnamomi* growth (Gallo *et al.*, 2007). The pH of pigs stomachs can be as low as 2.0 and stomach contents contain many enzymes which readily digest most organic substrates (Argenzio and Southworth, 1974; Manners, 1976). The cellulose matrix of the plant material is believed to have afforded protection against the core temperature, low pH and digestive enzymes within the pig stomach. This protection allowed *P. cinnamomi* within these ingested plant materials to remain viable, as demonstrated by the production of both mycelium and infective zoospores from each of the three plant substrates upon passage, and by the death of *B. baxteri* plants grown in container substrate colonised with the infected plant substrates recovered after passage through the pigs digestive tract.

The three inoculated plant materials used in this study were selected for their susceptibility to *P. cinnamomi* and varied digestive characteristics as analogues of the type of materials that are likely to be encountered during the natural foraging of feral pigs within native ecosystems. Pine plugs were used to demonstrate survival in coarse woody material, millet seed as a highly palatable medium and *B. leptophylla* as a demonstration of fine root material most likely to be consumed by feral pigs as bycatch when digging for subterranean roots and tubers. Comparison between these demonstrated that the risk of feral pig mediated spread of *P. cinnamomi* in ingested plant materials is dependent on the plant material in which the inoculum is present. Significant variation was observed in the reduction of inoculum concentration and viability from each of the inoculated plant materials with respect to their palatability and ease of digestion.

Pine plugs were the largest sized plant material and took the longest to totally passage through the pig digestive tract. The plugs fed were barely broken down in the stomach, retaining their structural integrity throughout passage, and the highest rates of *P. cinnamomi* re-isolation over time. This increased passage time poses a greater risk of *P. cinnamomi* being spread over longer distances in ecosystems around the world where both feral pigs and *P. cinnamomi* are both introduced and invasive species. In contrast, millet seed was readily digested and passaged much faster than pine with re-isolation levels also decreasing significantly. *Banksia leptophylla* roots represented a realistic plant material that feral pigs would encounter in the natural forest. Roots were mostly digested and passaged fastest amongst the three substrates.

Previous studies have demonstrated that a significant proportion (61-96%) of a feral pig's diet consists of organic plant material (Schley and Roper, 2003). Most notably, Gimenez-Anaya *et al*. (2008) demonstrated that 33% of pig diet in Spain was underground roots and rhizomes. Stomach contents analysis run in parallel to the present study has demonstrated this to be consistent within the jarrah (*Eucalyptus marginata*) forest of Western Australia (data not shown). Furthermore, feral pigs within this region have been observed to specifically target the subsurface structures of plants known to be susceptible to *P. cinnamomi* including *Macrozamia reidlei* (Masters, 1979). Given that roots and underground structures are primary sites for *P. cinnamomi* infection and colonisation, there is a strong likelihood of the pathogen being ingested as part of the pig's regular diet (Hardham, 2005; Hardy *et al.*, 2007). In the present study, only 10% of the ingested root material was passaged; however, 40% of this was found to contain viable *P. cinnamomi* indicating 4% survival through passage. While this is a significant reduction in inoculum load through digestion, transfer of passaged material into the soil surrounding susceptible host material was demonstrated to cause plant infection. In contrast to these study conditions, the diet of feral pigs means that they have the potential to encounter *P. cinnamomi* infected plant materials as a part of their daily food intake, resulting in a low, but sustained risk of pathogen dissemination.

This study demonstrates the inherent compounding impacts of these two invasive species within native ecosystems, both in Australia and throughout the temperate regions of the world where both species have been introduced. As the plant infection trials have demonstrated, *P. cinnamomi* infected plant materials are able to survive digestion and infect healthy susceptible plants. This is a cause for great concern as 40% of the 5710 plant species found in the South-west Botanical Province are susceptible to *P. cinnamomi* (Shearer et al., 2004). These results also have direct implications for the impact of feral pigs throughout the areas of the world where *P. cinnamomi* and other soil-borne plant pathogens are impacting native ecosystems and also for cultivated tree crops.

