PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Differences in gene expression in field populations of *Wolbachia*-infected *Aedes aegypti* mosquitoes with varying release histories in northern Australia

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Citation: Wimalasiri-Yapa BMCR, Huang B, Ross PA, Hoffmann AA, Ritchie SA, Frentiu FD, et al. (2023) Differences in gene expression in field populations of *Wolbachia*-infected *Aedes aegypti* mosquitoes with varying release histories in northern Australia. PLoS Negl Trop Dis 17(3): e0011222. https://doi.org/10.1371/journal.pntd.0011222

Editor: Adly M. M. Abd-Alla, International Atomic Energy Agency, AUSTRIA

Received: October 30, 2022

Accepted: March 7, 2023

Published: March 29, 2023

Data Availability Statement: Reads are available from the NCBI Short Read Archive under Accession Number PRJNA867516.

Funding: Funding for this study was provided to DW and AvdH by Forensic and Scientific Services, Department of Health, Queensland Government (ID:RSS19-001) and to AAH by the National Health and Medical Research Council (ID: 1132412, 1118640). The funders had no role in study design,

Abstract

Aedes aegypti is the principal mosquito vector of dengue, yellow fever, Zika and chikungunya viruses. The *w*Mel strain of the endosymbiotic bacteria *Wolbachia pipientis* was introduced into the vector as a novel biocontrol strategy to stop transmission of these viruses. Mosquitoes with *Wolbachia* have been released in the field in Northern Queensland, Australia since 2011, at various locations and over several years, with populations remaining stably infected. *Wolbachia* infection is known to alter gene expression in its mosquito host, but whether (and how) this changes over the long-term in the context of field releases remains unknown. We sampled mosquitoes from *Wolbachia*-infected populations with three different release histories along a time gradient and performed RNA-seq to investigate gene expression changes in the insect host. We observed a significant impact on gene expression in *Wolbachia*-infected mosquitoes versus uninfected controls. Fewer genes had significantly upregulated expression in mosquitoes from the older releases (512 and 486 from the 2011 and 2013/14 release years, respectively) versus the more recent releases (1154 from the 2017 release year). Nonetheless, a fundamental signature of *Wolbachia* infection on host gene expression was observed across all releases, comprising upregulation of immunity (e.g. leucine-rich repeats, CLIPs) and metabolism (e.g. lipid metabolism, iron transport) genes. There was limited downregulation of gene expression in mosquitoes from the older releases (84 and 71 genes from the 2011 and 2013/14 release years, respectively), but significantly more in the most recent release (509 from the 2017 release year). Our findings indicate that at > 8 years post-introgression into field populations, *Wolbachia* continues to profoundly impact expression of host genes, such as those involved in insect immune response and metabolism. If *Wolbachia*-

data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

mediated virus blocking is underpinned by these differential gene expression changes, our results suggest it may remain stable long-term.

Author summary

The *Aedes aegypti* mosquito is the main species responsible for urban transmission of dengue, Zika and chikungunya viruses. Control measures, including source reduction and insecticide treatment, have historically struggled to provide sustained control of this species to limit disease. An alternative approach involves releasing mosquitoes harbouring *Wolbachia* bacteria. *Wolbachia* inhibits virus transmission by *Ae. aegypti* and preliminary evidence indicates that dengue incidence is reduced in locations where it has been deployed. In this study, we found that *Wolbachia* significantly upregulates gene expression in *Ae. aegypti* at least 8 years after field deployment compared with uninfected controls, although some gene downregulation was also observed. We observed a more 'muted' response in mosquitoes from populations with older release histories, with far fewer genes being differentially regulated versus those from the most recent releases. Irrespective of release history, immune response and metabolism genes were significantly upregulated, and to a lesser extent genes related to behaviour. Our results, combined with previous studies that have revealed few changes in the *Wolbachia* genome post release, provide further evidence of the long-term stability of the effects of *Wolbachia* on introgressed mosquito populations in the field.

Introduction

The *Aedes aegypti* mosquito is the primary urban vector of dengue, yellow fever, chikungunya and Zika viruses [1]. *Aedes aegypti* has a close association with humans, who are its primary blood meal source [2]. It also preferentially blood-feeds and rests indoors, and utilizes water-filled receptacles proximal to human habitation as larval habitats. These behaviours make *Ae. aegypti* notoriously difficult to control, particularly in high-density urban areas [3]. Control of *Ae. aegypti* has relied on the reduction, elimination or insecticide treatment of receptacle habitats and/or application of adulticides, either as space sprays or targeted indoor residual application [4]. However, lack of sustainability of current government-administered control programs, coupled with insecticide resistance, compromises effectiveness of these control strategies. More sustainable approaches to controlling *Ae. aegypti* and its associated arboviruses are required, especially due to a lack of suitable vaccines (except for yellow fever) or antiviral therapies to limit disease.

Alternative strategies involving the targeted release of modified *Ae. aegypti* are at various stages of development and show excellent promise for sustained control of arbovirus transmission [5,6]. One of the most advanced involves the release of *Ae. aegypti* transinfected with the endosymbiotic bacterium *Wolbachia pipientis*, which confers phenotypes that can be exploited to limit arbovirus transmission [7]. High rates of maternal transmission and cytoplasmic incompatibility (CI) allow the bacteria to spread through and be maintained in the resident mosquito population [8,9]. Arbovirus replication and transmission is blocked in mosquitoes infected with *Wolbachia* [10–12]. Following initial success with driving the *w*Mel strain of *Wolbachia* into *Ae. aegypti* populations in urban centers of north Queensland, Australia [8,13,14], releases of *Wolbachia*-infected *Ae. aegypti* are being conducted in at least 12

countries [9,15]. Epidemiological evidence suggests a significant reduction in dengue incidence post deployment in dengue endemic locations [9,16].

