De novo transcriptome assembly and annotation of liver and brain tissues of New Zealand brushtail possums (*Trichosurus vulpecula*): transcriptome diversity after decades of population control

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Abstract: The New Zealand brushtail possum (*Trichosurus vulpecula*), introduced from Australia in the 1850s, is an invasive species that is widespread throughout New Zealand, and form the largest self-sustained reservoir of bovine tuberculosis (*Mycobacterium bovis*) in the wild. Conservation and agricultural authorities regularly apply a series of population control measures to suppress brushtail possum populations. The evolutionary consequence of more than half a century of intensive population control operations on the species genomic diversity and population structure is hindered by a paucity of available genomic resources for the species. This study is the first to characterise the functional content and diversity of brushtail possum liver and brain cerebral cortex transcriptomes. Raw sequences from hepatic cells and cerebral cortex were assembled into 58,001 and 64,735 transcripts in hepatic cells and cerebral cortex, respectively. Functional annotation and polymorphism assignment of the assembled transcripts demonstrated a considerable level of variation in the core metabolic pathways that represent potential targets for selection pressure exerted by chemical toxicants. This study suggests that the brushtail possum population in New Zealand harbours considerable levels of variation in metabolic pathways that could potentially promote the development of tolerance against chemical toxicants.

Keywords: De novo transcriptome assembly; brushtail possums; liver; brain cerebral cortex; drug and xenobiotic metabolism; chemical toxicants

1-Introduction

New Zealand separated from other landmasses approximately 85 million years ago and has remained largely isolated ever since [1]. Ecological isolation created distinctive evolutionary dynamics in the archipelago rarely seen anywhere else on the planet. By the time the first humans colonised in approximately 1300 AD [2,3], no native land mammals, other than three species of bats, were present [4]. The negative impacts of two consecutive waves of human colonisation triggered a series of fundamental alterations in the biological diversity of New Zealand. The

introduction of three species of rats (*Rattus* spp.), three species of mustelids (*Mustela* spp.), and the brushtail possum (*Trichosurus* vulpecula) coincided with a spiral of catastrophic events that drove fifty-nine bird species extinct and severely threatens the survival of other native species, including endemic marine mammals, bats, frogs, and much of the invertebrate fauna [5,6].

The brushtail possum was introduced into New Zealand from localities in Tasmania and the eastern Australian mainland in the 1850s to establish a profitable fur industry [7,8]. Soon after its introduction, the species established itself in the wild, proliferated, and spread across all habitats in the archipelago. In these new habitats, brushtail possums reached population densities far exceeding those in their native Australian range [4]. The phenomenal success of brushtail possums in New Zealand has been linked to the high abundance of palatable and nutritious food sources, and the presence of fewer parasites, predators, and competitors [9]. The scale of devastation upon New Zealand's endemic ecosystems prompted the brushtail possum's designation as both an agricultural pest and a major threat to New Zealand's unique flora and fauna [9,10]. Wild populations of brushtail possums also harbour a self-sustained reservoir of bovine tuberculosis (*Mycobacterium bovis*) that threatens New Zealand's dairy and livestock industries [4]. As a result, the brushtail possum has been subject to decades of targeted population control operations.

Conservation and agricultural authorities in New Zealand regularly apply a series of population eradication and control measures to mitigate the negative impacts of brushtail possums on native ecosystems. Since 1957, aerial dispersal of cereal baits containing the chemical toxicant sodium fluoroacetate (or "compound 1080") has constituted the cornerstone of management plans to control brushtail possums and other invasive mammals in New Zealand [10,11]. Synthetic compound 1080 is a water-soluble salt modelled on the naturally-occurring plant toxin fluoroacetate [12]. Several plant species across the globe, such as *Gastrolobium* spp. and *Oxyylobium* spp., use this toxicant as a deterrent against herbivory, including species within the possum's native Australian home range [13-15].

Brushtail possum populations in New Zealand face a combination of stressors and human-induced selection pressures that differ from those in their native Australia. The speed of local adaptation typically found in invasive species suggests that the genetic recipe for adaptation predominately arises from standing genetic variations present in the founder populations [16,17]. Triggs and Green [18] and Sarre, et al. [19] reported that current populations of brushtail possum in New Zealand originated from two distinct subspecies, *Trichosurus vulpecula fuliginosus* from Tasmania and *T. vulpecula vulpecula* from mainland Australia, and that extensive hybridisation took place following their introduction to New Zealand. Genetic variations in the founder populations propagated in the non-native range and most likely became reshuffled into new genotypes that do not occur in Australia.

Recent advances in DNA sequencing technology and high-performance computing have facilitated the comprehensive study of functional regions in eukaryote genomes. Variability in the genome has made it possible to address some fundamental questions in evolutionary genetics, such as the prediction of pathways associated with fruit colour [20], intraspecific variation in morphology and behaviour [21], susceptibility to pathogens [22-25], responses to chemicals [23], adaptation to environmental changes [26], species diagnostics markers in endangered shrimps [27], sex determination [28] and changes in the expression profiles of an organ during embryonic development [29].

Understanding brushtail possums' evolutionary dynamics in New Zealand is hindered by a paucity of available genomic resources for this species [30]. In particular, the evolutionary consequences of more than half a century of intensive population control measures on brushtail possums' genetic diversity and population structure merit in-depth investigation. To address these fundamental questions, genomic resources for brushtail possums are required.

In the current study, we characterise the functional content and genetic diversity of brushtail possums' liver and brain cerebral cortex transcriptomes, tissues of major importance in

responding to chemical toxicants and in modulating complex behaviour [31-34]. The core metabolic pathways in these tissue types are among the primary targets of selective pressures imposed by anthropogenic chemicals [35-37]. This study serves as a stepping-stone for a better understanding of brushtail possum evolutionary dynamics in New Zealand and will contribute towards the optimisation of a comprehensive population management plan for the species, in line with the New Zealand Predator Free 2050 horizon objectives.