In areas where both species are well established, ongoing transportation of *P. cinnamomi* has the potential to introduce and spread locally selected genotypes of the pathogen thus increasing the rate with which these are disseminated and established throughout infested and disease-free areas. Furthermore, this study demonstrates the potential for feral pigs to have an active role in the spread of other invasive species including *Phytophthora* and other soil-borne plant pathogens that infect and colonise plant roots. It is now well documented that hybrid *Phytophthora* species can become important plant pathogens (Brasier, 2008) and Burgess *et al*. (2010) discovered evidence of extensive and common hybridisation with both sexual and somatic hybridisation among *Phytophthora* isolates recovered from native forests, woodlands and waterways in Western Australia. Therefore, feral pigs could bring different *Phytophthora* species in contact with each other which in turn could result in viable pathogenic hybrid species being produced.

It remains unclear how the pathogen survives digestion with no indication of whether or not spore structures are of any significance or if it is simply the plant material which is offering protection from digestion. This may be elucidated with further investigation into determining how the pathogen survives (e.g. as mycelium, chlamydospores, hyphal swellings or oospores) in plant materials, especially in naturally infected materials such as the *B. leptophylla* roots. Furthermore, future studies should focus on the demonstration of the role of feral pigs in the spread of plant pathogens through field based studies within the feral pig populations within areas of high *Phytophthora* disease risk. Considering the ongoing persistence of both *P. cinnamomi* and feral pigs within ecosystems once introduced, this study demonstrates that feral pigs pose a significant threat for dispersal of the pathogen throughout the ecosystem. There is therefore a need to consider and apply stringent feral pig control within disease management programs.

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References

Andjic, V., Hardy, G. E. S., Cortinas, M. N., Wingfield, M. J. & Burgess, T. I. (2007) Multiple gene genealogies reveal important relationships between species of *Phaeophleospora* infecting *Eucalyptus* leaves. *Federation of European Microbiological Societies Microbiology Letters.* 268**,** 22-33.

Argenzio, R. A. & Southworth, M. (1974) Sites of organic acid production and absorption in gastrointestinal tract of the pig. *American Journal of Physiology.* 228**,** 454-460.

Asplund, J. M. & Harris, L. E. (1970) A technique and apparatus for the study of rate of passage of feed residues through the digestive tract of sheep. *Journal of Animal Science.* 31**,** 1199-1204.

Brasier, C. M. (2008) The biosecurity threat to the UK and global environment from international trade in plants. *Plant Pathology.* 57**,** 792-808.

Burgess, T. I., Stukely, M. J. C., Jung, T., White, D., Hüberli, D. and Hardy, G. E. St. J. (2010) Molecular characterisation of a *Phytophthora* hybrid swarm in native ecosystems and waterways in Western Australia. IN: *5th IUFRO Phytophthora Diseases in Forests and Natural Ecosystems, 7 - 12 March*, Auckland and Rotorua, New Zealand.

Carter, R. (2004) Arresting *Phytophthora* Dieback: The Biological Bulldozer. A report by the World Wildlife Fund and the WA Dieback Consultative Council.

Choquenot, D., McIlroy, J. & Korn, T. (1996) Managing vertebrate pests: Feral pigs. Bureau of Resource Sciences, Australian Government Publishing Service, Canberra.

Cooke, D. E. L., Drenth, A., Duncan, J. M., Wagels, G. & Brasier, C. M. (2000) A molecular phylogeny of *Phytophthora* and related oomycetes. *Fungal Genetics and Biology.* 30**,** 17-32.

Erwin, D. C. & Ribeiro, O. K. (1996) *Phytophthora* diseases worldwide, St. Paul, Minnesota, American Phytopathological Society.

Feio, C. L., Bauwens, L., Swinne, D. & De Meurichy, W. (1999) Isolation of *Basidiobolus ranarum* from ectotherms in Antwerp zoo with special reference to characterization of the isolated strains. *Mycoses.* 42**,** 291-296.

Gallo, L., Siverio, F. & Rodríguez-Pérez, A. M. (2007) Thermal sensitivity of *Phytophthora cinnamomi* and long-term effectiveness of soil solarisation to control avocado root rot. *Annals of Applied Biology.* 150**,** 65-73.

Giménez-Anaya, A., Herrero, J., Rosell, C., Couto, S. & García-Serrano, A. (2008) Food habits of wild boars (*Sus scrofa*) in a Mediterranean coastal wetland. *Wetlands.* 28**,** 197-203.

Graham, G. C., Mayers, P. & Henry, R. J. (1994) A simplified method for the preparation of fungal genomic DNA for PCR and RAPD analysis. *BioTechniques.* 16**,** 48 - 50.