Stability of the endosymbiont infection in *Ae*. *aegypti* will be critical for the long-term viability of *Wolbachia*-based arbovirus control programs. Any loss of the virus blocking phenotype could lead to increased virus transmission by mosquitoes. High temperatures could lead to the loss of *Wolbachia* from populations [17–19], but other factors (such as high fitness costs due to *Wolbachia* or the release strain and dry environmental conditions) could be responsible too [20,21]. Alternatively, there are three potential vulnerabilities for breakdown of virus blocking related to the evolution of microbe and mosquito: virus evolutionary escape, and changes to the *Wolbachia* or mosquito genomes. Hence, post-release long-term monitoring should include periodic genome sequencing and assessment of the virus, bacteria and mosquito. As dengue viruses have an RNA genome, they are subject to relatively high mutational rates compared to other DNA-based organisms and microbes, so selection of virus strains which escape from the effects of *Wolbachia* are a possibility [22].

Other evolutionary pressures may drive genetic changes in either the *Wolbachia* or the mosquito. In recent studies to explore the first possibility, mosquitoes collected from north Queensland had few changes in their *Wolbachia* genome sequences compared to the pre-release strain, indicating a high level of stability to date [23,24]. In addition, a comparison of the mosquito genomes also suggested that there have been few changes in the mosquito genome since *Wolbachia* replacement [25], whilst the frequency of *Wolbachia* in invaded populations has remained high [14,26] and most host effects of the *Wolbachia* have remained stable [26] with the exception of effects on egg quiescence [27]. Although few genomic changes have so far been detected and linked to structural changes in genes, it is possible that there have nevertheless been changes in the expression of host genes which are often sensitive indicators of adaptation, including in immune responses [28].

Changes in gene expression patterns of *Wolbachia*-infected mosquitoes could lead to loss of virus blocking abilities. Hypotheses on a virus blocking mechanism can be grouped into the two broad categories of mosquito immune gene activation and/or competition for host cell resources (reviewed in [29,30]). However, immune genes are activated by transinfected *Wolbachia* but are not required for blocking in naturally infected hosts, so may not be essential [31,32]. Important examples of antiviral pathways in the insect cell include the activation of signalling pathways such as Janus kinase-signal transducer and activator of transcription (JAK-STAT), reactive oxygen species (ROS), and Toll signalling. Various anti-microbial proteins and compounds can be induced such as the interferon-like Vago and Dnmt2 which may modify viral RNA, making it susceptible to methylation-mediated degradation. However, none of these pathways has so far been directly linked to the blocking effect [30]. The exonuclease XRN1 is induced in *Wolbachia*-infected cells and has been associated with viral RNA degradation [33]. RNA interference is also activated but may not be an important viral blocking pathway in *Wolbachia*-infected cells [34].

An alternative hypothesis to immune activation is that virus is in competition with *Wolbachia* for physical space and other host cell resources. *Wolbachia* density in some cases may be correlated with the virus blocking effect [35,36], and exclusion of virus from cells and tissues where *Wolbachia* is found at high density suggests competition for cellular resources. Cell nutrients are regulated by the host, and *Wolbachia* [37] and viruses may compete for amino acids. *Wolbachia* may also alter the metabolism of lipids such as cholesterol [38,39], a molecule which is also essential for dengue virus replication [40]. Alternatively, *Wolbachia* may physically exclude RNA viruses from cellular organelles necessary for viral replication [41]. The relative contributions of immune activation and host resource competition in virus blocking remains an open question. Elucidating whether immune activation persists in long-term *Ae*.

aegypti—*Wolbachia* associations would shed light on this question and inform considerations of the long-term stability of virus blocking.

The objectives of the study described here were threefold. First, we obtained samples from multiple locations in and around Cairns, Australia, the site of releases of *Wolbachia*-infected mosquitoes from 2011–2017 and compared their gene expression to uninfected mosquitoes to determine which genes were differentially expressed. Second, we established a baseline for gene expression in *Wolbachia*-infected mosquitoes for longer-term studies which will help monitor for any breakdown in virus blocking. Finally, *Wolbachia*-infected mosquitoes were present at some sites for up to eight years, so we determined if there were any trends or differences in mosquito gene expression between sites with older compared with more recent releases.

Results

Mosquito gene expression clusters according to *Wolbachia* infection status

Eggs were collected in 2019 from populations where *Wolbachia*-infected *Ae. aegypti* were released in years 2011 (Aae.wMel$_{2011}$), 2013–2014 (Aae.wMel$_{2013/2014}$) and 2017 (Aae.wMel$_{2017}$), as well as from a wild-type population (Aae.wt) where *Wolbachia*-infected mosquitoes had not been released (Fig 1). Eggs were hatched and reared under laboratory conditions and transcriptome sequencing (RNA-seq) of pools of 5 adult females at 4 days post emergence was performed (Table 1).

