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Phase variation in *latB* associated with a fatal *Pasteurella multocida* outbreak in captive squirrel gliders

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Highlights

- A septicaemic disease outbreak caused by *Pasteurella multocida* occurred in a resident group of squirrel gliders following the introduction of two squirrel gliders imported from another zoo
- Whole genome sequencing and phylogenomic analysis confirmed that the outbreak strain was introduced through animal movement between the zoos
- A comparison of *P. multocida* strains from fatal and asymptomatic cases revealed structural differences in their *latB*, a structural LPS gene
- Isolates collected from fatal cases always had a fully intact *latB* gene, which is predicted to code for an acyltransferase

Abstract

A septicaemic disease outbreak caused by *Pasteurella multocida* at a zoo in Western Australia (Zoo A) occurred in a resident group of squirrel gliders (*Petaurus norfolcensis*) following the introduction of two squirrel gliders imported from another zoo (Zoo B). *P. multocida* isolates obtained from the affected animals and asymptomatic, cohabiting marsupials at both zoos were typed via lipopolysaccharide outer core biosynthesis locus (LPS) typing, repetitive extragenic palindromic PCR (Rep-PCR) typing, and multilocus sequence typing (ST). Investigation of isolate relatedness via whole genome sequencing (WGS) and phylogenomic analysis found that the outbreak isolates shared the same genetic profile as those obtained from the imported gliders and the positive marsupials at Zoo B. Phylogenomic analysis demonstrated that these isolates belonged to the same clone (named complex one), confirming that the outbreak strain originated at Zoo B. As well, the carriage of multiple different strains of this pathogen in a range of marsupials in a zoo setting has been demonstrated. Importantly, the genomic investigation identified a missense mutation in the *latB*, a structural LPS gene, resulting in introduction of an immediate stop codon in the

isolates carried by asymptomatic squirrel gliders in Zoo B. The identified diversity in the *latB* gene of LPS outer core biosynthesis loci of these isolates is consistent with a novel phase variable mechanism for virulence in *P. multocida*.

Our study demonstrates the benefit of WGS and bioinformatics analysis in epidemiological investigations of pasteurellosis and its potential to reveal unexpected insights into bacterial virulence.

Keywords *Pasteurella multocida*, marsupials, whole genome sequencing, O-acetylation, *Petaurus norfolcensis*, *Bettongia pencillata*

Introduction

Pasteurella multocida is well recognised as the cause of economically important diseases of domestic livestock (Harper et al., 2006) and waterfowl (Blanchong et al., 2006). In captive wildlife, outbreaks of *P. multocida* septicaemia have been reported in zoo collections affecting a range of species including ungulates, birds and red kangaroos (*Macropus rufus*) during a multi-species outbreak (Okoh, 1980), long-nosed potoroos (*Potorous tridactylus*) (Wells and Montali, 1985) and squirrel gliders (*Petaurus norfolcensis*) (Australian Registry of Wildlife Health, 2006). Published reports of disease in marsupials caused by *P. multocida* are relatively few, with sudden death being reported most frequently (Australian Registry of Wildlife Health, 2006; Okoh, 1980; Wells and Montali, 1985). Other clinical manifestations of *P. multocida* infection in marsupials include neurological disease in squirrel gliders (Holz and Graham, 2008), oral and ocular lesions in red-necked wallabies (*Macropus rufogriseus*) (Bertelsen et al., 2012), pneumonia in macropods (Okoh, 1980;

Stern, 2010) and upper respiratory disease in koalas (*Phascolarctos cinereus*) (Blanshard, 1994).

Until recently, little was known of the carriage of *P. multocida* in the oral cavity of marsupials, but several studies have found that a number of Australasian species can harbour *P. multocida* as part of the oral flora of apparently healthy individuals including: Tasmanian devils (*Sarcophilus harrisii*) (Brix et al., 2015; Hansen et al., 2017), red-necked wallabies (*Macropus rufogriseus*) (Bertelsen et al., 2012), common wombats (*Vombatus ursinus*), common ring-tailed possums (*Pseudocheirus peregrinus*) and eastern quolls (*Dasyurus viverrinus*) (Hansen et al., 2017).

In the current study, we report on an outbreak of pasteurellosis in squirrel gliders. The outbreak occurred in a single enclosure at a zoo following the introduction of two new squirrel gliders imported from another zoo. The epidemiology of the outbreak was investigated by matching animal movements, clinical signs and pathological findings together with molecular characterisation, whole genome sequencing and phylogenomic analysis of *P. multocida* isolates obtained from both diseased and clinically healthy animals at both zoos.

Materials and methods

The outbreak

This study was undertaken at two zoos, coded A and B, in Perth, Western Australia. Mortalities occurred in the squirrel gliders from a single enclosure at Zoo A during October and November 2015. At the time of the outbreak, the affected enclosure contained three resident squirrel gliders (SG3, SG4, SG5), two newly introduced squirrel gliders (SG1 and SG2) and one woylie (W1) (also known as brush-tailed bettong, *Bettongia penicillata*) (Table 1). The three resident squirrel gliders died peracutely over a one-month period, following the introduction of the two new squirrel gliders from Zoo B. Complete necropsy examinations

were performed on the deceased gliders and formalin-fixed tissues were submitted for histopathology. Fresh frozen tissues from two animals (SG4, SG5) were subsequently submitted for bacteriology.

