

# A chromosome-level genome of *Antechinus flavipes* provides a reference for an Australasian marsupial genus with suicidal reproduction

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## Abstract

The 15 species of small carnivorous marsupials that comprise the genus *Antechinus* exhibit semelparity, a rare life-history strategy where death occurs after one breeding season. *Antechinus* males, but not females, age rapidly (demonstrate organismal senescence) during the breeding season and show promise as new animal models of ageing. Some antechinus species are also threatened or endangered. Here, we report chromosome-level genomes of the yellow-footed antechinus *Antechinus flavipes*. The genome assembly has a total length of 3.2 Gb with a contig N50 of 51.8 Mb and a scaffold N50 of 636.7 Mb. We anchored and oriented 99.7% of the assembly on seven pseudochromosomes and found that repetitive DNA sequences occupy 51.8% of the genome. Draft genome assemblies of three related species in the subfamily Phascogalinae, two additional antechinus species (*A. argentus* and *A. arktos*) and the iteroparous sister species *Murexia melanurus* were also generated. Preliminary demographic analysis supports the hypothesis that climate change during the Pleistocene isolated species in Phascogalinae and shaped their population size. A transcriptomic profile across the *A. flavipes* breeding season allowed us to identify genes associated with aspects of the male die-off. The chromosome-level *A. flavipes* genome provides a steppingstone to understanding an enigmatic life-history strategy and a resource to assist the conservation of antechinuses.

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## Abstract

The 15 species of small carnivorous marsupials that comprise the genus *Antechinus* exhibit semelparity, a rare life-history strategy where death occurs after one breeding season. *Antechinus* males, but not females, age rapidly (demonstrating organismal senescence) during the breeding season and show promise as new animal models of ageing. Some antechinus species are also threatened or endangered. Here, we report chromosome-level genomes of the yellow-footed antechinus *Antechinus flavipes*. The genome assembly has a total length of 3.2 Gb with a contig N50 of 51.8 Mb and a scaffold N50 of 636.7 Mb. We anchored and oriented 99.7% of the assembly on seven pseudochromosomes and found that repetitive DNA sequences occupy 51.8% of the genome. Draft genome assemblies of three related species in the subfamily Phascogalinae, two additional antechinus species (*A. argentus* and *A. arktos*) and the iteroparous sister species *Murexia melanurus* were also generated. Preliminary demographic analysis supports the hypothesis that climate change during the

Pleistocene isolated species in Phascogalinae and shaped their population size. A transcriptomic profile across the *A. flavipes* breeding season allowed us to identify genes associated with aspects of the male die-off. The chromosome-level *A. flavipes* genome provides a steppingstone to understanding an enigmatic life-history strategy and a resource to assist the conservation of antechinuses.

**Keywords :** *Antechinus* ; dasyurid; semelparity; genome assembly; chromosome-level; suicidal reproduction

## 1. INTRODUCTION

For every animal species, the most critical activity is mating and the raising of offspring. Reproduction is a costly process, and animals invest energy into strategies that maximize the number and survival of offspring (Collett, Baker, & Fisher, 2018). Broadly, these strategies span the so-called iteroparity-semelparity continuum – from a single (*semel*) to repeated (*itero*) reproductive episode(s) before death [reviewed in (Hughes, 2017; Krajewski, Woolley, & Westerman, 2000)]. In mammals, semelparity is observed in Australian dasyurids (males-only) and South American didelphids (both sexes) (Fisher, Dickman, Jones, & Blomberg, 2013; Leiner, Setz, & Silva, 2008). Obligate male semelparity is the most extreme form of semelparity, occurring in the marsupial subfamilies Phascogalinae (15 *Antechinus* species and three *Phascogale* species (A. J. Bradley, 2003; Naylor, Richardson, & McAllan, 2008)) and Dasyurinae (the kaluta, *Dasykaluta rosamondae* (Hayes et al., 2019)). In these dasyurid species, all males die soon after a 1-3 week mating period ('die-off' at 11.5 months of age), and a proportion of females survive to breed in the subsequent year (A. Baker & Dickman, 2018).

Breeding is a stressful life-history event, but the stress response enables most animals to cope and to mobilise energy (Romero, 2004). Hormones play a vital role in this process. While in antechinus, both sexes have elevated stress hormone levels (chiefly the corticosteroid cortisol) during breeding, males also exhibit surging plasma testosterone levels (A. J. Bradley, 2003; Braithwaite & Lee, 1979; Croft, 2003; McAllan, 2006; Naylor et al., 2008). This interferes with negative feedback mechanisms and reduces levels of corticosteroid binding globulin (CBG), increasing the levels of free (i.e., unbound), active corticosteroids in the blood (A. J. Bradley, 2003; Naylor et al., 2008). As the breeding season progresses, corticosteroid levels continue to rise; ultimately contributing to systemic collapse and death of all males. Corticosteroids are anti-inflammatory and, at high concentrations, immunosuppressive, triggering a decline of body condition (Manoli et al., 2007; Perogamvros, Ray, & Trainer, 2012). Stress, combined with malnutrition, results in a significant negative energy balance and is associated with numerous comorbidities (Argiles, Campos, Lopez-Pedrosa, Rueda, & Rodriguez-Manas, 2016). Cortisol is also elevated in females throughout the short breeding season, but in the absence of elevated testosterone, free cortisol levels remain relatively constant.