To ensure that the variation in gene expression described below was likely due to actual gene expression and not differences in *Wolbachia* density, the number of copies of *w*Mel *wsp* gene in *Ae. aegypti* was quantified using the method of Lee et al. [42]. There was no significant difference (Mann-Whitney test $P = 0.5926$) in *Wolbachia* density between mosquitoes collected from the 2013–14 and 2017 release locations (S1 Fig; note that there was no material available for density quantification from the 2011 release sites).

A total of 2,041,416,107 Illumina raw reads were obtained from sequencing on the NovaSeq at the Australian Genome Research Facility (AGRF). Among these, there was a total of 1,554,852,005 read pairs and overall 89.42% of the pairs were aligned to the *Ae. aegypti* reference genome GCF_002204515.2 (https://www.ncbi.nlm.nih.gov/genome/?term=txid7159[orgn]) (see S1 Table for mapping statistics). Following initial processing of raw reads (see Materials and methods), differentially expressed genes (DEGs) were identified by comparing gene expression in *Wolbachia*-infected versus uninfected mosquitoes using the DESeq2 tool. DEGs were considered statistically significant when the absolute fold change of the gene expression was > 2 and adjusted P-value was < 0.05. Comparison of gene expression between all infected mosquitoes (n = 28 pools) and uninfected mosquitoes (n = 5 pools) resulted in a total of 747 DEGs (656 upregulated and 91 downregulated). A heatmap of differential expression in these 747 genes indicated separate clustering of *Wolbachia*-infected and uninfected mosquitoes (Fig 2).

Elevated DEGs in mosquitoes from the most recent *Wolbachia* field release

An analysis of the gene expression of *Wolbachi*a-infected versus uninfected *Ae. aegypti* indicated that the highest number of DEGs was observed in the Aae.wMel$_{2017}$ mosquitoes (i.e. mosquitoes descended from releases that occurred 2 years prior to collection for this study (Table 2)). By contrast, a smaller number of DEGs was observed in mosquitoes from older releases, Aae.wMel$_{2011}$ and Aae.wMel$_{2013/2014}$ (Table 2), relative to *Wolbachia*-uninfected mosquitoes. The difference in number of DEGs from the Aae.wMel$_{2017}$ mosquitoes versus those from the Aae.wMel$_{2011}$ and Aae.wMel$_{2013/2014}$ mosquitoes was statistically significant (Chi-