To investigate the epidemiology of the outbreak, oropharyngeal swabs were collected from all squirrel gliders and other cohabiting marsupials at both zoos in the three months following the outbreak (Table 1). All subjects appeared clinically healthy at the time of sampling. All were anaesthetised with isoflurane delivered via facemask for swab collection, to ensure consistent swabbing technique. Swabs were placed in Amies transport medium with charcoal (Transwab®, Medical Wire & Equipment, Corsham, UK) and submitted on ice.

Initial bacteriology

All bacteriology samples were cultured onto agar containing 5% horse blood (HBA), and MacConkey agar (PathWest Media, Perth). Plates were incubated in a carbon dioxide incubator (5% CO₂, Contherm mitre 4000) at 36°C for a minimum of 48 h. Colonies were identified using matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF) and version 8 of the MBT Compass library (MALDI BioTyper, Bruker Daltonics, Germany). Isolates PM2183 and PM2186 from Zoo A, obtained from SG5 and W1, respectively, together with isolates PM2188, PM2192, PM2193 and PM2194 from Zoo B, obtained from SG6, SG15, W2 and RB1, respectively, were sub-cultured on HBA for biochemical identification using conventional methods (Bergey et al., 1974; Cowan and Steel, 1965). Testing for capsular type A or D was performed on PM2183, PM2186, PM2188 and PM2192 according to previously described methods (Carter and Rundell, 1975; Carter and Subronto, 1973), with previously typed porcine strains of *P. multocida* (P92-1432 type A and P92-1734 type D) as controls.

For initial genotyping, the repetitive extragenic palindromic polymerase chain reaction (rep-

PCR) was performed as previously described (Gunawardana et al., 2000), with the exception that the genomic DNA was prepared using the PrepMan Ultra Sample Preparation Reagent.

Whole genome sequencing

All fourteen *P. multocida* isolates were subjected to whole genome sequencing (WGS) and phylogenomic analysis. DNA was extracted using the DNeasy UltraClean Microbial Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions from overnight cultures on 5% sheep blood agar. The DNA samples were then sequenced on the Illumina NextSeq 500 platform (150 bp paired ends) by the Australian Centre for Ecogenomics at the University of Queensland (St Lucia QLD, Australia). The qualities of the paired end raw reads were assessed using FastQC v0.11.5 (Andrews, 2010), and hard trimmed using Nsoni v0.132 (<https://github.com/Victorian-Bioinformatics-Consortium/nesoni>). Quality-trimmed paired end reads were then assembled using SPAdes v3.10.1 (Bankevich et al., 2012). *In-silico* multi-locus sequence typing (MLST) (Jolley et al., 2004) profiling was performed using MLST v2.8 (<https://github.com/tseemann/mlst>) and the *P. multocida* RIRDC-MLST scheme (Subaaharan et al., 2010). A local database consisting of the gene sequences representing the 16 Heddlestone serovar LPS outer core biosynthesis loci was prepared and Blastn v2.2.31+ was used to perform *in-silico* LPS typing and extraction of the corresponding region from the carrying contigs, where the matching LPS type was selected based on the e-value. Geneious 10.0.6 (<https://www.geneious.com>) was used for visualisation and annotation of the LPS region and ClustalW v2.1 (Larkin et al., 2007) for the alignments. Abricate v0.5 (<https://github.com/tseemann/abricate>) was used to determine the antibiotic resistance gene profile.

Parsnp v1.2 (Treangen et al., 2014) was used for core genome alignments and SNP calling in the draft genome assemblies with *P. multocida* strain PM70 as the outgroup. A

phylogenetic tree was then constructed with RAxML x8.2.9 (Stamatakis, 2014) using the core genome SNP alignment after removal of the recombination sites by Gubbins v2.1.0 (Stamatakis, 2014). A general-time reversible nucleotide substitution model with a GAMMA correction for site variation was used for tree construction (bootstrap 1000 with Lewis ascertainment correction) (Stamatakis, 2014). The generated phylogenetic trees were visualised using FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>) and Evolvview (He et al., 2016).

Results

Animal movements, clinical signs and pathology

A schematic representation of animal movement and sampling is shown in Fig 1. The pasteurellosis outbreak at Zoo A resulted in the peracute deaths of three resident squirrel gliders in Enclosure A1 (SG3, SG4 and SG5). The deaths occurred 11, 27 and 45 days respectively after the introduction of the two imported squirrel gliders (SG1 and SG2) from Zoo B into Enclosure A1. Histopathology confirmed septicaemia as the cause of death in all three cases, with bacterial colonisation of multiple organs (Table 1; Fig 2, Panels D and E). Once the common aetiological agent was identified, the remaining two squirrel gliders (SG1 and SG2) were treated prophylactically for eight days with oral amoxicillin clavulanate at 15 mg/kg twice a day and the enclosure was stripped of furniture and substrate and thoroughly disinfected.