2-Materials and Methods

2-1 Animal ethics statement

Animal ethics approval for this study was obtained from the Lincoln University Animal Ethics Committee (AEC 586). Approved standard operation procedures were used for both animal husbandry and euthanasia. All necessary steps were taken to minimise the pain and suffering of the subject animals.

2-2 Specimen collection

Four brushtail possums (two males and two females) were live trapped on the east and west coasts of New Zealand's two main islands (the Banks Peninsula and Spring's Junction, South Island, and Taranaki and Hawkes Bay, North Island). The animals were transferred to the wild animal husbandry facilities (Johnstone Memorial Laboratory) at Lincoln University. On the day of specimen collection, sex, weight and health status of each animal were recorded by Lincoln University's wildlife animal facility staff. Brushtail possums were humanely euthanised by intracardiac administration of 300 mg of Pentobarbital. The dissection and collection of the liver and cerebral cortex biopsies were performed after the death of the animals was confirmed on site. Approximately $1mm^3$ tissue from liver and brain cerebral cortex were excised transferred to 10:1 volume of RNAlater solution (Qiagen-Hilden) and stored at -80°C until RNA was extracted within one

week after specimen collection. Liver cells are assumed to be more homogeneous in their gene

expression profiles [but see 38], whereas brain cell expression profiles often differ considerably

between different brain regions [39,40]. To account for variations in the gene expression profiles in

the possum cerebral cortex, brain biopsies from three individuals were excised from different

sections of the proximal cerebral cortex in the left and right cortical lobes. The remaining biopsy

was taken from deeper white matter in the cerebral cortex. A wildlife histologist examined all brain

biopsies to validate their origin.

2-3 Nucleic acid extraction, genomic library preparation, and sequencing

Total RNA was purified from liver and brain tissue using Qiagen's RNeasy Mini Kit (Hilden,

Germany). The extracted RNA was eluted in 50–100 µl of water. The quality of the purified RNA was

measured using Agilent's Bioanalyzer (Santa Clara, United States) to obtain an RNA Integration

Number score before proceeding with complementary DNA preparation. Nugen's Ovation RNA-Seq

System (Redwood City, United States), automated on an Apollo324 liquid handler (Takara Bio,

Kyoto, Japan), was used to create complementary DNA using a single primer isothermal

amplification method and 100 ng of template RNA. The resulting complementary DNA was

quantified on a Nanodrop spectrophotometer (ThermoFisher Scientific, Massachusetts, United

States) and sheared to 200 bp fragments using the Covaris M220 ultrasonicator (Woburn, Unites

States). Illumina-compatible sequencing libraries were prepared using KAPA Biosystem's LTP library

preparation kit (Pleasanton, United States). Individual sequencing adapters with sample-specific

barcodes (Bioo Scientific, Austin, United States) were ligated to the end-repaired fragments, the

resulting libraries were cleaned using AMPure beads (Beckman-Coulter, Brea, United States) and

amplified for ten PCR cycles with KAPA's HiFi polymerase. The concentration of each library was

quantified separately using qPCR on the Quantstudio 5 (Applied Biosystems, Foster City, United

States) and the fragment sizes were verified using the Agilent Bioanalyzer. All sequencing libraries were normalised to 2 nM concentration before pooling for multiplex sequencing on two 1x50 flow cells on an Illumina HiSeq2000 platform (San Diego, United States) at the Arizona State University genomics core facility.

2-4 Transcriptome assembly, variant calling, and functional annotation

Leading and trailing low quality or N bases (Phred quality below three), all base pairs with Phred score < 20, and Illumina adapter contaminations were removed using Trimmomatic v0.39 [41]. A reference transcriptome for each tissue type was assembled denovo in Trinity v2.8.6 (Inchworm, Chrysalis, and Butterfly modules) [42] using default settings. Resulted transcripts were clustered into unique clusters by utilising cd-hit-est from the CD-HIT v4.7 package and setting identity parameters to 98% [43]. The longest reading frame within each transcript was predicted in TransDecoder v5.5.0 [44]. All transcripts were functionally annotated using the Trinotate pipeline [45]. In Trinotate, all transcripts were searched against known proteins and core metabolic pathways deposited in Swiss-Prot protein (https://www.uniprot.org/), NCBI non-redundant protein (https://www.ncbi.nlm.nih.gov/refseq/), and Kyoto Encyclopedia of Genes and Genomes (KEGG)(https://www.genome.jp/kegg/) databases. In addition, the protein domains for all transcripts were predicted in HMMER v3.1 [46] based on the similarity to the known proteins in the Pfam (https://pfam.xfam.org/) database. Transmembrane proteins and ribosomal RNA were separately identified using TmHMM v2 [47] and RNAMMER v1.2 [48], respectively. The Benchmarking Universal Single-Copy Orthologs (BUSCO V3) pipeline [49] was executed in protein mode to evaluate the completeness of the gene content of the reference transcriptome of each tissue compared to the core mammalian gene content (Mammalia-0db-10, created on 2019-11-20).

Quality-filtered sequences from all specimens were aligned against the corresponding assembled transcriptome in Bowtie2 v2.3.5 short read aligner [50]. A combination of SAMtools v1.9

mpileup command [51] and Varscan2 v2.3.7 [52] was used to identify single nucleotide polymorphic sites (SNPs) in each tissue type. Default settings were used for variant calling, an exception being the p-values in Varscan2, which were lowered to 0.001 to increase prediction accuracy. Functional impacts of the polymorphic sites on protein sequences were predicted using KisSplice2reftranscriptome v1.3.3 pipeline [53].

Two data sets were created and investigated in parallel to unravel variability in the functional content of each tissue type. The first consisted of ten transcripts within each tissue's reference transcriptomes that showed the highest number of polymorphic sites, as reported in the Varscan2 output files. In the second data set, the abundance of each transcript was estimated in RSEM v1.3.1 [54], and 5% of the transcripts with the highest normalised level of expression (estimated as per million mapped reads, TPM) were sub-selected for further functional analysis.

The enrichments of the ten most polymorphic transcripts in each tissue type for particular biological functions in terms of Gene Ontology categories (GO terms) [55] were investigated using the g: Profiler online server [56] and the annotated genome of the opossum, *Monodelphis domestica*, was used as the closest high quality annotated gene set.