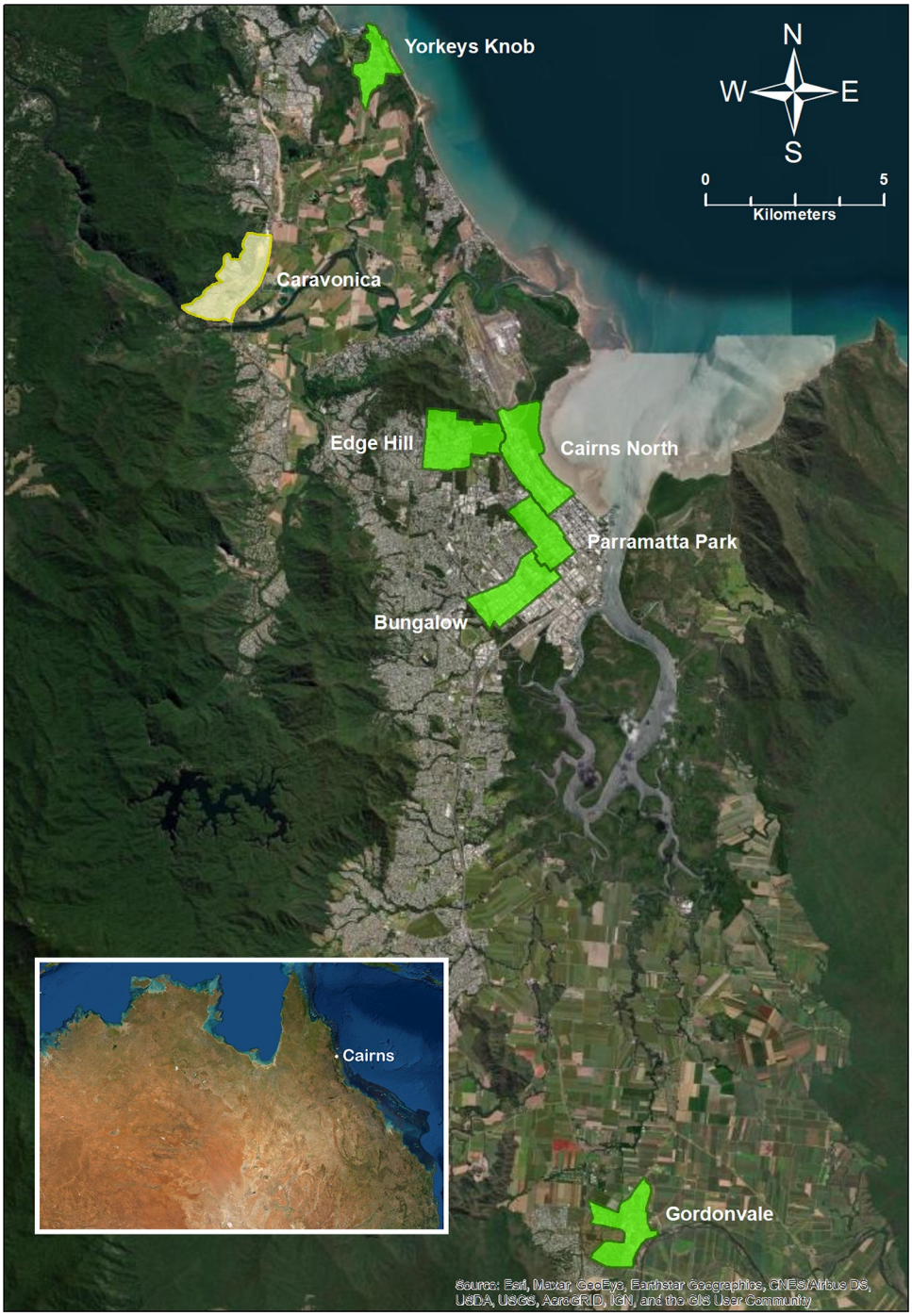

Fig 1. Map of mosquito collection locations in the Cairns Regional Council area, northern Queensland, Australia. *Wolbachia*-infected mosquitoes were released in the suburbs shaded green. There had been no releases of *Wolbachia* infected mosquitoes in Caravonica (shaded yellow) up until at least April 2019, when the collections for the current study were undertaken. Source of baselayer is https://www.arcgis.com/home/item.html?id=10df2279f9684e4a9f6a7f08febac2a9. This map was created using ArcGIS software by ESRI (www.esri.com). ArcGIS and ArcMap are the intellectual property of Esri and are used herein under license. For more information about Esri software, please visit www.esri.com.

https://doi.org/10.1371/journal.pntd.0011222.g001

Table 1. *Aedes aegypti* collected in April 2019 from Cairns, Queensland, Australia, for mosquito transcriptome sequencing.

Suburb	Release date[a]	No. sample sites	No. mosquitoes	No. pools
Gordonvale	January 2011	10	50	10
Yorkeys Knob	January 2011	2	10	2
Edge Hill	January 2013	8	40	8
Parramatta Park	January 2013	1	5	1
Cairns North	August 2014	1	5	1
Bungalow	July 2014	1	5	1
Cairns North	March 2017	3	15	3
Parramatta Park	March 2017	2	10	2
Caravonica	No release	5	25	5

[a]Date when releases of *Wolbachia*-infected *Ae. aegypti* commenced in the suburb.

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square $P < 0.001$). A principal component analysis (PCA) of all genes indicated a clear demarcation of gene expression between infected and uninfected mosquitoes descended from 2017 releases (Fig 3A). This demarcation was less apparent in mosquitoes with earlier release histories (Fig 3B and 3C).

Comparison of DEGs from the three *Wolbachia*-infected mosquito release times identified 357 common upregulated genes and 23 common downregulated genes (Fig 4). There were 76, 37 and 1029 unique DEGs for the mosquitoes from *w*Mel releases in the Aae.wMel$_{2011}$, Aae.wMel$_{2013/2014}$ and Aae.wMel$_{2017}$ mosquitoes, respectively, compared with the uninfected mosquitoes.

We identified 594 genes to be uniquely upregulated in the mosquitoes from locations of *w*Mel releases in 2017 (that is, not found in any of the earlier releases). The comparison of expression level of these 594 genes across all populations using the Kruskal-Wallis test showed that expression of these genes was significantly associated ($P < 0.001$) with time point (the year that mosquito population acquired *w*Mel). Post-hoc analysis revealed that the median gene expression (counts per million) in Aae.wMel$_{2017}$ mosquitoes was significantly higher than those originating from the earlier releases ($P < 0.001$; S2 Table).

Upregulated DEGs with the highest fold change belong to immune response

Immune function genes, genes associated with stress response and non-coding genes comprised the 10 most upregulated genes, with the highest fold change values in comparisons involving *Wolbachia*-infected from all release populations versus uninfected mosquitoes. Among these, the majority (70–80%, depending on release history) were immune genes (Fig 5). Three immune genes were significantly upregulated in all *w*Mel-infected mosquito populations, irrespective of release year. These were CTLGA8 (LOC5575053), alpha-2-macroglobulin (LOC23687443) and leucine-rich repeat-containing protein 40-like (LOC110677030). There were 7, 8 and 8 DEGs related to immune response in mosquitoes from Aae.wMel$_{2011}$, Aae.wMel$_{2013/2014}$ and Aae.wMel$_{2017}$, respectively. The topmost upregulated gene in Aae.wMel$_{2011}$ and Aae.wMel$_{2013/2014}$ mosquitoes was the transferrin gene (LOC5579417), with this gene being the second-most upregulated in Aae.wMel$_{2017}$ mosquitoes. Interestingly, this gene was upregulated in all mosquito samples from *Wolbachia*-infected populations, across all years of release, compared to uninfected mosquitoes. Two out of three remaining topmost upregulated genes of Aae.wMel$_{2011}$ mosquitoes were CLIP (LOC5578693) and leucine-rich repeat (LOC5575814), both pathogen recognition receptor (PRR) molecules that are important for