SG1 and SG2 had been introduced into Enclosure A1 following a 30 day quarantine period during which they were isolated from all other animals at Zoo A. During quarantine, SG1 developed a soft tissue swelling on its right muzzle, which subsequently developed a draining sinus five days after transfer into Enclosure A1. Treatment was initiated at this time with orally administered amoxicillin clavulanate at 12 mg/kg twice a day for 7 days, which

resulted in apparent resolution. Following recurrence of the swelling two months later, advanced osteomyelitis of the right maxilla was diagnosed, and the squirrel glider was euthanized. Maxillary sinusitis and osteomyelitis were confirmed at necropsy (Fig 2, panels A, B and C).

A woylie (W1), which shared the same enclosure as the squirrel glider group showed no signs of ill health during the outbreak, but was euthanized due to dental disease and other age-related health problems two months after the last squirrel glider death. Two years after the outbreak, another woylie from Zoo A (W3) housed in the same nocturnal house precinct, but with no prior history of exposure to squirrel gliders, was euthanized due to severe pneumonia and pulmonary abscessation. Bacteriology confirmed *P. multocida* as the cause of disease.

Bacteriology

Zoo A

Of the dead squirrel gliders at Zoo A, heavy dominant growth of *P. multocida* was obtained from the liver of SG4, and colonies with the same morphology were obtained from the liver, spleen and kidney of SG5 (Table 1). No tissue from SG3 was submitted for bacteriology.

Oropharyngeal swabs were then collected from the two introduced squirrel gliders SG2 and SG1, 12 and 14 days respectively after completion of the antibiotic course. Light growth of *P. multocida* was obtained from SG2 and moderate growth from SG1, despite the course of antibiotic. Following euthanasia of SG1, culture of the maxillary sinus lesions yielded a heavy growth of *P. multocida* along with mixed anaerobes. Also included from Zoo A were *P. multocida* isolates from the two woylies, W1 (cultured from oropharyngeal swabs in 2016) and W3 (cultured from lung tissue in 2017).

Zoo B

At Zoo B, swabs were taken from five squirrel gliders and two rufous bettongs (*Aepyprymnus rufescens*), from Enclosure B1 (the enclosure from which SG1 and SG2 originated), four squirrel gliders from Enclosure B2 (a separate but adjacent enclosure), one squirrel glider at Enclosure B3 which was previously a resident of B1, and one woylie from Enclosure B4 which had no history of contact with squirrel gliders (Fig 1). *P. multocida* was obtained from four of the five squirrel gliders and one of the two rufous bettongs in Enclosure B1. Swabs from the four squirrel gliders housed in the adjacent Enclosure B2 were negative for *P. multocida*. *P. multocida* was also cultured from the squirrel glider as well as the woylie from Enclosures B3 and B4, respectively (Table 2).

The tested isolates were confirmed as capsular type D as they autoagglutinated when acriflavine was added to a trypticase soy broth culture. Information on the phenotypic characterisation of the isolates are provided in the supplementary file.

Genotyping and phylogenomic analysis of *P. multocida* isolates

The rep-PCR typing (Supplementary Fig 1) as well as the *in-silico* MLST and LPS typing resulted in the recognition of three different profiles in the fourteen *P. multocida* isolates obtained from the two zoos. The two *P. multocida* isolates obtained from the outbreak at Zoo A (PM2182 and PM2183), shared the same ST, rep-PCR and LPS type as those obtained from the newly introduced squirrel gliders as well as those obtained from the Zoo B residents; namely ST 10, rep-PCR 1 and LPS 4 (termed clonal complex one) (Table 2 and Fig 3). However, isolates obtained from woylies W1 (ST 306, rep-PCR 2 and LPS 6 - clonal complex two) and W3 (ST 24, rep-PCR3, LPS type 3 – clonal complex 3) at Zoo A had different genetic profiles compared to each other and the other isolates and were not considered part of the outbreak.

Phylogenetically, the clonal complex 1 isolates obtained from squirrel gliders at Zoo A and Zoo B were very closely related with an overall diversity of 0 to 10 core genome SNPs (relative to the *P. multocida* reference strain PM70) (Fig 3). At Zoo A, *P. multocida* isolates PM2184 and PM2185, obtained from the imported squirrel gliders SG1 and SG2, respectively, were indistinguishable at the core genome level to *P. multocida* isolates collected from squirrel gliders (SG7 and SG15) inhabiting enclosures B1 and B3 at Zoo B. The two outbreak isolates from Zoo A (PM2182 and PM2183 from squirrel gliders SG4 and SG5, respectively) and the second isolate (PM2187) recovered from the primary case, squirrel glider SG1, are separated from the other clonal complex 1 isolates by 4 core genome SNPs. PM2187 and PM2183 were very closely related, differing by only 2 core genome SNPs. The two additional *P. multocida* isolates from Zoo B, PM2193 from woylie W2 and PM2194 from rufous bettong RB1, differed from the squirrel gliders isolates by 18 and 26 SNPs, respectively.

Analysis of lipopolysaccharide (LPS) biosynthesis loci

The LPS outer core biosynthesis loci of the isolates were explored by blasting the *de-novo* assemblies of each isolate against the *in-house* LPS database, as well as using the quality trimmed reads for read mapping against the complete genome of *P. multocida* isolates PM70 and OH1095 (obtained from oral cavity of *Canis lupus* in California). The *in-silico* LPS typing identified three different LPS types between the fourteen *P. multocida* isolates with LPS type L3 in PM2346, L6 in PM2186 and L4 in the remaining twelve isolates.