There is mounting evidence that testosterone and cortisol contribute to differences in ageing and related diseases (Austad & Fischer, 2016; Gems, 2014; Lemaitre et al., 2020; Martocchia et al., 2016). Moreover, ageing (Childs et al., 2017; Froy et al., 2019) and obligate semelparity manifest as a decline in immune function – immunosenescence. In contrast to current mammal models of ageing, antechinuses are ‘natural’ models of physiological senescence (see (Diamond, 1982; McAllan, 2006)). For example, the brain of the ageing wild-type mouse and rat does not accumulate amyloid-beta (A $\beta$ ) (Jucker, 2010). A $\beta$  plaques, which are associated with chronic stress and elevated cortisol levels [e.g., see (Lesuis, Weggen, Baches, Lucassen, & Krugers, 2018)], accumulate at the end of the antechinus lifespan in antechinuses – mirroring Alzheimer’s disease neuropathology and potentially making them a suitable disease model (McAllan, 2006; Naylor et al., 2008). A high-quality genome assembly is critical for future genetic experiments (e.g., CRISPR). Moreover, considering the strong effects of cortisol and testosterone on male obligate semelparity, identifying the expression of genes associated to these hormones and its consequences may help to understand the evolution of this extreme reproductive strategy.

Recent work has uncovered several new antechinus species, bringing the total number of species to 15 (Mutton, Phillips, Fuller, Bryant, & Baker, 2019). In 2018, *A. arktos* and *A. argentus* were listed as Endangered on the Australian Government’s threatened species list and were included in the 20 Australian mammals most likely to go extinct in the next two decades (Geyle et al., 2018). Moreover, areas of their

habitat were recently ravaged by extensive wildfires and drought. Genomic resources are urgently required to support research on the population structure, speciation, and recovery of antechinus species.

To investigate the phylogeny, demographic history, and genomic diversity of *A. flavipes* and to pave the way to understanding the evolution of semelparity, high-quality genomic data is required. A genome assembly of the brown antechinus (*A. stuartii*) was recently reported (Brandies, Tang, Johnson, Hogg, & Belov, 2020a). However, a chromosome-level genome is not yet available. PacBio long-read sequencing combined with the chromosome conformation capture method Hi-C resolves complex regions (such as repeat regions) and can greatly improve downstream analyses (Schloissnig et al., 2021). Here, we employed third-generation sequencing technologies to generate the first chromosome-level genome of an antechinus, that of the yellow-footed antechinus (*A. flavipes*), as well as draft genomes of three related dasyurids (**Figure 1a**). We highlight genome features, phylogeny and historical demography, and the gene expression profile of *A. flavipes* male die-off.

## 2. MATERIALS AND METHODS

### 2.1 Sample collection

‘AdamAnt’, an adult male yellow-footed antechinus (*Antechinus flavipes*) was sampled in Samford Valley (greater Brisbane), QLD, Australia ( $27^{\circ} 22' S$ ,  $152^{\circ} 52' E$ ) in September 2018. A second male individual (‘Ant2’), sampled at the same location in 2017, was not used for genome assembly but employed in demographic analysis. Animals were euthanised by inhalation of CO<sub>2</sub>. Once death was confirmed, animals were placed directly on dry ice in the field and transferred to a -80 degC freezer for later dissection. Tissue specimens for short-insert whole-genome sequencing were obtained from the black-tailed dusky antechinus *A. arktos* (ear tissue; Lamington National Park, QLD, Australia,  $28^{\circ} 22' S$ ,  $153^{\circ} 15' E$ ; specimen AA100 sampled by A.M.B. in 2015), the silver-headed antechinus *A. argentus* (ear tissue; from Blackdown Tableland National Park, QLD, Australia;  $23^{\circ} 82' S$ ,  $149^{\circ} 07' E$ ; specimen BD-17-5A sampled by A.M.B. in 2017), and the black-tailed dasyure (*Murexia melanurus*; liver tissue; Wau, Morobe, Papua New Guinea,  $7^{\circ} 20' S$ ,  $146^{\circ} 42' E$ ; specimen ABTC46020 sampled by the South Australian Museum in 1985). All DNA and RNA were extracted by BGI Hong Kong.

### 2.2 Reference genome sequencing and assembly

We used a combination of long-fragment sequencing, short-insert library sequencing for error correction and gapfilling, and chromatin conformation capture (Hi-C) to generate chromosome-level semelparous mammal reference genomes. High-molecular weight (HMW) DNA extracted from the testis of the *A. flavipes* individual ‘AdamAnt’ was used to generate long-read (PacBio) sequencing data by Annoroad Gene Technology (Beijing, China). Paired-end (2 x 100 bp) BGI-SEQ500 data were generated from cerebrum, liver, heart, and lung tissue from the same individual by BGI-Qingdao. A total of 323.85 Gb (~100x) *A. flavipes* PacBio reads were assembled using Canu v1.7 (Koren et al., 2017) with the error correction module. The corrected subreads were used for initial draft assembly using Wtdbg2 v1.2.8 (Ruan & Li, 2020). To reduce base errors, the assembly was ‘polished’ using Pilon v1.23 (Walker et al., 2014) and 151.43 Gb (50x) 100 bp paired-end BGISEQ-500 reads [mapped to the initial PacBio assembly using Minimap2 v2.10 (Li, 2018) and SAMtools v1.9 (Li et al., 2009)].

Genome sizes were estimated by *k*-mer frequency analysis (Liu et al., 2013). Briefly, 100 bp paired-end WGS reads were used as input into the GCE (Genomic Character Estimator) v1.0.0 (Marcais & Kingsford, 2011) to obtain the *k*-mer frequency and the genome size was estimated using the equation ‘Genome size = *k*-mer number / *k*-mer depth’, where the ‘*k*-mer number’ is the total number of *k*-mers and ‘*k*-mer depth’ denotes the peak frequency that occurred more than any other frequencies. Genome length was estimated on the basis of total scaffold length of the assembly. Using the frequency distribution of 17-mers of short paired-end reads (**Figure S1**), the *A. flavipes* genome was estimated to be 3.2 Gb.