The tissue-specific expression profiles and intra-individual variations in the functional content of polymorphic sites were statistically tested using the WEGO v2.0 online tool [57] and visualised using a combination of the REVIGO online server [58] and CirGO [59]. The signal of pervasive selection (the ratio of non-synonymous to synonymous amino acid substitution, dN/dS ratio) in one particular liver transcript with an exceptionally high number of polymorphic sites was tested using a fixed-effects likelihood model [60] implemented in the online server Datamonkey [61].

The functional content of the assembled transcripts and two generated datasets, in terms of core metabolic pathways, was reconstructed by mapping transcripts against the KEGG database using the KEGG mapper online server [62] and results were summarized in GAEV [63]

3-Results

The histological reports confirmed that three of the brain cerebral cortex biopsies originated from the proximal cortex, while the remainder originated from deeper within cerebral cortex white matter (Supplementary Materials Figure S1, S2, S3, S4).

The RNA sequencing run yielded 183,517,606 and 178,725,338 raw sequences from liver and brain cerebral cortex, respectively. Liver tissue sequences were assembled into 58,001 transcripts (candidate genes and isoforms), with a mean contig length of 813.37 and a GC content of 42.57%. From the cerebral cortex brain tissue, Trinity assembled sequences with a total of 64,735 transcripts, a mean contig length of 738.13, and a GC content of 42.68% (Table 1). CDHIT subsumed all transcripts into 57,221 and 63,679 unique clusters with more than 98% sequence identity.

Table 1. Summary statistics of the reference transcriptome assembly of the New Zealand brushtail possum's liver and brain cerebral cortex tissue as estimated in QUAST v.4.0[64]

Assembly name	Liver cells	Brain cells
Number of contigs	58,001	64,735
Number of contigs >=1000 bp	13,361	12,385
Number of contigs >=5000 bp	605	612
Number of contigs >=10000 bp	19	24
Total length bp	47,176,165	47,783,051
Total length >=1000 bp	29,238,918	27,088,346
Total length >=5000 bp	3,994,586	4,080,570
Total length >=10000 bp	245,362	318,579
Largest contig	18,159	21,422
GC %	42.57	42.68
N50	1,447	1,247
N75	607	508
L50	8,660	9,524
L75	21,240	24,749

The overall alignment rate of quality-filtered sequences against assembled transcriptomes in Bowtie2 was estimated at 90.93% for liver sequences and 79.80% for brain cerebral cortex sequences. The alignment rate in both tissue types lies within the recommended range in the Trinity

pipeline for a complete reference transcriptome assembly

(https://github.com/trinityrnaseq/trinityrnaseq/wiki/RNA-Seq-Read-Representation-by-Trinity-

Assembly). From a total of 9,226 searched BUSCOs, 3,290 and 2,446 of the core mammalian's

complete BUSCOs were identified in the hepatic and brain cerebral cortex transcriptomes,

respectively (Supplementary Materials Table S1). Tissue-specific transcriptomes, especially in non-

model organisms, are very unlikely to produce complete mammalian BUSCOs and that should be

interpreted within the biological context of the study, rather than as a direct metric for estimating

the completeness of the assembled transcriptomes.

Varscan2 identified 35,300 (one SNP for every 1,339 bp) and 23,805 (one SNP for every

2,007 bp) variable sites in liver and cerebral cortex transcripts, respectively. The number of

polymorphic sites with transitions was estimated to be three times higher than the number of

transversions in both tissue types. The polymorphism landscape in brushtail possum cerebral cortex

was more heterogeneous compared to hepatic cells. Only 25% (5,939 out of 23,805) of the

polymorphic sites in cerebral tissues were successfully genotyped across all four sampled tissues

compared to 70% shared polymorphisms (24,924 out of 35,225) among hepatic tissues.

KisSplice identified 34,329 and 598 shared amino acid substitutions in hepatic and cerebral

cortex tissues, respectively. The number of polymorphic sites resulting in a synonymous amino acid

substitution in hepatic cells was two times higher than non-synonymous amino acid substitution

(22,887 and 11,442, respectively). However, shared polymorphic sites in brain cerebral cortex

demonstrated two times higher non-synonymous substitutions than synonymous substitutions (413

and 185, respectively). The reported high levels of heterogeneity in gene expression profiles among

different sections of the cerebral cortex that only retained amino acid substitution early in the brain

development and before fine-scale specialisation or as a methodological artefact of the algorithm

applied in the KisSplice pipeline that only assembles transcriptomes around variable sites, provide

the most likely explanation for the observed pattern of amino acid substitutions.

In the hepatocytes, mutations resulting in the non-synonymous substitution of the amino acids valine to isoleucine, isoleucine to valine, and alanine to threonine had the highest occurrence. In the brain, cortical cells, non-synonymous substitutions of threonine to isoleucine, arginine to histidine, and cysteine to tyrosine were prevalent. The highest rate of synonymous amino acid substitutions was observed for leucine, threonine, and serine in hepatocytes, and for leucine, serine, and glycine in the cerebral cortex.

A transcript in the liver was of particular interest because of an exceptionally large number of polymorphic sites (127 SNPs). The BLASTX search of this transcript showed high sequence identity (88% similarity) to various isoforms of multidrug resistance proteins reported in koala, *Phascolarctos cinereus* (NCBI reference XP_020843128.1) and wombat, *Vombatus ursinus* (NCBI Reference Sequence: XP_027703707.1). In all four brushtail possums, alignment of the protein-coding sequences of this transcript demonstrated 19 candidate sites that were under pervasive purifying selection (dN/dS <1, p-value <0.05). The majority of the non-synonymous amino acid substitutions in these transcripts (6 out of 30) were in the form of valine to isoleucine substitutions. The synonymous substitution for amino acid alanine and isoleucine constituted the most common substitution pattern in the same transcript.

Searching all assembled transcripts against multiple databases showed a considerable number of uncharacterised transcripts within both tissue types (Supplementary Materials Figure S5, S6).