The sequences of the six transferase genes of the LPS outer core biosynthesis loci type L3 (*natC*, *gatG*, *natB*, *gatF*, *gctC*, *hptE*), carried by isolate PM2346 obtained from W3 were identical to that from *P. multocida* type strain PM70 using both techniques.

The sequences of the four glycosyltransferase genes (*gatL*, *gatK*, *natD*, and *natE*) as well as the acyltransferase gene *latB*, from the squirrel gliders in Zoo A (PM2182, PM2183, PM2185, PM2184 and PM2187 from SG4, SG5, SG2 and SG1, respectively) together with those from isolates PM2193 and PM2190 obtained from W2 and SG8 in Zoo B, were identical to those of Heddleston serovar 6 type strain, P2192. However, the *P. multocida* isolates obtained from the remaining five squirrel gliders in Zoo B (PM2188, PM2189, PM2190, PM2191, and PM2192 obtained from SG6, SG7, SG8, SG9, and SG15, respectively) also carrying LPS outer core biosynthesis loci L4 carried a nucleotide substitution at position 361 of their *latB* gene (C 361A), resulting in introduction of an immediate stop codon (G121X). Isolate PM2194 obtained from the rufous bettong, had a T insertion (position 1511) in its *latB* gene resulting in a frame shift and early termination of translation

Discussion

Whole genome sequencing is a rapidly emerging tool for bacterial epidemiology. The single nucleotide resolution offered by WGS enables transmission pathways between individuals and environments to be readily delineated. Here, WGS was used to support an epidemiological investigation to determine the source and likely transmission routes of a *P. multocida* outbreak in a Western Australia Zoo (Zoo A).

Comparison of isolates collected from squirrel gliders at Zoo A and Zoo B clearly indicates that the *P. multocida* outbreak isolate originated at Zoo B and the transfer of animals (in this case squirrel gliders SG1 and SG2) from Zoo B to Zoo A was responsible for introducing the outbreak strain into Zoo A with subsequent onward transmission to resident

squirrel gliders (SG4 and SG5). The full discussion on the epidemiology of the disease in provide in supplementary file.

In Zoo B, clinically normal squirrel gliders, a rufous bettong and a woylie were all positive by culture for *P. multocida*, with all isolates found to belong to the same clade (clonal complex one) as that causing the outbreak at Zoo A. The phylogenetic analysis of the core genome SNPs of the fourteen *P. multocida* isolates indicated that most squirrel glider clonal complex one isolates were identical at the core genome level, consistent with recent inter-animal transmission. In contrast, the *P. multocida* genomes obtained from the woylie and rufous bettong at Zoo B, each differed by more than 20 core genome SNPs from the squirrel glider *P. multocida* genomes. These genetic distances are consistent with a common source of *P. multocida* in the Zoo B environment, although a more temporally diverse dataset would be needed to estimate divergent dates.

Each of the clonal complexes carried different LPS outer core biosynthesis loci with the outbreak clone carrying LPS type 4 and the isolates obtained from the woylies in 2016 and 2017 carrying LPS type 6 (PM2186) and LPS type 3 (PM2346), respectively. LPS type 3 is the most prevalent LPS type carried by the Australian fowl cholera isolates (Harper et al., 2013), has been found in diseased pigs in Australia (Turni et al., 2018) and has been recently found in association with pasteurellosis in captive pinnipeds (Crawford et al., 2019).

Historically, LPS type 4 has been associated with avian isolates of *P. multocida* with the Heddlestone serovars 6 and 7 type strains obtained from a chicken and a herring gull, respectively (Brogden and Packer, 1979). In Australia, an ST 10, LPS type 4-carrying isolates has been previously found in association with chickens (Turni et al., 2018). The fact that this type has been previously found in an avian host, suggests that LPS 4-bearing strains might be found in different geographical regions as a result of wild bird movements.

The LPS outer core biosynthesis locus type 4 consists of four glycosyltransferase coding genes named *gatL*, *gatK*, *natD* and *natE* as well as one acetyltransferase gene, *latB*. *P. multocida* serovar 6 type strain has the capacity to produce a full length LPS outer core while serovar 7 type strain only produces a truncated LPS as a result of a mutation in the *natE* gene resulting in early termination of translation of this glycosyltransferase gene (Harper et al., 2015). Four of the five *P. multocida* isolates obtained from the squirrel gliders in Zoo B and a single isolate from a rufous bettong at the same zoo, carried a nucleotide change in their *latB* gene, predicted to result in an early termination of translation. The *latB* gene is predicted to code for an acyltransferase which shares 34% amino acid identity with the *O*-acetylase OafA in non-typable *Haemophilus influenzae* (NTHi) (Harper et al., 2015). Hence, *latB* is predicted to be responsible for the *O*-acetylation of the first two *N*-acetyl-galactosamine (GalNAc) residues in the LPS outer core of *P. multocida* serovar 6 reference strain (Harper et al., 2015). The fact that the outbreak-associated isolates carried the full-length *latB* gene, hence *O*-acetylated LPS outer core, whilst 80% of the *P. multocida* isolates carried by squirrel gliders of Zoo B had a non-functional *latB*, is in agreement with the recently published data on the variation of expression of OafA in NTHi (36). In NTHi, *oafA* is a phase variable gene which is switched off in the isolates obtained from the nasopharynx of healthy carriers, hence the inner core of LPS of those non-invasive isolates were not *O*-acylated, while it was fully functional in the majority of the disease associated isolates of NTHi (Phillips et al., 2019). The acetylation of the outer surface oligosaccharides has been suggested to result in increased virulence of bacterial pathogens, perhaps through higher resistance to the host complement-mediated innate immunity (Phillips et al., 2019). Therefore, turning on this gene in *P. multocida* may be a novel phase variable virulence factor that could contribute to a switch from colonisation to infection.