Assembly quality was assessed using BUSCO (Benchmarking Universal Single-Copy Orthologs) v5.0.0\_cv1 (Seppey, Manni, & Zdobnov, 2019), employing the gene predictor AUGUSTUS v3.2.1 (Stanke & Waack,

2003) and the 9,226-gene BUSCO mammalian lineage data set (mammalia\_odb10). Although, gene centric, the BUSCO Score is a good predictor of genome completeness (Seppey et al., 2019).

### 2.3 Assignment of scaffolds to chromosomes

*A. flavipes* Hi-C libraries from liver tissue were prepared, as described previously (Lieberman-Aiden et al., 2009). Hi-C libraries were sequenced on the BGISEQ-500 platform and quality controlled using the HiC-Pro (v2.8.0\_devel) (Servant et al., 2015) pipeline, resulting in 51.0 Gb uniquely aligned read pairs. Reads validated by HiC-Pro were next used to scaffold contigs into seven chromosome clusters ( $2n = 14$  karyotype) using the 3D-DNA v1.12 pipeline (O. Dudchenko et al., 2017). The assembly was further improved by interactive correction using Juicebox v1.11.08 (Olga Dudchenko et al., 2018; Durand et al., 2016). A chromatin contact map can be found in **Figure S2**.

### 2.4 Draft genomes assemblies of dasyurids

Voucher specimens of the Australian dasyurids *A. arktos* and *A. argentus*, and the New Guinean dasyurid *M. melanurus* were sequenced by short-insert whole-genome sequencing (WGS) (see **Table S1**). Reference-based (i.e., ‘pseudo’) nuclear genomes were obtained by mapping to the *A. flavipes* reference genome [see (Feigin et al., 2018) and Supplemental Methods].

### 2.5 RNA-seq and transcriptome data processing

*A. flavipes* were obtained by overnight live-trapping in Samford Valley, QLD, Australia (27° 22' S, 152° 52' E). Animals were euthanised by CO<sub>2</sub> and tissues frozen at -80 degC. Eight specimens collected in 2017 were sequenced by BGI Hong Kong. The RNA of the remainder [seven males and six females collected in 2018] was extracted by BGI Hong Kong and sequenced by BGI Qingdao. Only the samples collected during the 2018 breeding season in Samford Valley were used for differential gene expression analysis (the samples collected in 2017 were used for gene annotation only). Raw data was filtered using Flexbar v3.4.0 (Dodd, Roehr, Ahmed, & Dieterich, 2012; Roehr, Dieterich, & Reinert, 2017) with default settings (removes reads with any uncalled bases). BGISEQ-500 adapters were obtained from a comparative study of Illumina and BGI sequencing platforms (Mak et al., 2017). Any residual ribosomal RNA reads (the majority removed by poly(A) selection prior to sequencing library generation) were removed from the reads using SortMeRNA v2.1b (Kopylova, Noe, & Touzet, 2012) against the SILVA v119 ribosomal database (Quast et al., 2013). Tissue transcriptomes were de novo assembled using Trinity v2.8.4 (Grabherr et al., 2011; Haas et al., 2013; Henschel et al., 2012) with default parameters. The *A. flavipes* transcriptome assemblies were assessed using BUSCO (Seppey et al., 2019). Details of transcriptome sequencing data used in study are listed in **Tables S2-S4** (also see Supplemental Methods).

### 2.6 Genome annotation

#### Repeat element annotation

We identified repetitive elements by integrating homology and de novo predictions. Homology-based transposable elements (TEs) annotation were obtained by interrogating a genome assembly with known repeats in the Repbase database v16.02 (Bao, Kojima, & Kohany, 2015) using RepeatMasker v4.0.5 (DNA-level) (Tarailo-Graovac & Chen, 2009) and RepeatProteinMask (protein-level; implemented in RepeatMasker). De novo TE predictions were obtained using RepeatModeler v1.1.0.4 (Smit & Hubley, 2010) and LTRharvest v1.5.8 (Ellinghaus, Kurtz, & Willhoefft, 2008) to generate database for a RepeatMasker run. Tandem Repeat Finder (v4.07) (Benson, 1999) was used to find tandem repeats (TRs) in the genome. A non-redundant repeat annotation set was obtained by combining the above data. Genome sequences of other marsupials were also interrogated using the repeat element annotation pipeline. The following were downloaded from DNA Zoo [Virginia opossum (*Didelphis virginiana*; dv-2k (Olga Dudchenko et al., 2018))], the NCBI Reference Sequence Database (Release 86) (Pruitt, Tatusova, & Maglott, 2007) [gray short-tail opossum (*Monodelphis domestica*; MonDom5; GCF\_000002295.2), koala (*Phascolarctos cinereus*; phaCin-unsw\_v4.1; GCA\_002099425.1), Tasmanian devil (*Sarcophilus harrisii*; Devil\_ref v7.0, also known as sarHar1; GCA\_000189315.1), Tammar wallaby (*Notamacropus eugenii*; Meug\_1.1; GCA\_000004035.1), and common

wombat (*Vombatus ursinus* ; GCF\_900497805.2)], and the GigaScience Database [brown antechinus (*Antechinus stuartii* ; (Brandies et al., 2020a; Brandies, Tang, Johnson, Hogg, & Belov, 2020b)].