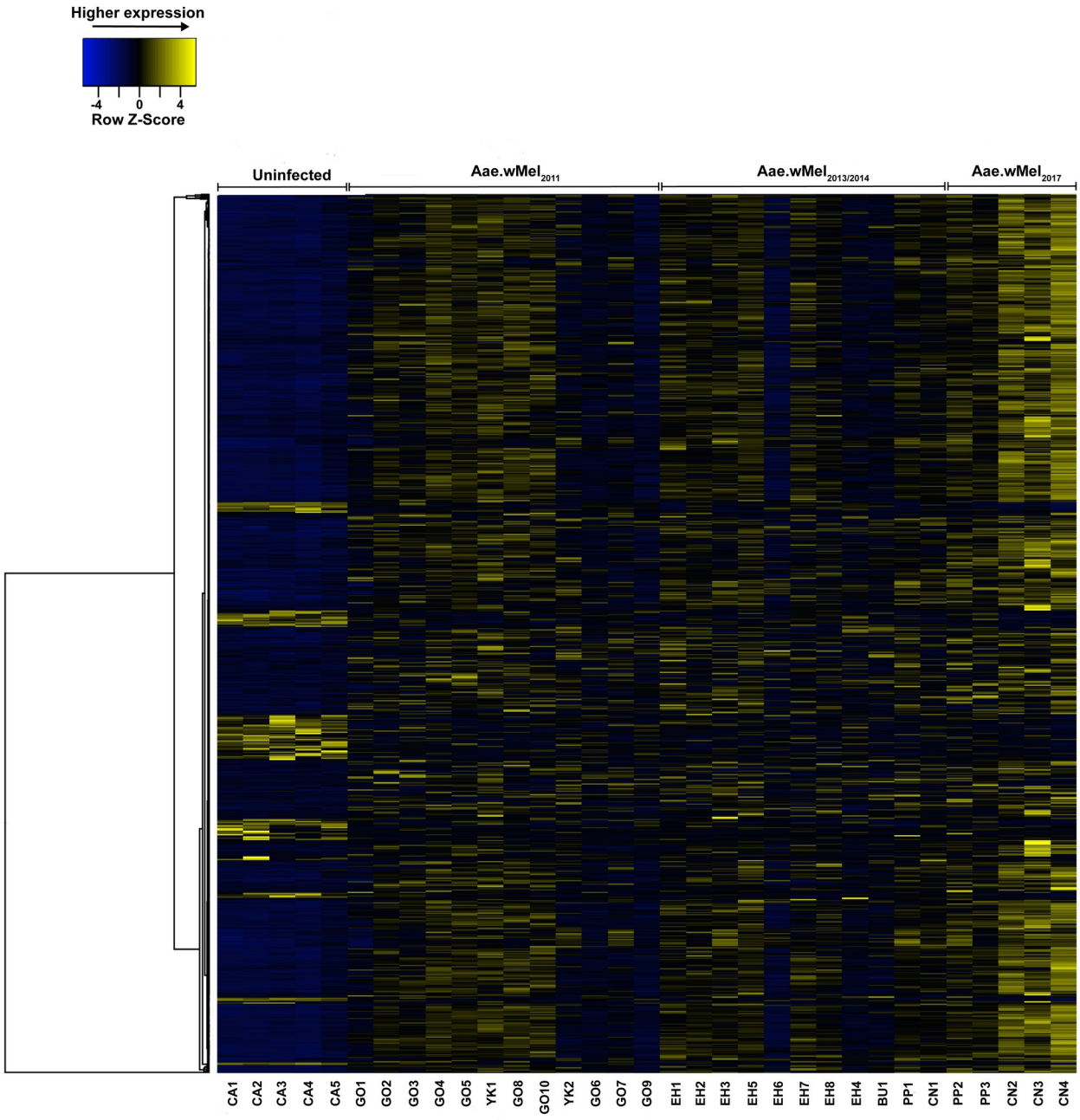

Fig 2. Heatmap of normalised read counts of differentially expressed genes (DEGs) showing clustering of transcriptomic response in *w*Mel *Wolbachia*-infected *Aedes aegypti* from locations with different release years versus uninfected *Ae. aegypti*. Hierarchical clustering was performed using the complete linkage method and the distances between columns were computed by the Euclidean method (http://www.heatmapper.ca/expression/). Acronyms for the individual mosquito pool identifiers on the X-axis denote suburb names (CA = Caravonica, GO = Gordonvale, YK = Yorkeys Knob, EH = Edge Hill, BU = Bungalow, PP = Parramatta Park, and CN = Cairns North).

https://doi.org/10.1371/journal.pntd.0011222.g002

immune function. The other was arrestin C-terminal-like domain-containing protein 3 (LOC5570224) which plays a role in regulation of the olfactory system. Similarly, Aae. wMel$_{2013/2014}$ and Aae.wMel$_{2017}$ mosquitoes also showed two PRRs each including LOC5575053 and LOC110677006 in the earlier, and LOC5570871 and LOC5576315 in the later release. Additionally, mosquitoes from these releases showed differential expression of

Table 2. Number of DEGs (absolute fold change ±2 and adjusted *P*-value (false discovery rate (FDR)) ≤ 0.05) in mosquitoes from populations with different *w*Mel release years versus *Wolbachia*-uninfected mosquitoes.

	Aae.wMel$_{2011}$	Aae.wMel$_{2013/2014}$	Aae.wMel$_{2017}$
Upregulated	512	486	1154
Downregulated	84	71	509
Total	597[a]	557[b]	1664[a, b]

[a] Chi square (number of DEGs in Aae.wMel$_{2011}$ vs Aae.wMel$_{2017}$) $P < 0.001$

[b]Chi-square (number of DEGs in Aae.wMel$_{2013/2014}$ vs Aae.wMel$_{2017}$) $P < 0.001$. Comparisons between Aae.wMel$_{2011}$ and Aae.wMel$_{2013/2014}$ were not significantly different (Chi square $P > 0.05$).