Gurevitch, J. & Padilla, D. K. (2004) Are invasive species a major cause of extinctions? *Trends in Ecology & Evolution.* 19**,** 470-474.

Hardham, A. R. (2005) *Phytophthora cinnamomi*. *Molecular Plant Pathology.* 6**,** 589-604.

Hardy, G. E. S. J., Huberli, D., Dunstan, W. & Dell, B. (2007) The dynamics and management of *Phytophthora* in the jarrah (*Eucalyptus marginata*) forest of Western Australia. *Brazilian Phytopathology.* 32 (Supplement)**,** S87-S88.

Hüberli, D., Tommerup, I. & Hardy, G. (2000) False-negative isolations or absence of lesions may cause mis-diagnosis of diseased plants infected with *Phytophthora cinnamomi*. *Australasian Plant Pathology.* 29**,** 164-169.

Keast, D. & Walsh, L. G. (1979) Passage and survival of chlamydospores of *Phytophthora cinnamomi Rands*, the causal agent of forest dieback disease, through the gastrointestinal tracts of termites and wild birds. *Applied and Environmental Microbiology.* 37**,** 661-664.

Kliejunas, J. T. & Ko, W. H. (1976) Dispersal of *Phytophthora cinnamomi* on the Island of Hawaii. *Phytopathology.* 66**,** 457-460.

Knudsen, K. E. B., Jensen, B. B. & Hansen, I. (1993) Digestion of polysaccharides and other major components in the small and large intestine of pigs fed on diets consisting of oat fractions rich in β-D-glucan. *British Journal of Nutrition.* 70**,** 537-556.

Manners, M. J. (1976) The developement of digestive function in the pig. *Proceedings of the Nutrition Society.* 35**,** 49-55.

Martin, H. L. & Dale, M. L. (2001) Potential of *Cactoblastis cactorum* as a vector for fungi pathogenic to pricklypear, *Opuntia inermis*. *Biological Control.* 21**,** 258-263.

Masters, K. B. (1979) Feral pigs in the south-west of Western Australia - Final report to Feral Pig Committee. Department of Agriculture, Perth.

McCauley, I., Matthews, B., Nugent, L., Mather, A. & Simons, J. A. (2005) Evaluation of wireless sensors to measure the effect of microclimate on pig body temperature. IN PATERSON, J. E. (Ed.) *Manipulating pig production X. Proceedings of the Tenth Biennial Conference of the Australasian Pig Science Association (APSA).* Christchurch, New Zealand.

McLeod, R. (2004) Counting the cost: Impact of invasive animals in Australia 2004. Cooperative Research Centre for Pest Animal Control. Canberra

Schley, L. & Roper, T. J. (2003) Diet of wild boar *Sus scrofa* in Western Europe, with particular reference to consumption of agricultural crops. *Mammal Review.* 33**,** 43-56.

Shearer, B. L., Crane, C. E. & Cochrane, A. (2004) Quantification of the susceptibility of the native flora of the South-West Botanical Province, Western Australia, to *Phytophthora cinnamomi*. *Australian Journal of Botany.* 52**,** 435-443.

Teague, H. S. & Hanson, L. E. (1954) The effect of feeding different levels of a cellulosic material to swine. *Journal of Animal Science.* 13**,** 206-214.

Tierney, T. & Cushman, J. (2006) Temporal changes in native and exotic vegetation and soil characteristics following disturbances by feral pigs in a California grassland. *Biological Invasions.* 8**,** 1073-1089.

van der Putten, W. H., Klironomos, J. N. & Wardle, D. A. (2007) Microbial ecology of biological invasions. *International Society for Microbial Ecology.* 1**,** 28-37.

White, T. J., Bruns, T., Lee, S., Taylor, J., Innis, M., Gelfand, D. & Shinsky, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications.* Academic Press.

Zevenhuizen, L. P. T. M. & Bartnicki-Garcia, S. (1969) Structure of the insoluble hyphal wall glucan of *Phytophthora cinnamomi*. *Biochemistry.* 8**,** 1496-1502.