The analysis of the functional content of both transcriptomes in terms of Gene Ontology illustrated that GO terms associated with biochemical pathways involved in cellular components (cells, organelle and organelle parts, membrane, and extracellular regions), molecular functions (catalytic activity, binding, molecular function regulators, and molecular transducer activity), and biological processes (response to stimulus, cellular process, biological regulation, metabolic process, and biogenesis) dominated the functional diversity of both tissue types (Figure 1, 2).

The hepatic cells were more homogeneous in the Gene Ontology profiles across individuals. Only two metabolic pathways involved in the biological process, anatomical structure developments and multicellular organism developments, showed statistically significant differences in the number of associated polymorphic sites (χ^2 test p-value <0.05) across the four individuals (Figure 3). The brain biopsies taken from different sections of the cerebral cortex demonstrated more heterogenous profiles in their polymorphism landscape. Twelve metabolic pathways involved in cellular components (neuron part, extracellular organelle-complex, and extracellular space), molecular functions (catalytic, hydrolase, transferase, and protein-binding activities), and a biological process linked to transposition showed a significant level of variation across different parts of the cerebral cortex (χ^2 test p-value <0.05) (Figure 4).

Comparing the liver and brain datasets consisting of ten most polymorphic sites against the closely related opossum complete gene set illustrated significant enrichment for various metabolic pathways (Figure 5, 6).

The dataset of 5% highly expressed transcripts illustrated that the expression profile between two tissue types differs significantly (χ^2 test p-value <0.05) in the metabolic pathways linked to 58 GO terms (Supplementary Materials Figure S7).