### Protein-coding gene annotation

Protein-coding genes of *A. flavipes* and *Didelphis virginiana* were annotated using homology-based prediction, de novo prediction, and RNA-seq-assisted prediction methods. Sequences of homologous proteins from five mammals [human (*Homo sapiens* ),*M. domestica* ), *P. cinereus* , *S. harrisii* , and *V. ursinus* ] were downloaded from NCBI. These protein sequences were aligned to the repeat-masked genome using BLAT v0.36 (Kent, 2002). Genewise v2.4.1 (Birney, Clamp, & Durbin, 2004) was employed to generate gene structures based on the alignments of proteins to the genome assembly. De novo gene prediction was performed using AUGUSTUS v3.2.3 (Stanke et al., 2006), GENSCAN v1.0 (Burge & Karlin, 1997), and GlimmerHMM v3.0.1 (Majoros, Pertea, & Salzberg, 2004) with a human training set. Transcriptome data were mapped to the assembled genome using HISAT2 v2.1.0 (Kim, Paggi, Park, Bennett, & Salzberg, 2019) and SAMtools v1.9 (Li et al., 2009), and coding regions were predicted using TransDecoder v5.5.0 (Grabherr et al., 2011; Haas et al., 2013). A final non-redundant reference gene set was generated by merging the three annotated gene sets using EVidenceModeler v1.1.1 (EVM) (Haas et al., 2008) and excluding EVM gene models with only ab initio support. The gene models were translated into amino acid sequences and used in local BLASTp (Camacho et al., 2009) searches against the public databases Kyoto Encyclopedia of Genes and Genomes (KEGG; v89.1) (Kanehisa & Goto, 2000), Clusters of Orthologous Groups (COG) (Tatusov, Galperin, Natale, & Koonin, 2000), NCBI non-redundant protein sequences (NR; v20170924) (O’Leary et al., 2016), Swiss-Prot (release-2018\_07) (UniProt, 2012), TrEMBL (TRanslation of EMBL [nucleotide sequences that are not in Swiss-Prot]; release-2018\_07) (O’Donovan et al., 2002), and InterPro (v69.0) (A. L. Mitchell et al., 2019). A total of 18,068 (93.5%) of *A. flavipes* genes could be functionally annotated. Where specific genes are named in this manuscript, human nomenclature assignments are used unless otherwise noted.

### 2.7 Phylogeny and divergence time estimation

Marsupials (metatherians) and eutherians diverged ~160 million years ago, long before the radiation of extant eutherian clades (~100 million years ago) (Luo, Yuan, Meng, & Ji, 2011; Phillips, Bennett, & Lee, 2009). We employed the platypus (*Ornithorhynchus anatinus* ) as an outgroup as this species is frequently used as an outgroup for both eutherian and marsupial mammals and it has a high-continuity, well-annotated genome (contig N50 15.1 Mb; scaffold N50 83.3 Mb). In contrast, the genomes of the most closely related eutherian mammals, the edentates (sloth, armadillo and anteaters) currently have relatively poor-quality genome assemblies. These include, for example, the armadillo *Dasyurus novemcinctus*(contig N50 0.03 Mb, scaffold N50 1.7 Mb) and the sloth *Choloepus hoffmanni* (contig N50 0.06 Mb; scaffold N50 0.4 Mb).

We identified 7,116 high-confidence 1:1 orthologs by interrogating the predicted proteins from the gene models of 11 species (nine marsupials and the platypus) using SonicParanoid v1.3.0 (Cosentino & Iwasaki, 2019). Because the *A. arktos* , *A. argentus* , and *M. murexia* pseudo-genomes were derived from *A. flavipes* , their genes were extracted using the *A. flavipes* gene annotation file. The corresponding coding sequences (CDS) for each species were aligned using PRANK v100802 (Loytynoja & Goldman, 2005) and filtered by Gblocks v0.91b (Talavera & Castresana, 2007) to identify conserved blocks (removing gaps, ambiguous sites, and excluding alignments less than 300 bp in size), leaving 7,116 genes. Maximum-likelihood (ML) phylogenetic trees were generated using RAxML v7.2.8 (Stamatakis, 2006) and FastTree v2.1.10 (Price, Dehal, & Arkin, 2010) with three CDS data sets: the whole sequence, first codon positions, and fourfold degenerate (4d) sites. Identical topologies and similar support values were obtained (1,000 bootstrap iterations were performed). The divergence time between species was estimated using MCMCTree [Bayesian molecular clock model implemented in PAML v4.7 (Yang, 2007)] with the JC69 nucleotide substitution model (Jukes & Cantor, 1969), and 4d ML tree and concatenated supergenes of first codon positions and fourfold degenerate (4d) codons as inputs. We used 100,000 iterations after a burn-in of 10,000 iterations. MCMCTree calibration points (million years ago; Mya) were obtained from TimeTree (Kumar, Stecher, Suleski, & Hedges, 2017): *O. anatinus* -*P. cinereus* (~167-192 Mya), *V. ursinus* -*D. virginiana* (~72-86 Mya), *V. ursinus* -*M. melanurus* (~56-64 Mya), *V. ursinus* -*P. cinereus* (~31-39 Mya), *M. domestica* -*D. virginiana* (~24-39 Mya), and *S.*

*harrisii* -*M. melanurus* (~4-22 Mya). For comparison, phylogenetic trees of marsupials in the marsupial orders Dasyuromorphia and Didelphimorphia was obtained by querying the PHYLACINE (The Phylogenetic Atlas of Mammal Macroecology) resource (Faurby et al., 2018).

## 2.8 Genomic diversity

Genome-wide heterozygosity ( $\pi$ ) was calculated from ( $2 \times 100$  bp) WGS read mapped to the *S. harrisii* genome. For details, see Supplemental Methods.