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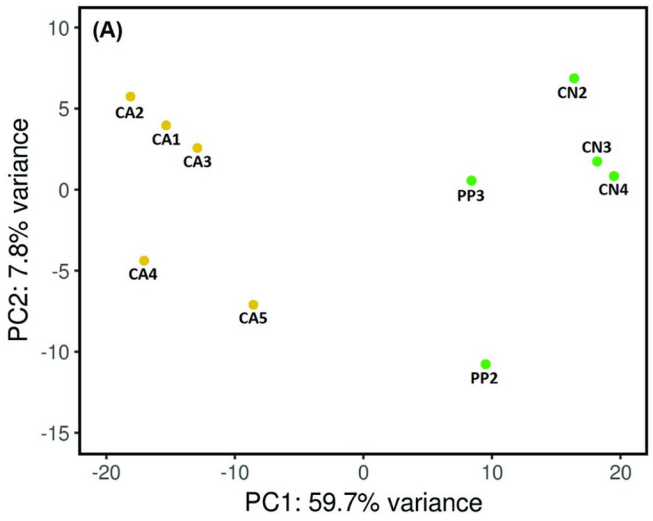
putative defense protein 1 (LOC5572918) and a negative regulator of translation (LOC5569955). Altogether, four genes (2011: LOC5567033, LOC5566857; 2013–14: LOC110673980; and 2017: LOC5563952) out of the 10 topmost upregulated genes across all three mosquito groups are regarded as involved in stress response whilst two genes were non-coding (Fig 5).

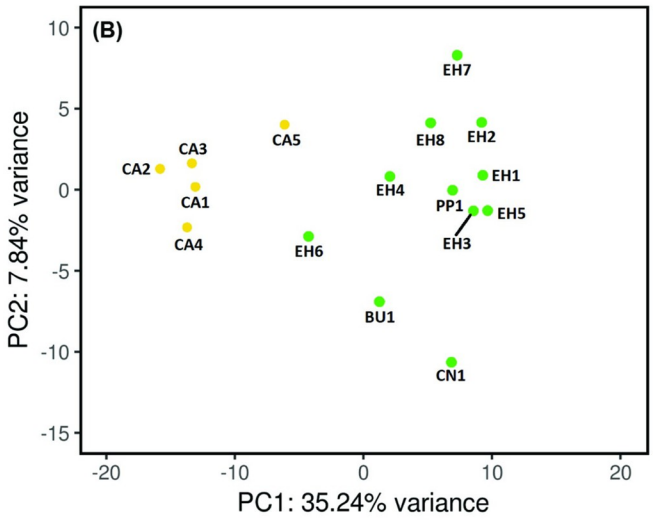
Topmost downregulated genes belong to non-coding RNAs, cell proliferation and host behaviour

The highest fold downregulated gene categories included non-coding RNAs, genes involved in cell replication, and host behaviour related genes (Fig 6). Unlike upregulated genes with highest fold changes mentioned above, none of the downregulated genes were common to mosquitoes from all three *Wolbachia* release histories. However, four downregulated DEGs common to Aae.wMel$_{2011}$ and Aae.wMel$_{2013/2014}$ mosquitoes were CFI06_mgr02, LOC5574234, LOC5576517 and LOC5572259. Aae.wMel$_{2011}$ had the majority (6/10) of downregulated genes with the highest fold change in either non-coding RNA genes (LOC110676610, LOC110679144, LOC5574600, LOC110676459) or uncharacterized genes (LOC5572259, LOC5568345). Three out of 10 topmost downregulated genes with highest fold change from Aae.wMel$_{2013/2014}$ were either ncRNA or uncharacterized proteins. Genes that were downregulated with highest fold change in Aae.wMel$_{2017}$ mosquitoes were unique except for histone-lysine N-methyltransferase Suv4-20 (LOC5569935) which was shared with Aae.wMel$_{2013/2014}$ mosquitoes. Some of these genes included three ncRNA genes (LOC110679860, LOC5564187, LOC5563860), a neurotransmitter gene (LOC23687658), a gene responsible for promotion of micropinocytosis (LOC110674232) and three genes playing a role in cell replication. No single gene was observed to be significantly downregulated in all *Wolbachia*-infected mosquito samples, from across all years, versus uninfected samples.

*w*Mel is associated with upregulation of pathways related to immunity, amino acid and lipid metabolism, and behaviour, across all mosquito release time points

Gene ontology (GO) analysis performed using the DAVID bioinformatics tool [43] revealed that 357 upregulated genes common to all releases resulted in six significantly enriched (Fisher's exact $P < 0.05$) biological processes including innate immune response (GO:0045087), fatty acid biosynthetic process (GO:0006633), phototransduction (GO:0007602), defense response to bacterium (GO:0042742), visual perception (GO:0007601) and urea cycle (GO:0000050) (Fig 7). Moreover, two cellular locations comprising extracellular space (GO:0005615) and extracellular region (GO:0005576) were significantly enriched at the same


(A)
CA2
CA1
CA3
CA4
CA5
CN2
CN3
CN4
PP3
PP2
PC2: 7.8% variance
PC1: 59.7% variance


(B)
EH7
EH8
EH2
EH1
EH5
EH4
PP1
EH3
EH6
BU1
CN1
CA5
CA3
CA2
CA1
CA4
PC2: 7.84% variance
PC1: 35.24% variance