The metabolic pathways analysis of the transcripts against the KEGG database indicated that the liver transcriptome was linked to 394 core metabolic pathways and 23 complete metabolic modules. Brain cerebral cortex transcriptome was involved in 379 core metabolic pathways and eight complete metabolic modules (Supplementary Materials Table S2, S3).
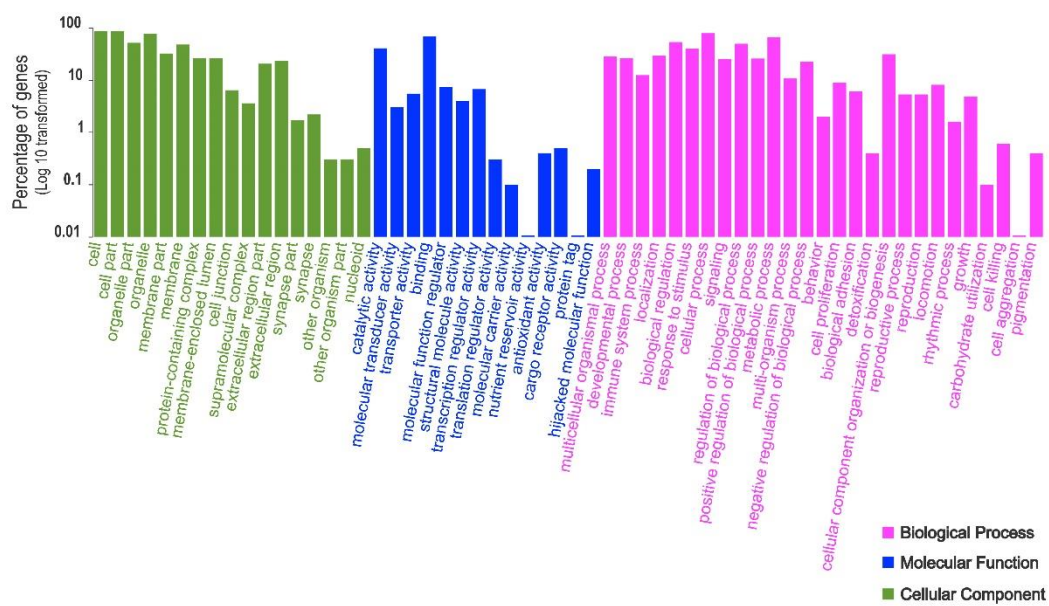
Figure 1. Functional annotation of all polymorphic transcripts in brushtail possum liver cells based on Gene Ontology (GO) categorisation. The X-axis indicates GO functions and the Y-axis shows the percentage of transcripts in log(10) scales.
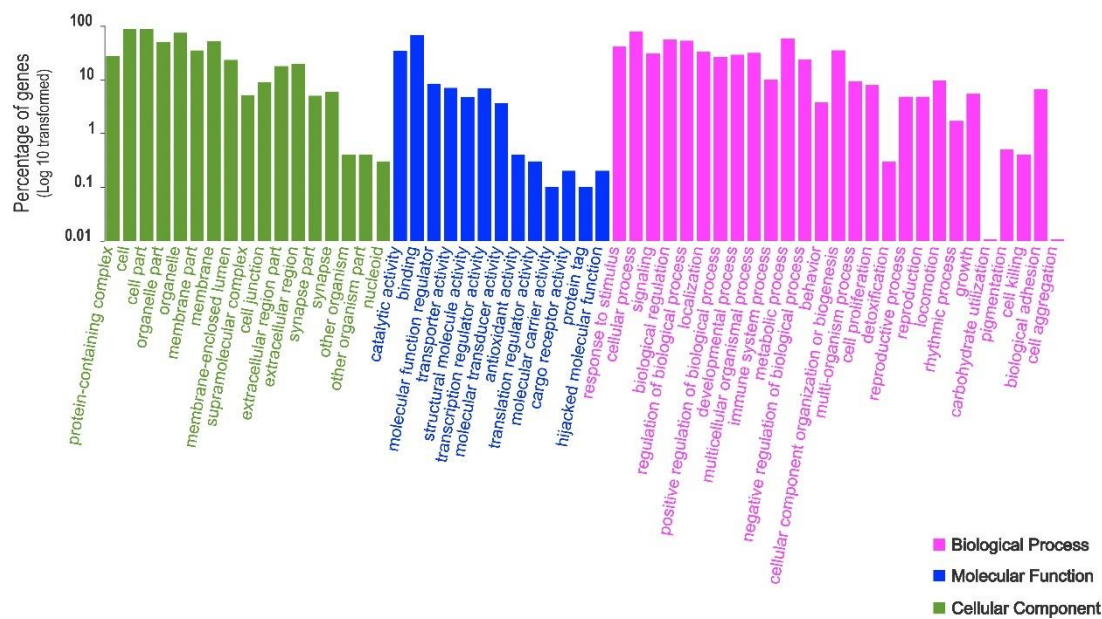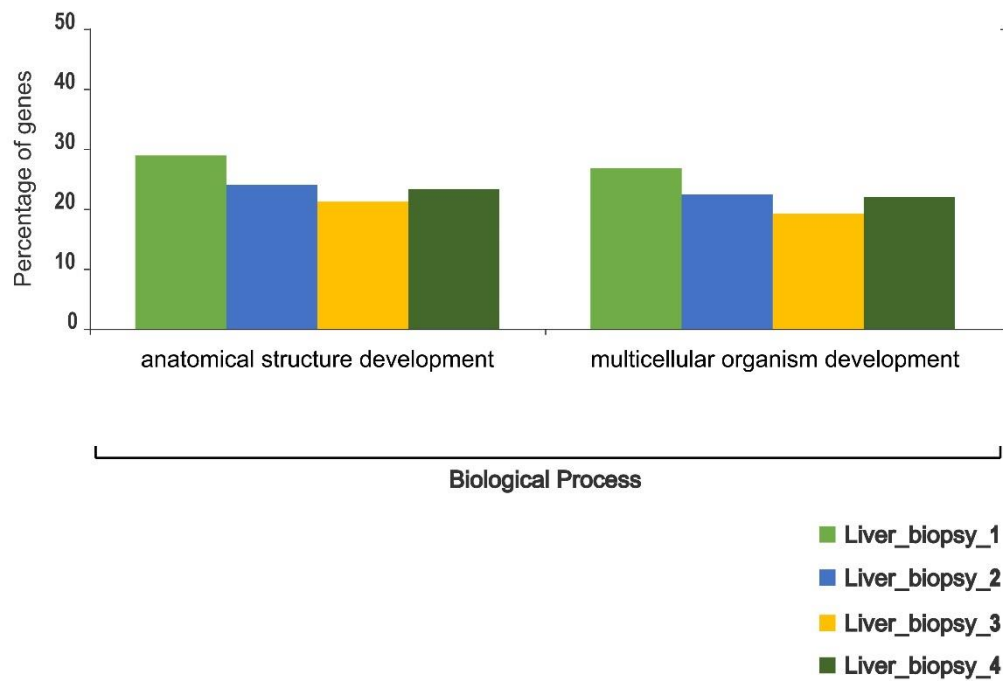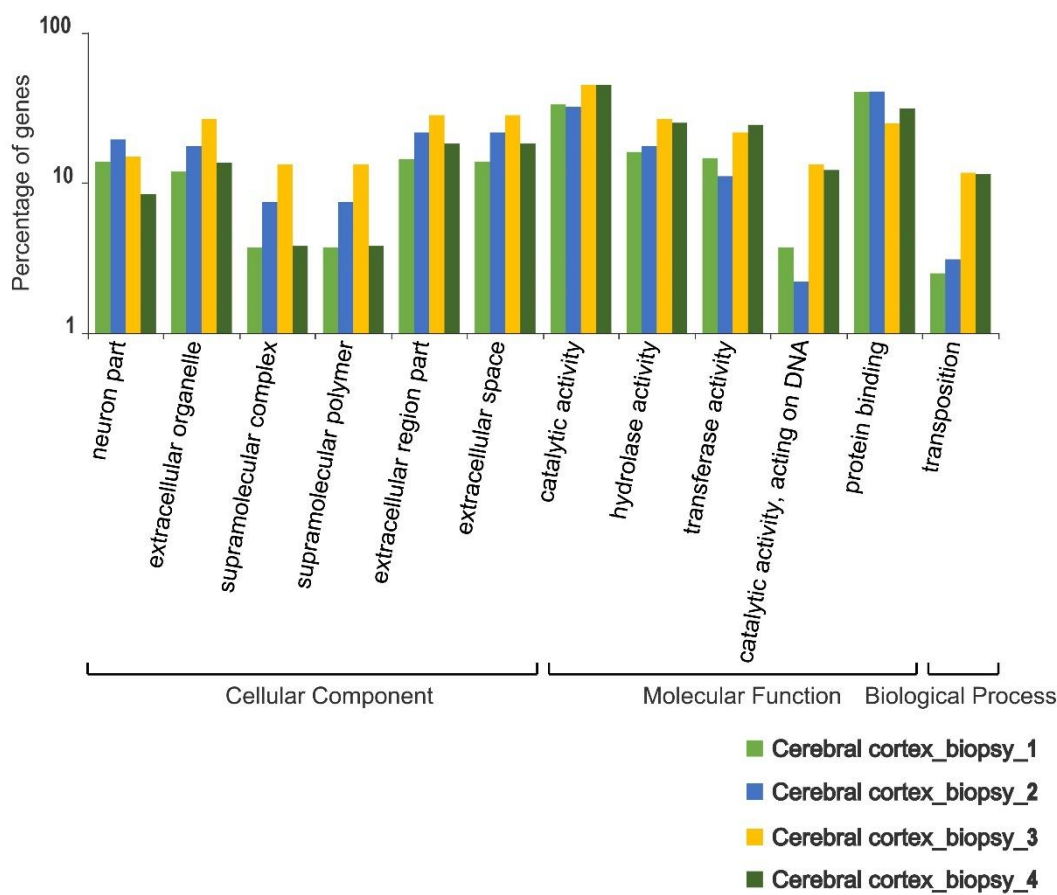
Figure 2. Functional annotation of all polymorphic transcripts in brushtail possum brain cerebral cortex cells based on Gene Ontology (GO) categorisation. The X-axis indicates GO functions and the Y-axis shows the percentage of transcripts in log(10) scales.

Figure 3. Intra-individual variation in the functional content of polymorphic transcripts in brushtail possum liver cells based on Gene Ontology (GO) categorisation. The X-axis indicates GO functions and the Y-axis shows the percentage of transcripts.