## 2.9 Demographic history

We inferred demographic histories by applying the Pairwise sequentially Markovian coalescent (PSMC) method (Li & Durbin, 2011). Short-insert paired-end WGS reads of *A. flavipes* (two individuals), *A. arktos*, *A. argentus*, and *M. melanurus* were mapped to a repeat-masked *S. harrisii* genome. Putative X-linked scaffolds in the *S. harrisii* assembly were excluded (see Supplemental Methods). Maps were generated using the R package ‘ggmap’ (Kahle & Wickham, 2013).

## 2.10 Transcriptome profiling of the *Antechinus flavipes* breeding

To search for transcripts associated with obligate semelparity, we performed differential gene expression analysis of a single *A. flavipes* population (Samford Valley, Queensland, Australia) from August to October 2018. Every two days, we microscopically examined female urine for cornified, anuclear vaginal epithelial cells, a sign of peak oestrus (Selwood, 1980). Concurrent visual inspections of captured males for behavioural changes (e.g., agitation) revealed evidence of stress beginning in the last week of September, followed by progressive loss of body condition (Figure S3). The practical and ethical challenges of longitudinal sampling from a wild population limited the number of samples that could be obtained. In this study, we present transcriptomes of eight male and six female antechinuses [liver, kidney, skeletal muscle, cerebrum, and reproductive tissues (testis and ovary)]. See Supplemental Methods and Figure S4 for data processing and differential expression procedures, and File S2 for normalised counts.

# 3. RESULTS AND DISCUSSION

## 3.1 Genome assembly and annotation

We used PacBio sequencing and Hi-C (Burton et al., 2013) to create a 3.2 Gb ( $2n = 14$ ) chromosome-level *A. flavipes* nuclear genome assembly with a contig N50 of 51.8 Mb and scaffold N50 of 636.7 Mb (Table 1 and Figure 1). The seven chromosomes ranged in size from 86.6 to 727.3 Mb (Table 2) and covered ~99.7% of the assembly. We also generated *A. flavipes* reference-based assemblies of *A. arktos*, *A. argentus*, and *M. melanurus* from short-insert whole-genome sequencing reads. Most *A. flavipes* genome regions had a GC-depth of about 50×, while a small peak at 25× depth revealed heterozygous sex chromosomes (Figure S5). Hi-C was able to recover a ~86.5 Mb X chromosome (chromosome 7), while the Y chromosome could not be obtained (see Supplemental Methods). This is unsurprising, as marsupial Y chromosomes are small (~10-12 Mb; akin to microchromosomes of birds), repeat-rich, and refractory to assembly (Deakin & O'Neill, 2020). The chromosomes of *A. flavipes* were highly homologous to the related Tasmanian devil (*Sarcophilus harrisii*) (Figure 1C), supporting the quality of the assemblies.

The GC content of *A. flavipes* (~36%) (Table 1) is similar to the brown antechinus (*A. stuartii*) and *S. harrisii*. As indicated by 17-mer frequency analysis (Figure S1), the *A. flavipes* genome is repeat rich. Repetitive elements account for 51.8% (~1.7 Gb) of the assembly, with long interspersed elements (LINEs; ~45%), long terminal retrotransposons (LTRs; 15.4%) and short interspersed elements (SINEs; 6.6%) being the major classes of transposable elements (TEs) (Tables S5 and S6). We annotated 24,708 *A. flavipes* protein-coding genes (82.2% supported by transcriptome data from 13 tissues). The *A. flavipes* reference assembly obtained a BUSCO (Benchmarking Universal Single-Copy Orthologs) (Seppey et al., 2019) genome completeness score of 92.4% – comparable to *A. stuartii* (92.2% and 92.3% for a female and male assembly, respectively) (Brandies et al., 2020a), the koala (*Phascolarctos cinereus*; 93.9%) (Johnson et al., 2018), and the *S. harrisii* (91.6%; 2019 assembly mSarHar1.1) (Table S7). The scores of the three dasyurid reference-

based assemblies, here used for phylogenetic and demography analyses, ranged from 74.2% to 90.0% recovered complete BUSCO genes (**Table S8** ).

### 3.2 Phylogeny and demographic history

To construct a time-calibrated species tree (**Figure 2a** ), we identified 7,116 high-confidence single-copy orthologs from nine marsupial species and a monotreme, the platypus (*Ornithorhynchus anatinus* ). Extant Australasian and American marsupials diverged from a South American ancestor approximately 80 million years ago (Kumar et al., 2017; Nilsson et al., 2010), emphasising the deep evolutionary history of marsupials. We estimated that the lineages leading to extant Dasyuridae (e.g., *Antechinus* ) and Didelphidae diverged 81.7 million years ago [77.7-85.8 Mya 95% credibility interval (CI)]. The dasyurid subfamily Phascogalinae comprises three genera: the semelparous Australian *Antechinus* and *Phascogale* , which have male die-off, and the iteroparous New Guinean sister genus *Murexia* . Small dasyurids from mainland Australia are likely to have dispersed into New Guinea 9-11 million years ago (K. J. Mitchell et al., 2014). We found the divergence time of *Antechinus* and *Murexia* to be about 7.1 Mya (95% CI 5.9-7.3). Our analysis show that the three antechinus species split from a common ancestor ~4.6 Mya (95% CI 3.8-4.8), while *A. flavipes* and *A. argentus* share an ancestor ~2.4 Mya (95% CI 2.0-2.6). Our phylogeny and divergence times concord with trees generated from the PHYLACINE database (Faurby et al., 2018) (**Figure S6** ) and with recent estimates from a small set of mitochondrial and nuclear genes (Mutton et al., 2019).