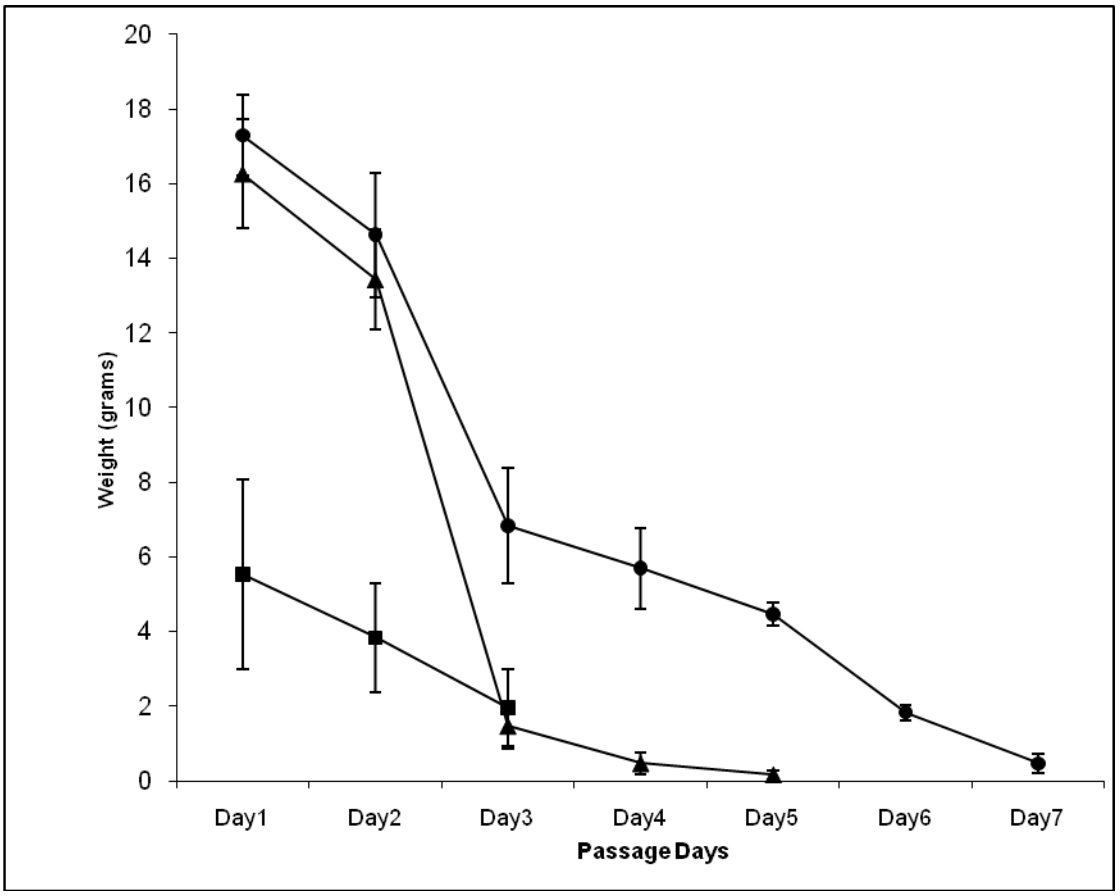

Figure 1: Daily recovery of different ingested plant materials (g) within pig faeces over time (days). Millet seeds (▲), pine plugs (●), *Banksia leptophylla* roots (■). Bars represent standard error of mean mean across the samples of each individual plant material type on day of collection.