Figure 4. Intra-individual variation in the functional content of polymorphic transcripts in brushtail possum cerebral cortex cells based on Gene Ontology (GO) categorisation. The X-axis indicates GO functions and the Y-axis shows the percentage of transcripts.
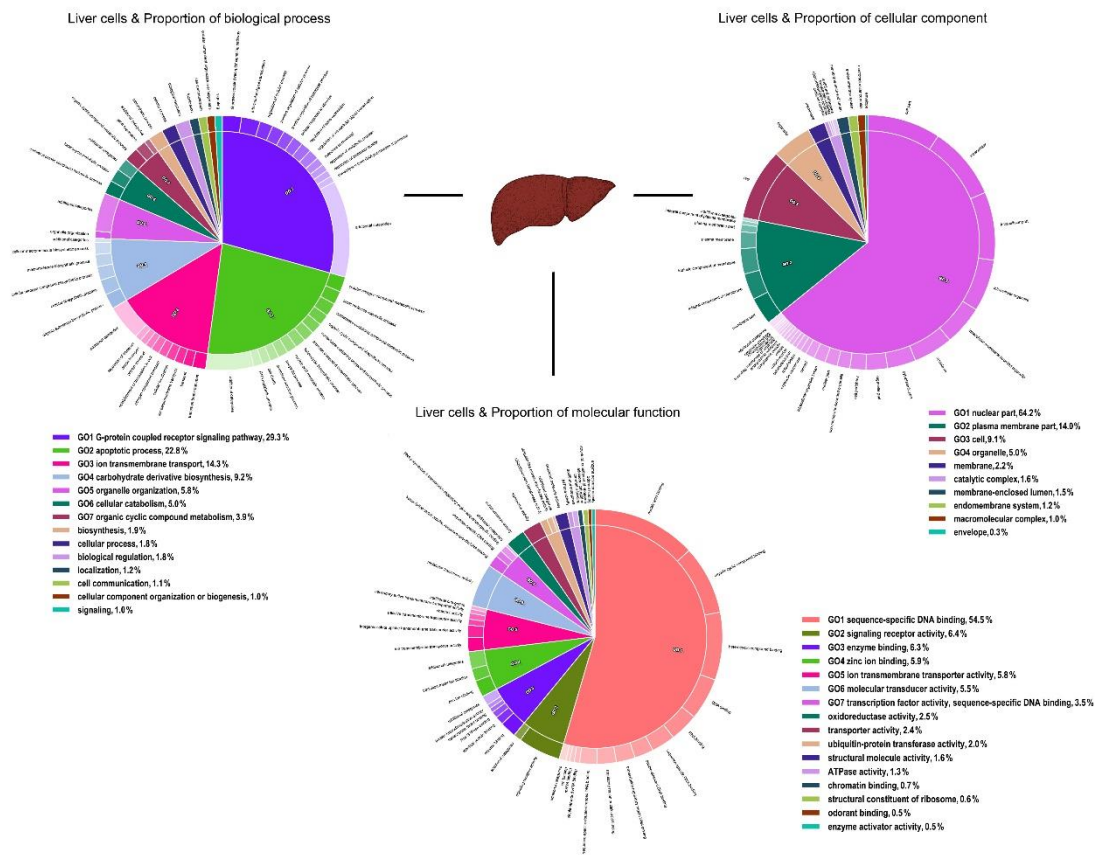
Figure 5. Functional enrichment of 10 transcripts with the highest levels of polymorphism, based on Gene Ontology (GO) categorization, in brushtail possum liver cells compared to the opossum's (*Monodelphis domestica*) complete gene set.
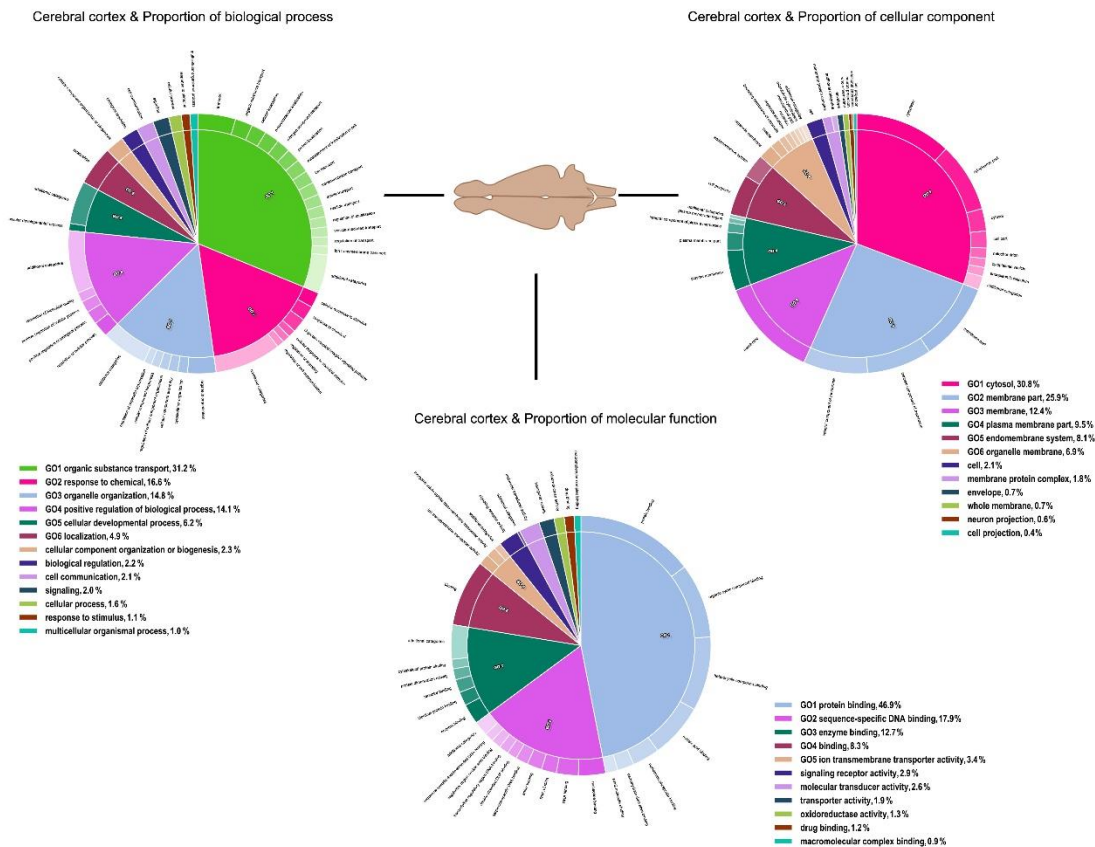
Figure 6. Functional enrichment of 10 transcripts with the highest levels of polymorphism, based on Gene Ontology (GO) categorization, in brushtail possum cerebral cortex cells compared to the opossum's (*Monodelphis domestica*) complete gene set.