The pairwise sequentially Markovian coalescent (PSMC) method (Li & Durbin, 2011), which can infer changes in the effective population size ( $N_e$  ) over the Pleistocene, was used to evaluate the four sequenced dasyurid species (**Figure 2b,c** ). Until the end of the Pleistocene, approximately 11,700 years ago, mainland Australia, New Guinea, and Tasmania formed a single continent, Sahul (Barrows, Stone, Fifield, & Cresswell, 2002). Glaciation of Sahul was restricted to the Snowy Mountains of southeastern mainland Australia and the Tasmanian highlands, and the continent experienced cycles of aridity and sea-level fluctuations (Barrows et al., 2002). Until about 250,000 years ago, the species in higher-elevation, wetter habitats; *A. argentus* , *A. arktos* , and *M. melanurus* , had larger effective population sizes than *A. flavipes* , which were found in lower-elevation, drier habitats than today (A. M. Baker & Van Dyck, 2013). After 250,000 years ago, the species in higher-elevations declined in population size, while *A. flavipes* populations expanded (**Figure 2a,b** ). This pattern persisted into the Late Pleistocene ~11,700 to 126,000 years ago, a period when temperature fluctuations were large, and many mesic-adapted species declined, became restricted to wet refugia such as mountain-tops or became extinct, and more arid-adapted species radiated (Saltre et al., 2016). While the PSMC method cannot infer population dynamics after ~20,000 years ago, *A. flavipes* had the largest  $N_e$  in the most recent PSMC estimate, consistent with the status of the yellow-footed antechinus being one of the most common and broadly distributed species in genus *Antechinus* today (A. M. Baker & Van Dyck, 2013; Lada, Thomson, Mac Nally, & Taylor, 2008; Mutton et al., 2019). This conclusion is in agreement with a recent study showing that aridity and geographical isolation led to dasyurid speciation (García-Navas, Kear, & Westerman, 2020). In conclusion, our analysis suggests that climate change during the Early (~2.5-0.78 Mya) (Lisiecki & Raymo, 2005) and Mid Pleistocene (0.78-0.126 Mya) (Elderfield et al., 2012) shaped the evolution of the dasyurid subfamily Phascogalinae by isolating populations in different habitats.

We found that all small dasyurid taxa have much higher (100 orders of magnitude) genome-wide heterozygosity ( $\pi$  from 0.048 to 0.096) than the endangered *S. harrisii* ( $\pi=0.00032$  (Miller et al., 2011)) (**Figure S7** ). Moreover, the heterozygosity of *A. flavipes* twofold higher than *A. arktos* , *A. argentus* , and *M. melanurus* . This may reflect its larger historical and current population size (**Figure 2b,c** ). Although we are comparing individuals from single populations, combined with factors such as environmental change, loss of habitat, and population fragmentation, the lower genetic diversity of *A. arktos* and *A. argentus* may reflect their present-day low population and threatened species status (Mutton et al., 2019; Riordan, Pearce, McDonald, Gynther, & Baker, 2020). Future studies, including assessment of geographic population structures and the inclusion of historical and current specimens (Diez-Del-Molino, Sanchez-Barreiro, Barnes, Gilbert, & Dalen, 2018) are required to assess the threat level of extant small dasyurids. Our high-quality *Antechinus flavipes* assembly serves as an ideal reference genome for population genomic studies of *A. flavipes* and the 14 other

antechinus species.

### 3.3 A gene expression profile of male die-off in *Antechinus*

We profiled gene expression during antechinus semelparity by surveying multiple tissues from an *A. flavipes* population in Samford Valley in Queensland, Australia during the 2018 breeding season (**File S1**). We observed reproductive behaviour and peak oestrus on September 15, 2018 (see Supplemental Methods for details). Adult male *A. flavipes* disappear from this site after the first week of October. Antechinus male die-off is characterised by a dramatic increase in free cortisol (**Figure 3a**), mediated by a surge in testosterone which decreases the production of corticosteroid-binding globulin (Boonstra, 2005; Naylor et al., 2008). The potent effects of cortisol mean that the associated gene expression and its consequences (McDowell et al., 2018; Phuc Le et al., 2005) become a suitable backdrop against which comparisons can be made. While corticosteroids regulate a large number of genes via the glucocorticoid receptor (GR) (McDowell et al., 2018; Phuc Le et al., 2005), complicating gene expression analyses, chronic (i.e., breeding male antechinus) exposure to free corticosteroids has distinct outcomes (Manoli et al., 2007). The steroid hormone mediates a stress response, alters metabolism and mobilises glucose as energy from a series of stores, initially enhancing short-term survival. Because of a limited number of samples in our current data set, we identified genes differentially expressed by moribund antechinus males; individuals sampled near the conclusion of breeding, in early October, when all males die (right panel in **Figure 3b**).