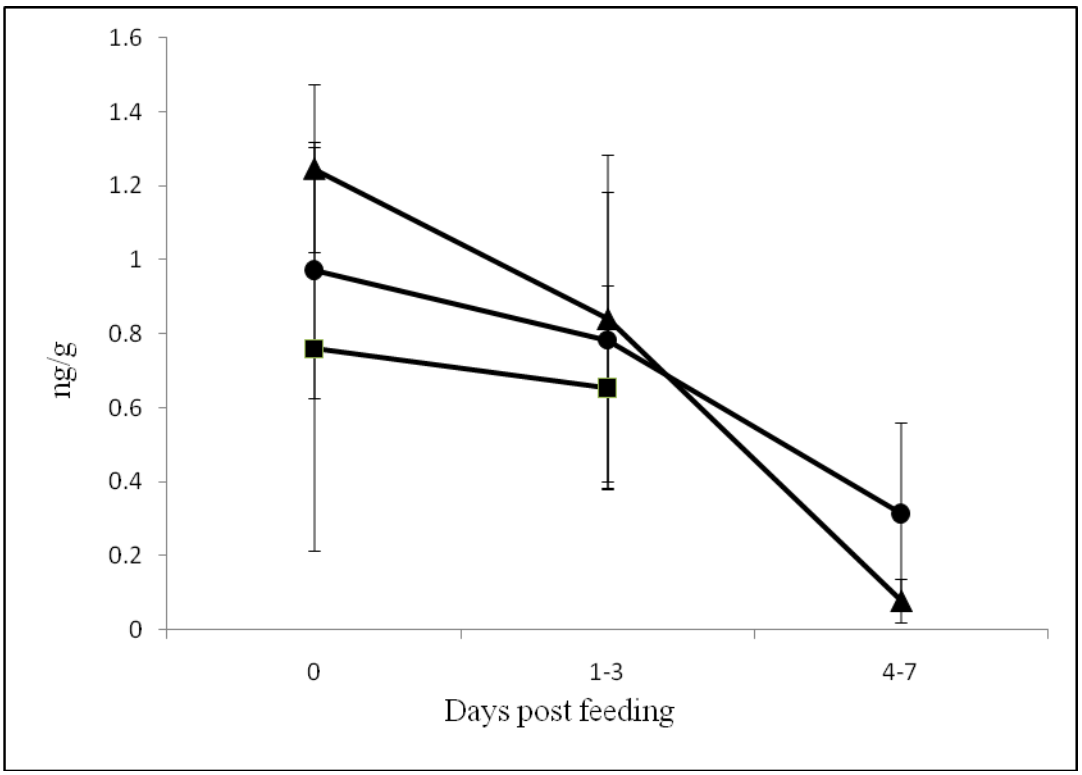

Figure 2: Amount of *P. cinnamomi* DNA concentration (ng) detected by quantitative PCR from *P. cinnamomi* colonised plant substrates passaged through pig digestive tract over time (days) compared to Day 0, the control unfed material. Millet seeds (▲), pine plugs (●), *Banksia leptophylla* roots (■). Bars represent standard errors of the mean across the samples of each individual plant material type on day of collection. No *B. leptophylla* was available for the 4-7 day sample as they were either completely digested or passaged by the 4th day of sampling.

Table 1: Pooling of recovered materials from pig bolus for the detection of *Phytophthora cinnamomi* using different analyses.

Day	Baiting for zoospore production			Plating for mycelium production			PCR detection		
	Millet	Pine	Root	Millet	Pine	Root	Millet	Pine	Root
1	M_{1-3}	P_{1-3}	R_{1-3}	M_{1-3}	P_{1-3}	R_{1-3}	M_{1-3}	P_{1-3}	R_{1-3}
2									
3									
4	NA	P_{4-7}	NA	M_{4-5}	P_{4-7}	NA	M_{4-5}	P_{4-7}	NA
5									
6				NA			NA		
7									

Note: NA- no material or insufficient material available. M_{1-3}: Pooled sample of millet seeds collected from first 72 h after bolus feeding. M_{4-5}: Pooled sample of millet seeds collected after 72 h till last passage. P_{1-3}: Pooled sample of pine plug collected from first 72 h after bolus feeding. P_{4-7}: Pooled sample of pine plug collected after 72 h til last passage. R_{1-3}: Pooled sample of *B. leptophylla* roots collected.

Table 2: Viability of *P. cinnamomi* from the plant materials recovered after passage for different times through pig digestive tracts as assessed by direct plating onto NARPH agar plates and baiting with *L. angustifolius* cv. Mandelup cotyledons.

Sample	Direct Plating on NARPH (% viability)	Baiting
M_{1-3}	25.5% (n=611)	Yes
M_{4-5}	36.6% (n=164)	NA
P_{1-3}	98.3% (n=60)	Yes
P_{4-7}	43.9% (n=57)	Yes
R_{1-3}	41.3% (n=80)	Yes

Note: NA - insufficient material for testing. M_{1-3}: Pooled sample of millet seeds collected from first 72 h after bolus feeding. M_{4-5}: Pooled sample of millet seeds collected after 72 h till last passage. P_{1-3}: Pooled sample of pine plugs collected from first 72 h after bolus feeding. P_{4-7}: Pooled sample of pine plugs collected after 72 h till last passage. R_{1-3}: Pooled sample of *B. leptophylla* roots collected.