P. multocida was cultured from the introduced squirrel gliders after prophylactic antimicrobial treatment indicating that treatment had not eliminated the organism from the oral cavity. Moreover, our phylogenetic analysis of the outbreak isolates showed that a second isolate (PM2187) from the primary case (SG1), obtained post antibiotic treatment, and two outbreak isolates (PM2182 and PM2183) form a nested sub-clade separate from the other clonal complex one isolates. Selection pressure, associated with antimicrobial treatment, could be driving the rapid accumulation of SNPs and microevolution within the primary patient. Alternatively, the primary patient may have been colonised with a population of very closely related *P. multocida* strains. It would be necessary to collect a number of single colonies from the primary isolation plates to determine if any within-host diversity of *P. multocida* can be found in either an apparently healthy or a diseased marsupial. Our laboratory has previously reported within-host variations in the core genome of *P. multocida* isolates obtained from cases of pasteurellosis in captive pinnipeds (Crawford et al., 2019). Advances in shotgun metagenome sequencing such as strain-level discrimination and long-read technologies hold great promise for better integrating *P. multocida* ecology with epidemiology.

Our genomic data has conclusively confirmed that the introduction of the animals from Zoo B (with one of these animals displaying recurrent facial swelling with maxillary sinusitis and osteomyelitis) was the cause of this outbreak. This is the first report of the use of WGS and bioinformatics analysis in epidemiological investigations into pasteurellosis outbreaks in captive marsupials. The results provide clear evidence of the power of these techniques to trace the source of the infection. We recommend that specific diagnostic testing for *P. multocida* is considered prior to the introduction of new squirrel gliders into established groups, including wild translocations. Additionally, cross-species transmission of *P.*

multocida should be considered as a potential biosecurity risk to marsupial collections in zoos.

Supplementary information

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Ethics

The animal ethics committee of the Perth Zoo does not require ethics approval for disease outbreak investigations undertaken by the zoo veterinary staff.

Availability of data and materials

The datasets supporting the conclusions of this article are available from NCBI Sequence Read Archive (SRA) repository [PRJNA542763, raw Illumina reads from the SAMN11638060 to SAMN11638073 <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA542763>].

Competing interests

The authors declare that they have no competing interests.

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Table 1 Full details of the original outbreak at Zoo A and the subsequent investigations at Zoo A and Zoo B

Animal ID	Age ^b	Sex	Zoo-Enclosure	Sample collection date	Clinical signs & Pathology	Culture results	PM code
SG3	10	F	A-1	23 Oct 15	Moribund. Died shortly after presentation. Suppurative endometritis, glomerulonephritis and splenitis with intralesional bacteria	Not performed	-
SG4	1	F	A-1	8 Nov 15	Sudden death. Suppurative endometritis, intravascular bacterial colonisation in heart, brain, kidney and liver	<i>P. multocida</i> from liver	PM2182
SG5	11	M ^c	A-1	26 Nov 15	Sudden death. Acute nephritis, splenitis and hepatitis with intralesional bacteria, incidental renal cyst	single morphology colonies from liver, kidney and spleen	PM2183
SG1 ^a	2	F	A-1				
sample A				16 Dec 15	Recurrent facial swelling with draining sinus	<i>P. multocida</i> from oral swab	PM2184
sample B				5 Feb 16	Maxillary sinusitis and osteomyelitis. Euthanized	<i>P. multocida</i> & mixed anaerobes from maxillary sinus	PM 2187
SG2 ^a	2	F	A-1	18 Dec 15	None	<i>P. multocida</i> from oral swab	PM2185
W1	15	F	A-1	28 Jan 16	Severe dental disease; euthanized	<i>P. multocida</i> from oral swab	PM2186
SG6	NR	M	B-1 ^d	17 Feb 16	None	<i>P. multocida</i> from oral swab	PM2188
SG7	NR	M	B-1	17 Feb 16	Mild dental disease	<i>P. multocida</i> from oral swab	PM2189
SG8	NR	F	B-1	17 Feb 16	Mild dental disease	<i>P. multocida</i> from oral swab	PM2190 ¹⁹

SG9	NR	M	B-1	17 Feb 16	None	<i>P. multocida</i> from oral swab	PM2191
SG10	NR	F	B-1	17 Feb 16	None	Negative (oral swab)	-
RB1	1	M	B-1	17 Feb 16	None	<i>P. multocida</i> from oral swab	PM2194
RB2	1	F	B-1	17 Feb 16	None	Avibacterium gallinarum	-
SG11	1	F	B-2 ^c	17 Feb 16	None	Negative (oral swab)	-
SG12	1	F	B-2	17 Feb 16	None	Negative (oral swab)	-
SG13	1	F	B-2	17 Feb 16	None	Negative (oral swab)	-
SG14	NR	F	B-2	17 Feb 16	None	Negative (oral swab)	-
SG15 ^f	NR	F	B-3	17 Feb 16	Severe dental disease, previously housed at B-1	<i>P. multocida</i> from oral swab	PM2192
W2 ^g	6	M	B-4	17 Feb 16	None	<i>P. multocida</i> from oral swab	PM2193
W3	9	F	A-2	28 Aug 17	Severe bronchopneumonia, septic arthritis, euthanized.	<i>P. multocida</i> from lung	PM2346

Abbreviations; RB - rufous bettong; SG - squirrel glider; W - woylie; PM code - *P. multocida* code; NR - not recorded.