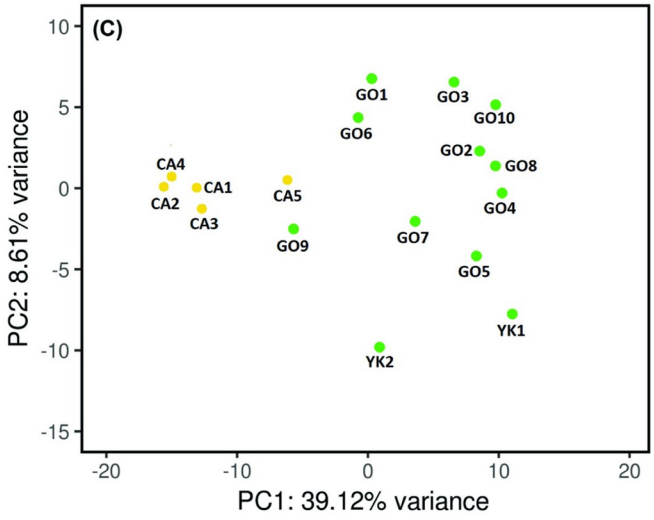

(C)
GO1
GO3
GO10
GO6
GO2
GO8
GO4
GO7
GO5
GO9
YK1
YK2
CA4
CA1
CA2
CA3
CA5
PC2: 8.61% variance
PC1: 39.12% variance

Fig 3. Principal component analysis (PCA) of gene expression in *Wolbachia*-infected mosquitoes (green shading) originating from different release histories: A) Aae.wMel$_{2017}$ vs uninfected; B) Aae.wMel$_{2013/2014}$ vs uninfected; and C) Aae.wMel$_{2011}$ vs uninfected. Uninfected mosquitoes (yellow shading) were from the suburb of Caravonica where releases of *Wolbachia*-infected mosquitoes had not been conducted up until the time of our collections in April 2019. Acronyms for the individual mosquito pool identifiers denote suburb names (CA = Caravonica, GO = Gordonvale, YK = Yorkeys Knob, EH = Edge Hill, BU = Bungalow, PP = Parramatta Park, and CN = Cairns North).

https://doi.org/10.1371/journal.pntd.0011222.g003

cut-off criterion. Molecular functions, namely serine-type endopeptidase activity (GO:0004252) and endopeptidase inhibitor activity (GO:0004866) were significantly enriched (Fisher's exact $P < 0.05$).

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (FDR < 0.05) of these proteins further revealed that metabolic pathways (aag01100), glycine, serine and threonine metabolism (aag00260), biosynthesis of amino acids (aag01230), arginine biosynthesis (aag00220), tyrosine metabolism (aag00350), arginine and proline metabolism (aag00330), caffeine metabolism (aag00232), carbon metabolism (aag01200) and phototransduction–fly (aag04745) were upregulated (see S3 Table for enrichment analysis data).

Uniquely upregulated DEGs in Aae.wMel$_{2017}$ mosquitoes are involved in stress response, membrane transport and iron metabolism

Following the observation of higher levels of DEGs in the Aae.wMel$_{2017}$ mosquitoes versus other release histories, we explored what types of genes were present in this group, starting with upregulated genes. For this purpose, first we evaluated upregulated DEGs (n = 594) unique to Aae.wMel$_{2017}$ mosquitoes. Three GO terms of biological process, namely sodium ion transport (GO:0006814), ion transport (GO:0006811) and metabolic process (GO:0008152), were significantly enriched at Fisher's exact $P < 0.05$. Moreover, four GO