In the liver tissue, the most polymorphic transcripts were involved in eight KEGG metabolic pathways including lipid metabolism, nucleotide metabolism, amino acid metabolism, cofactor and vitamin metabolism, xenobiotics biodegradation and metabolism, and the endocrine system. Mapping the ten most polymorphic transcripts in the brain cerebral cortex against the KEGG database indicated their involvement in 14 core biological pathways, including core metabolic pathways, biosynthesis of secondary metabolites, metabolism of cofactors and vitamins, signal transduction, signalling molecules and interaction, transport and catabolism, the cellular community, and the excretory system.

The dataset consisting of 5% of the most abundant transcripts of hepatic cells was linked to 354 biological pathways and eight complete metabolic modules (Table 2). The highly expressed transcripts in the brain cerebral cortex were associated with 302 biological pathways and one complete metabolic module (Table 3). A total of 22 genes in the liver transcriptomes and six genes in the cerebral cortex were directly linked to the metabolic pathways that involved drug and xenobiotic substances metabolism (Table 4, 5).

Table 2. KEGG metabolic pathway names and number of instances identified in the top 5% of the highly expressed genes within the brushtail possum liver transcriptome.

Metabolic pathway	Number
Global and overview maps	591
Carbohydrate metabolism	134
Energy metabolism	65
Lipid metabolism	118
Nucleotide metabolism	12
Amino acid metabolism	152
Metabolism of other amino acids	25
Glycan biosynthesis and metabolism	17
Metabolism of cofactors and vitamins	54
Metabolism of terpenoids and polyketides	10
Biosynthesis of other secondary metabolites	12
Xenobiotics biodegradation and metabolism	60
Transcription	8
Translation	98
Folding, sorting and degradation	70
Replication and repair	3
Membrane transport	5
Signal transduction	228
Signalling molecules and interaction	35
Transport and catabolism	116
Cell growth and death	60
Cellular community - eukaryotes	54
Cellular community - prokaryotes*	2
Cell motility	23
Immune system	181
Endocrine system	185
Circulatory system	24
Digestive system	67
Excretory system	18
Nervous system	81
Sensory system	14
Development and regeneration	21
Aging	23
Environmental adaptation	49

Table 2. KEGG metabolic pathway names and number of instances identified in the top 5% of the highly expressed genes within the brushtail possum cerebral cortex transcriptome.

Metabolic pathway	Number
Global and overview maps	127
Carbohydrate metabolism	27
Energy metabolism	10
Lipid metabolism	29
Nucleotide metabolism	9
Amino acid metabolism	18
Metabolism of other amino acids	5
Glycan biosynthesis and metabolism	12
Metabolism of cofactors and vitamins	6
Biosynthesis of other secondary metabolites	1
Xenobiotics biodegradation and metabolism	8
Transcription	11
Translation	31
Folding, sorting and degradation	29
Replication and repair	11
Membrane transport	3
Signal transduction	190
Signalling molecules and interaction	18
Transport and catabolism	47
Cell growth and death	38
Cellular community - eukaryotes	37
Cell motility	12
Immune system	67
Endocrine system	79
Circulatory system	19
Digestive system	22
Excretory system	8
Nervous system	48
Sensory system	16
Development and regeneration	24
Aging	8
Environmental adaptation	14

Table 4. Name and description of the putative genes involved in drug and xenobiotic substance metabolic pathways in brushtail possum liver cells as predicted using the KEGG database.

Gene name	Description
frmA, ADH5, adhC	S-(hydroxymethyl)glutathione dehydrogenase / alcohol dehydrogenase
AOX	aldehyde oxidase
DPYD	dihydropyrimidine dehydrogenase (NADP+)
MAO, aofH	monoamine oxidase
FMO	dimethylaniline monooxygenase (N-oxide forming)
UGT	glucuronosyltransferase
GST, gst	glutathione S-transferase
ndk, NME	nucleoside-diphosphate kinase
CES1	carboxylesterase 1
DPYS, dht, hydA	dihydropyrimidinase
dut, DUT	dUTP pyrophosphatase
PIK3R1_2_3	phosphoinositide-3-kinase regulatory subunit alpha/beta/delta
CES2	carboxylesterase 2
CYP1A2	cytochrome P450 family 1 subfamily A polypeptide 2
CYP2E1	cytochrome P450 family 2 subfamily E polypeptide 1
GSTK1	glutathione S-transferase kappa 1
ADH1_7	alcohol dehydrogenase 1/7
CYP3A4	cytochrome P450 family 3 subfamily A polypeptide 4
CYP3A5	cytochrome P450 family 3 subfamily A polypeptide 5
CYP2B6	cytochrome P450 family 2 subfamily B polypeptide 6
CYP2C8	cytochrome P450 family 2 subfamily C polypeptide 8
CYP2C19	cytochrome P450 family 2 subfamily C polypeptide 19

Table 5. Name and description of the putative genes involved in drug and xenobiotic metabolic pathways in brushtail possum brain cerebral cortex cells as predicted using the KEGG database.