^a Gliders moved from Zoo B to Zoo A. ^b years. ^c castrated male. ^d the enclosure where SG1 and SG2 originated. ^e This enclosure was separate from B1 but adjacent to it. ^f SG15 was a previous resident of B1. ^g this woylie had no previous contact with the squirrel gliders. Zoo A:

enclosures A1 and A3 are 10 meters apart. Zoo B: B1/B2 are adjacent (less than 1 meter apart); B3 and B4 are approximately 10 meter from B1/B2.

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Table 2 Summary of the disease investigations undertaken in this study. The key information on the animals examined, their location in terms of the zoo and the enclosure as well as the outcome of the key typing methods are provided.

Study	Host species	Zoo, Enclosure	Number ^a	<i>P. multocida</i> ID	Clonal complex	<i>latB</i>	Notes
Zoo A							
2015 outbreak	SG	A1	3 (2)	PM2182, PM2183	One	Full-length	Sudden death
2016 swabs	SG	A1	2 (2)	PM2184, PM2185, PM2187 ^b	One	Full-length	2 sample were collected from the squirrel glider with sinusitis
	W	A1	1 (1)	PM2186	Two		Severe dental disease
2017 death	W	A2	1 (1)	PM2346	Three		Pneumonia
Zoo B							
2016 swabs	SG	B1	5 (4)	PM2188, PM2189, PM2190 ^c , PM2191	One	G121X	-
	RB	B1	2 (1)	PM2194 ^d	One	1151insT	-
	SG	B2	4 (0)	-	-	-	No history of contact with B1 residents
	SG	B3	1 (1)	PM2192	One	G121X	Previous resident of B1
	W	B4	1 (1)	PM2193	One	Full-length	

Abbreviation: ID – identification number; RB – rufous bettong; SG – squirrel glider; W – woylie; . Clonal complex one; ST10- repPCR1- LPS4.

Clonal complex two; ST306-repPCR2- LPS6. Clonal complex three; ST24 - repPCR3-LPS3. LPS – lipopolysaccharide genotype; repPCR –

Repetitive Element Palindromic PCR; ST = sequence type within the RIRDC multi-locus sequence typing scheme. ^a Number of marsupials affected/sampled, the numbers in brackets indicate the number of marsupials from which *P. multocida* was cultured. ^b PM2182 was obtained from SG4, PM2183 from SG5, PM2184 and 2187 from SG1 and PM2185 from SG2. ^cPM2190 obtained from SG8 in Zoo B carried a full *latB* gene. ^d the *latB* gene from RB1 in Zoo B had a T insertion resulting in a frame shift and early termination of translation.

Figure legends

Figure 1. Schematic diagram representing animal movement between the zoos and the outbreak timing as well as sample collection dates. Alternating grey and white columns represent periods of approximately 10 days. The Zoo B marsupials marked by a red arrow carried a non-functional (phase variant) *latB* gene.