There were more differentially expressed genes (limma-modified Student's *t*-test  $P \leq 0.01$ ) in the liver and skeletal muscle than the kidney and cerebrum (**Figure 3c** and **File S3**), and most of these were downregulated. Genes upregulated in moribund male livers showed enrichment of biological process gene sets attributed to the innate immune response (bootstrap  $P \leq 0.0001$ ) (**Table S9**). However, several cytokine genes (*PDGFRA*, *PDGFRB*, *PRNP*, *CD248*, *CD79A*, *CXCR5*, and *CXCR6*) were downregulated in the moribund liver (**File S3**). Characteristic liver pathology of the moribund male antechinus includes infections by bacteria such as *Listeria monocytogenes* (Barker, Beveridge, Bradley, & Lee, 1978; A. Bradley, McDonald, & Lee, 1980), viruses (e.g., dasyurid herpesvirus 1 (Amery-Gale et al., 2014)), and parasitic infestation (Poskitt et al., 1984). Cortisol-mediated immunosuppression in the preceding two weeks is likely to lead to poor late-stage resistance to invading pathogens. We identified eight genes that were downregulated and two genes that were upregulated by both liver and skeletal muscle of moribund males (**Figure 3d**). This included decreased expression of the glycophagy receptor STBD1 (Johansen & Lamark, 2020) and increased expression of *ASNS* – a gene induced by amino acid starvation stress (Balasubramanian, Butterworth, & Kilberg, 2013). Downregulation of *SLC2A4* (encodes the glucose transporter GLUT4 (Amoasii et al., 2019)) and *NUR4A2* (NUR11; a regulator of GLUT4 expression (Amoasii et al., 2019)) indicates reduced glucose uptake by the skeletal muscle of moribund males. KEGG pathway and biological process enrichment analysis revealed metabolic dysregulation of skeletal muscle in moribund males, with marked downregulation of genes associated with glycolysis, gluconeogenesis, and insulin signalling – indicating insulin resistance (bootstrap  $P \leq 0.0001$ ) (**Figure 3e**, **Tables S9 and S10**). These genes included *PRKAG3* (-4.7-fold; limma-modified Student's *t*-test,  $P = 3.7 \times 10^{-4}$ ), which encodes a subunit of a key energy-sensing molecule, AMP-activated protein kinase, and its regulator LKB1 (*STK11*; -2.0-fold,  $P = 2.4 \times 10^{-3}$ ) (**File S3**). AMPK responds to energetic stress to regulate multiple metabolic pathways. Reduced expression of LKB1 was limited to the skeletal muscle of moribund *A. flavipes* males (**Figure 3f**). LKB1 has a tissue-specific role in energy metabolism. Complete or partial loss of *Stk11* expression in mouse skeletal muscle prevents AMPK activation and causes metabolic defects such as acceleration of ageing-induced myopathy (Bujak et al., 2015; Koh et al., 2006; Sakamoto et al., 2005; Thomson et al., 2007). Our work is the first gene expression profile of a semelparous mammal. In agreement with observational data from *A. flavipes* and other antechinuses (A. Bradley et al., 1980; Naylor et al., 2008; Woppard, 1971), our results indicate near-complete depletion of fat stores and skeletal muscle atrophy – leading to a catastrophic energy crisis and an inability to maintain cognitive and physical function in moribund males.

Further experimental work on the endocrine system of semelparous mammal species is needed to characterise the role of testosterone and the dysregulation of the hypothalamic-pituitary-adrenal axis and negative feed-

back by cortisol. We were not able to capture gene expression changes in brain regions other than the whole cerebrum of *A. flavipes* during the breeding season, limiting our insights into the effects on major organs. Nevertheless, the *A. flavipes* reference genome permits future studies on gene expression and regulation (e.g., glucocorticoid receptor ChIP-seq).

## 4.0 CONCLUSION

Our study and accompanying data sets, including the first chromosome-level genome of a semelparous mammal, provide a critical resource for future studies on semelparity and its association with the universal ageing process. Complementary genomic data, including the genomes of additional Australasian and South American semelparous and iteroparous marsupials, is forthcoming and promises to shed light on whether semelparity in lineages with a common ancestor ~80 million years ago evolved by independent, lineage-specific molecular changes or by shared molecular convergence. The genomic resources also provide tools for broader studies on the ecology, evolution, and conservation of marsupials.

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## Data Accessibility

*Antechinus flavipes* PacBio reads are available at China National GenBank (CNGB) Project ID CNP0001147. Short-read whole-genome sequencing and RNA sequencing data (BGISEQ-500) generated in this project is available at NCBI BioProject PRJNA565840. The *A. flavipes* genome assembly is available at NCBI Genomes (JADWMD0000000000). Pseudo (reference-based) and de novo genome assemblies of *Antechinus arkos*, *Antechinus argentus* and *Murexia melanurus* (Seim, 2020a, 2020b), and a ROPUS Singularity image (Seim, 2020c) are available at Zenodo. Mitochondrial genome assemblies of *A. flavipes*(MN447797), *A. arkos* (MK977601), and *M. melanurus*(MK977600) are available at GenBank. Various scripts used for data processing and analyses are available on GitHub at <https://github.com/sciseim/semelparity-genomics>.

## Author contributions

I.S. and G.F. initiated and coordinated the project. C.P., K.M, and A.B.M. collected *Antechinus flavipes* samples. L.K.C. dissected *Antechinus flavipes*. A.M.B. provided voucher specimens of *Antechinus arkos* and *Antechinus argentus*. P.B.T., L.K.C. provided frozen tissue samples for whole-genome, Hi-C, and transcriptome sequencing. C.S. led the sequencing and assembly efforts with G.F. R.T., K.H., and C.S. contributed to genome assembly and annotation. R.T., K.H., C.S., and I.S. performed comparative genomics analyses. The de novo and reference-based assemblies of *A. arkos*, *A. argentus*, and *M. melanurus* were generated by I.S. I.S. performed population history analysis. K.H. performed diversity analysis. I.S. assembled transcriptomes and performed gene expression analysis. R.T. performed molecular evolution analyses. L.K.C., D.O.F., N.O.L., and A.M.B. provided direction on marsupial ecology and physiology. R.T., L.K.C., D.O.F., A.M.B., and I.S. wrote the paper with input from all authors.