Gene name	Description
frmA, ADH5, adhC	S-(hydroxymethyl)glutathione dehydrogenase / alcohol dehydrogenase
PIK3CA_B_D	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha/beta/delta
ndk, NME	nucleoside-diphosphate kinase
REV3L, POLZ	DNA polymerase zeta
MSH3	DNA mismatch repair protein MSH3
UMPS	uridine monophosphate synthetase

Discussion

The potential of pests, pathogens, and invasive species to evade conventional population control measures represents a major threat across the globe [65]. Resistance against antibiotics and anthropogenic toxicants occurs in all domains of life, from bacteria to arthropods and vertebrates [66-70]. The genomic basis of developing resistance in non-rodent vertebrates continues to be underrepresented in research and suffers from a lack of enough functional genomic studies. The current study is the first to characterise the functional content and diversity of the liver and brain cerebral cortex transcriptomes of a non-rodent mammalian pest, the brushtail possum in New Zealand.

The functional content and polymorphism landscape of liver and brain cerebral cortex in brushtail possums are involved in a diverse array of biological processes. The functional content of these tissue types plays a critical part in the detoxification of xenobiotic substances [71,72] and modulation of complex behaviours [73,74] that potentially promote the development of physiological and behavioural tolerance against chemical compounds.

Enzymes produced from cytochrome P450 gene groups (such as CYP1A2, CYP2E1, CYP3A4, CYP3A5, CYP2B6, CYP2C8, and CYP2C19) dominate the biochemical pathways involved in drug and xenobiotic substance metabolism in brushtail possum hepatic cells. Polymorphisms in this group of heme-containing proteins influence the speed and efficiency of the chemical compound breakdown in the liver and other organs [75,76].

The detoxification of the harmful component of compound 1080, fluoroacetate, takes place in the hepatocytes [14,77-79]. Polymorphism in the liver transcripts for the metabolic pathways involved in response to drugs and xenobiotic substances is not unexpected. Nonetheless, an

exceptionally high number of polymorphic sites associated with these pathways, particularly a contig with high similarity to the multidrug resistance protein family reported from other marsupials, suggest the potential of standing genomic variation within New Zealand populations to respond against population control chemicals effectively.

Multidrug resistance proteins confer increased drug resistance through a decrease in drug accumulations and an increase in drug efflux [80,81]. Members of this ATP-binding cassette protein super-family function as active transporters of various organic substances, anionic conjugates, and xenobiotic substances, including glutathione, glucuronide, and sulfate conjugates [82,83]. Metabolic pathways that involve glutathione and a unique glutathione transferase are closely linked to the detoxification of compound 1080 and other similar-acting toxicants in hepatic cells [14,77-79]. Moreover, the estimated ratio of synonymous to non-synonymous amino acid substitutions in this transcript indicates that parts of the multi-drug resistance metabolic pathways in brushtail possum hepatic cells may be subject to a significant level of purifying selection against less beneficial genotypes. The pre-adaptations of founder populations with natural exposure to this toxicant in Australia, and the fitness implication of the observed evolutionary dynamics, are yet to be fully understood and require comparative functional genomic studies between a larger sample of brushtail possums in their native range in Australia and invaded habitat in New Zealand.

In the current study, several enriched metabolic pathways were identified in the brushtail possums' cerebral cortex transcriptome that regulates a wide range of core neurobiological processes, such as organic substance transport, response and regulation of biological processes, cell communications, neurochemical signaling, and locomotion. The biochemical pathways involved in drug and xenobiotic substance metabolism in the cerebral cortex are different from those identified in liver cells. The products of several genes in the brushtail possum cerebral cortex (such as PIK3CA_B_D, NME, REVE3L, MSH3, and UMPS) are associated with the development of the resistance against platinum-based anti-malignancy drugs in humans. The complex dynamics of

resistance involve a combination of decreased drug influx, increased drug efflux, intracellular detoxification by glutathione, decreased binding, increased DNA repair, decreased mismatch repair, and defective apoptosis [84,85]. However, the physiological significance of these metabolic pathways in the brushtail possum cerebral cortex in response to the chemicals remains to be investigated.

The delicate interplay between brain cerebral cortex polymorphisms and modulation of complex behaviours is an active area of research in neurobiology [86,87]. While addressing these associations lies beyond the scope of the current study, our results suggest a considerable level of neurobiochemical variation in the brushtail possum cerebral cortex transcriptomic repertoire that is necessary to modulate complex behaviours. Ross*, et al.* [88] suggested that a single exposure to a sub-lethal dose of chemical toxicants in brushtail possums triggers the development of long-lasting bait-shyness behaviours in wild and captive populations. To what extent bait-shyness represents learned behaviour within an individual subsequent to sub-lethal exposure, and its heritability across multiple generations more neurogenomic research.

Instances of developing tolerance against 1080 have already been reported in some wild and laboratory-reared populations with natural exposure to fluoroacetate [77], including wild populations of rabbit (*Oryctolagus cuniculus*) in Australia [69], brushtail possums in south-western Australia [89], and laboratory-reared rats (*Rattus norvegicus*) [90]. The current study does not aim to identify the development of practical resistance against toxins in New Zealand's brushtail possum population. Rather, we demonstrate that these populations harbour considerable levels of functional variation in the metabolic pathways that could potentially promote the development of tolerance against chemicals, such as compound 1080 and other similar acting toxicants. To determine the sensitivity of the current populations to long-term toxin exposure requires formal toxicology trials to measure the lethal concentrations of toxicants in the laboratory and over time.

The power of genetic data to conclusively predict the development of tolerance against toxins remains limited.

The small scale of this study only allows for some preliminary conclusions which are consistent with what we would expect to see from a species that has been under intense anthropogenic selection pressures including both trapping and toxins for generations.

As conservation efforts to eradicate invasive mammals intensify towards the objectives of the New Zealand Predator Free 2050 horizon, we strongly advocate combined and multifaceted population control strategies that simultaneously target different aspects of an invasive species' life history, behaviour, and physiology in time and space, and thus prevent or delay the potential development of resistance against any specific population control measure [65,91]. The ecological and economic impacts of brushtail possums potentially developing tolerance against population control measures necessitate constant monitoring of current populations and their response to management plans.

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