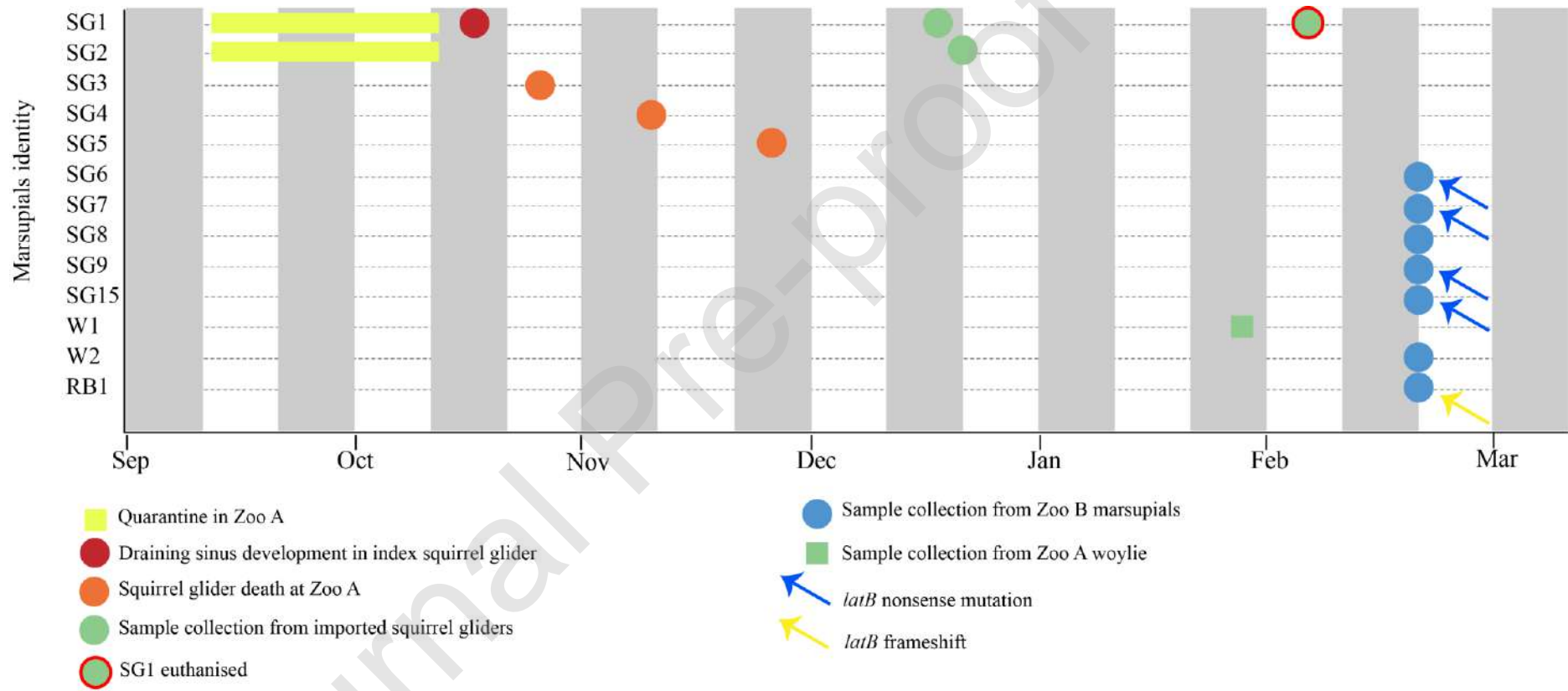


Figure 2. Histopathology of lesions associated with pasteurellosis in squirrel gliders. Panels A, B, and C; sinusitis in the squirrel glider with maxillary osteomyelitis (SG1) Panel A, Longitudinal bisection of the skull showing purulent exudate in the right maxillary sinus, panels B and C severe sinusitis with sinus lumen plugged with mixed cell exudate within which numerous bacterial colonies could be observed. Panels D (Gram's stain) and E; Gram-negative bacterial thrombi in glomerulus of SG3.