## Tables and Figures

**Figure 1 Overview of sequenced species and reference genome assemblies generated in this study.** (a) Photographs of the five marsupial species sequenced. A yellow-footed antechinus (*Antechinus flavipes*), silver-headed antechinus (*Antechinus argentus*), and black-tailed dusky antechinus (*Antechinus arkos*) from Australia; a black-tailed dasyure (*Murexia melanurus*) from Papua New Guinea. *Antechinus spp.* and *M. melanurus* photographs are courtesy of A.M. Baker and John Hornbuckle, respectively. (b) Circos plot of the reference genome assembly of *A. flavipes*. The outermost segment represents chromosome sequences, with the numbers on the external surface indicating genome size in Mb. Chr7 in *A. flavipes* corresponds to the X chromosome. The line plots, from outside to inside represent the distribution of CDS density (from 0 to 0.15), GC content (from 0.30 to 0.65) and TE ratio (from 0.2 to 1.0). Frequencies were calculated in 500-kb sliding windows. (c) Circos plot showing shared synteny of *Antechinus flavipes* (chr1-chr7) and *Sarcophilus harrisii* (NC\_045426.1-NC\_045432.1). Aligned using LASTZ. The synteny blocks are linked using lines coloured in accordance with the *A. flavipes* chromosomes. Aligned blocks with length shorter than 20 kb are not shown. Chr7 in *A. flavipes* corresponds to the X chromosome of *Sarcophilus harrisii*.

**Figure 2 Evolutionary history of semelparous mammals.** (a) Inferred phylogeny of ten marsupials and the platypus (outgroup) based on fourfold degenerate sites from 6,431 1:1 orthologs. Blue numbers at nodes represent the estimated divergence time from present (million years ago; Mya) between lineages. Semelparous lineages are indicated in red. (b) Demography of the dasyurid subfamily Phascogalinae. Historic population sizes ( $N_e$ ) were estimated using the pairwise sequentially Markovian coalescent (PSMC) method (Li & Durbin, 2011) and diploid genome sequences. The  $x$ -axis shows the time before present in years on a log scale; the dashed gray line shows human arrival in Australia 59.3 to 65 kya (Clarkson et al., 2017); red dots indicate the extinction times of 15 species of Pleistocene Sahul (Saltre et al., 2016). The  $y$ -axis shows  $N_e$ , with bootstrap estimates indicated by lighter lines. Plots were scaled using a mutation rate ( $\mu$ ) of  $1.37 \times 10^{-9}$  substitutions nucleotide $^{-1}$  generation $^{-1}$  and species-specific generation times ( $g$ ) of one year. Two *A. flavipes* individuals were interrogated (coloured in orange and blue). (c) Map showing the location (coloured circles) and elevation (mamsl; metres above sea level) of sampled dasyurids (see subfigure b).

**Figure 3 Gene expression profiling of the *Antechinus flavipes* breeding cycle.** (a) Schematic overview of the yellow-footed antechinus (*Antechinus flavipes*) breeding season in Samford Valley, Queensland, Australia. Free-CORT illustrates an increase in the level of free (unbound) stress hormones (chiefly the corticosteroid cortisol) in males during the breeding season of antechinuses (based on three studies illustrated in (Naylor et al., 2008)). (b) Rapid organismal senescence of male *A. flavipes*. Left, male (~11 months of age) prior to the breeding season. Right, late breeding stage male (~11.5 months of age). Note the loss of fur clumps and deterioration of body condition. (c) In the moribund state, when other energy stores have been depleted, skeletal muscle catabolism generates amino acids for gluconeogenesis, induced by the corticosteroid, cortisol. (d) Venn diagram for the overlap of significantly up-regulated (left) or down-regulated (right) genes in moribund male antechinuses. Called by limma v3.22.1 ([?]1.5-fold change and  $P$  [?]0.01). (e) Heat map of selected energy metabolism genes differentially expressed in the skeletal muscle of moribund male antechinuses. Moribund samples are indicated by a red bar below the map. Scaled TMM-normalised

read counts (denoted as the row *Z* score) are plotted, with red indicating high expression and blue indicating low expression. *BPGM*, 2,3-bisphosphoglycerate mutase; *FBP2*, fructose-bisphosphatase 2; *GPI*, glucose-6-phosphate isomerase; *LDHA*, lactate dehydrogenase A; *PFKM*, phosphofructokinase, muscle; *PGK2*, phosphoglycerate kinase 2; *PKM*, pyruvate kinase M1/2; *GPD1*, glycerol-3-phosphate dehydrogenase 1; *GPD2*, glycerol-3-phosphate dehydrogenase 2; *SLC2A5*, solute carrier family 2 member 5; *SLC37A4*, solute carrier family 37 member 4; *PRKAG3*, protein kinase AMP-activated non-catalytic subunit gamma 3; *STK11*, serine/threonine kinase 11. (f) Expression pattern of the AMPK regulator LKB1 (*STK11*) in male (left) and female (right) tissues throughout the breeding season. Counts per million TMM-normalized counts, with standard error bars.

**Table 1.** *Antechinus flavipes* genome assembly statistics

Assembly methods:	PacBio (100×) + BGISEQ-500 (50×) polishing	PacBio + BGISEQ-500 polishing+
Contig number	1,107	1,107
Contig length (bp)	3,192,388,871	3,192,388,871
Contig N50 (bp)	51,843,704	51,843,704
Contig max length (bp)	168,884,005	168,884,005
Scaffold number	1,105	487
Scaffold N50 (bp)	51,843,704	636,717,922
GC content (%)	36.21%	36.21%

**Table 2.** Summary of assembled *Antechinus flavipes* pseudochromosomes

Chr ID	Sequence number	Sequence Length (bp)
Chr 1	88	727,308,865
Chr 2	83	686,337,178
Chr 3	146	636,717,922
Chr 4	50	486,195,733
Chr 5	104	300,479,852
Chr 6	63	264,590,327
Chr 7	91	86,570,782