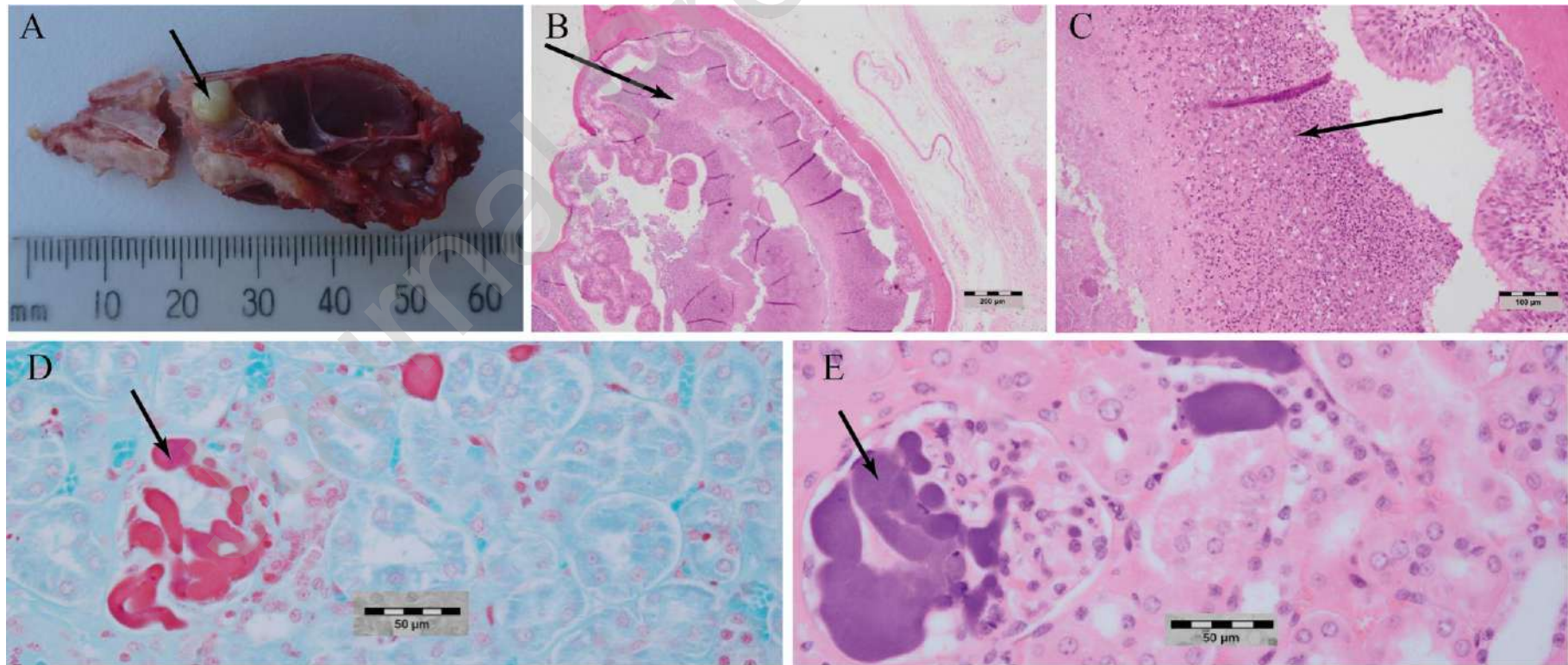
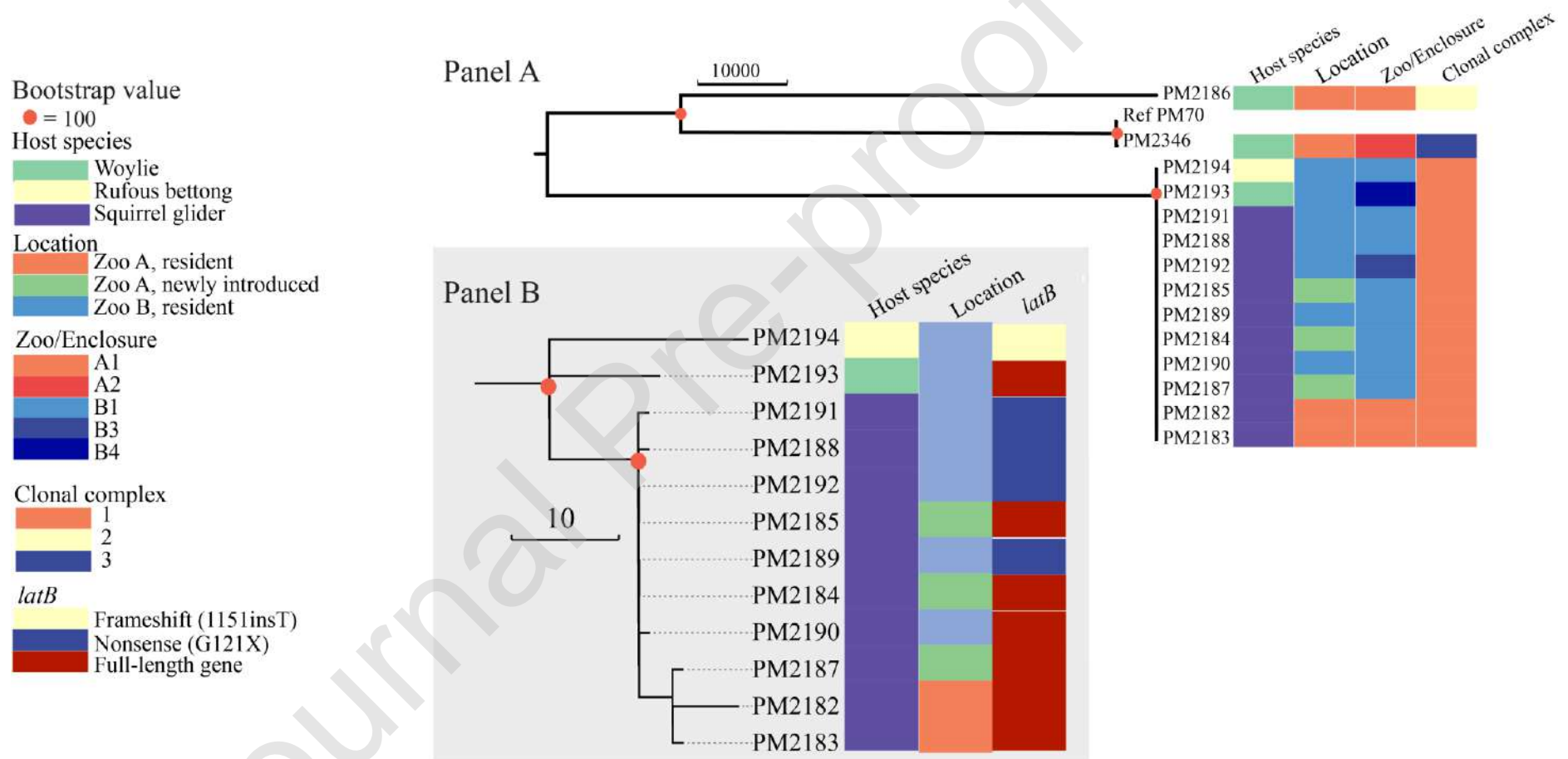


Figure 3. Core SNP maximum likelihood analysis of the *P. multocida* isolates obtained in this study. *P. multocida* strain PM70 was used as the reference and the tree has been midpoint rooted. Panel A; SNP analysis of all 14 isolates. Panel B; zoomed in look at the clonal complex one isolates. Clonal complex one; Rep1, MLST 10, LPS 4. Clonal complex two; Rep 2, MLST 306, LPS 6. Clonal complex three; Rep 3, MLST 24, LPS 3.



Supplementary Fig 1. Representatives of the three rep-PCR profiles found in the fourteen *P. multocida* isolates in this study. Lane 1 – Molecular weight marker; lane 2 PM 2186 – rep-PCR pattern 2; lane 3 PM 2190 – rep-PCR pattern 1; lane 4 PM 2192 – rep-PCR pattern 1; lane 5 PM 2193 – rep-PCR pattern 1; lane 6 PM 2346 – rep-PCR pattern 3; Lane 7 – Molecular weight marker.

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