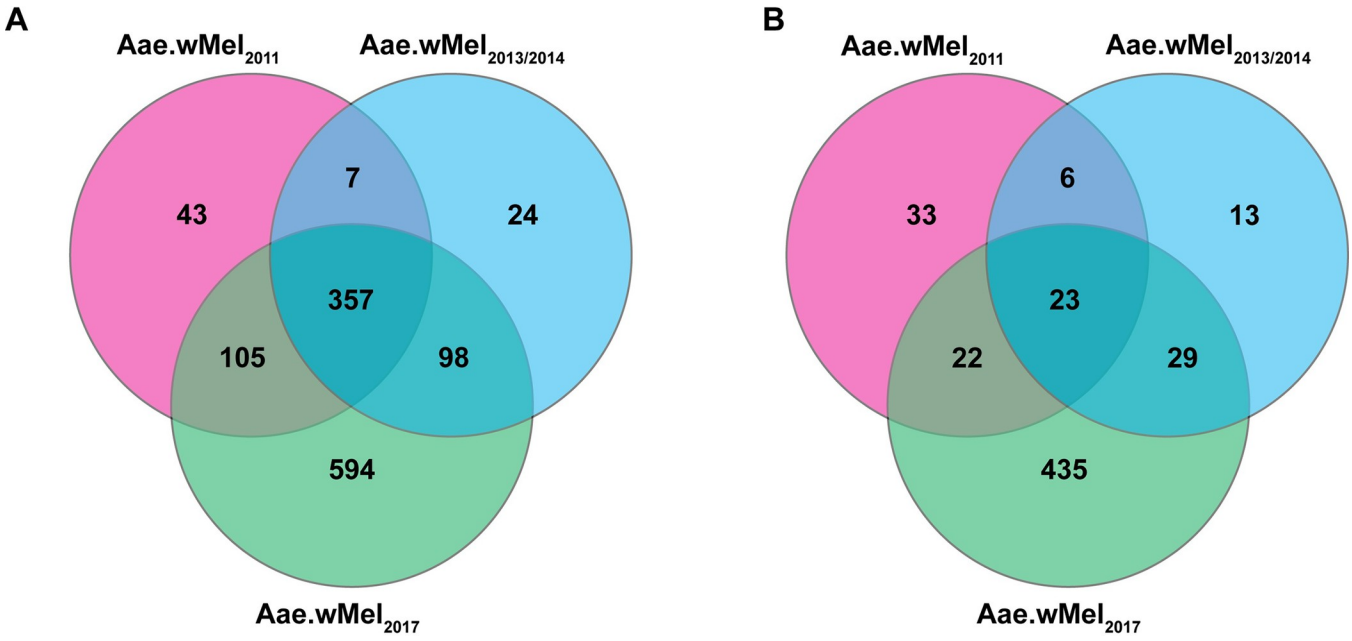


Fig 4. Numbers of shared and uniquely (A) upregulated and (B) downregulated genes in mosquitoes from populations with different *w*Mel release histories.

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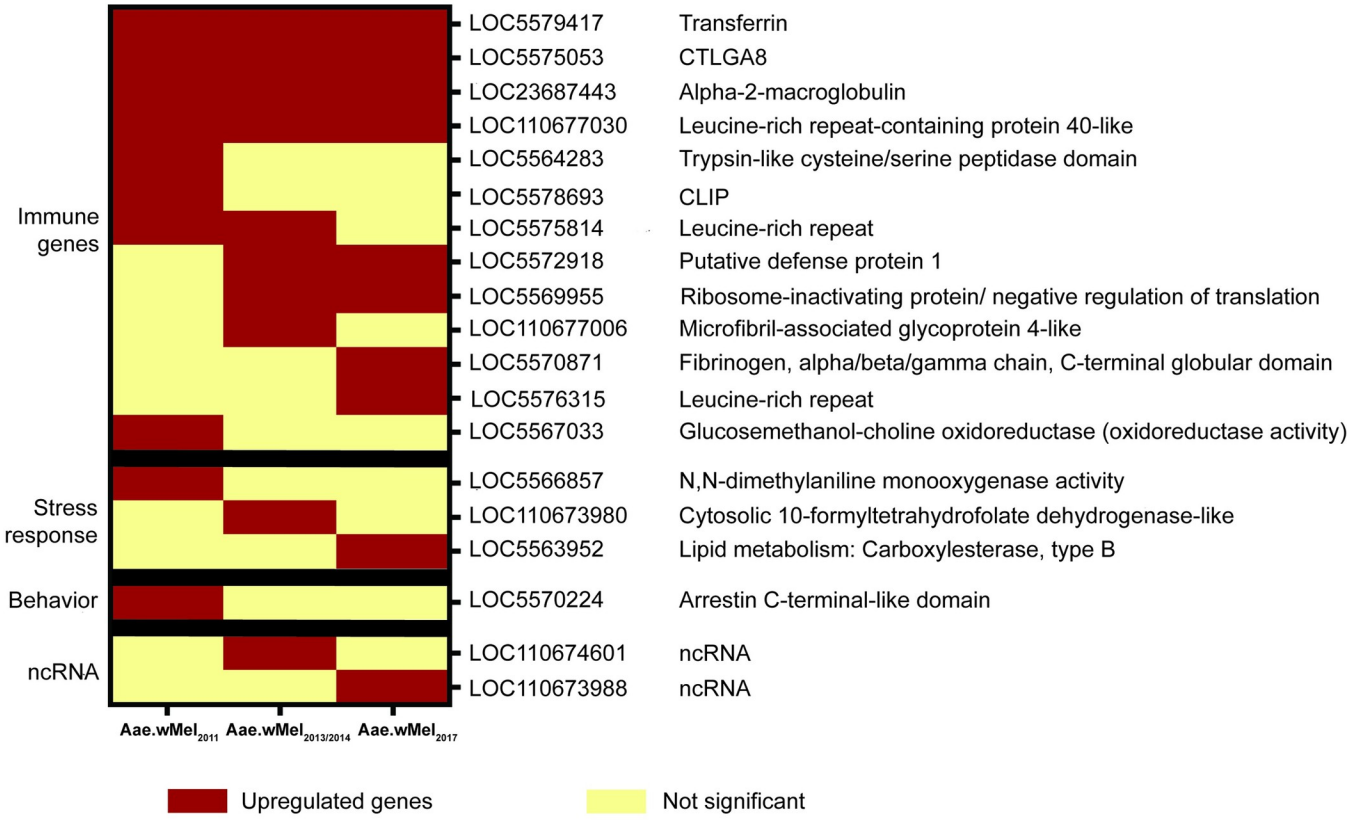

Fig 5. The top 10 upregulated genes with highest fold difference between *Wolbachia*-infected and uninfected *Aedes aegypti*, according to release year. The type of upregulated gene (immune genes, stress response, ncRNA, and behaviour) is shown.

https://doi.org/10.1371/journal.pntd.0011222.g005

terms of cellular locations: integral component of membrane (GO:0016021), extracellular region (GO:0005576), gap junction (GO:0005921) and extracellular space (GO:0005615) were also significantly enriched. Cytochrome-c oxidase activity (GO:0004129), oxidoreductase activity (GO:0016491), monooxygenase activity (GO:0004497), oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen (GO:0016705), iron ion binding (GO:0005506), serine-type endopeptidase activity (GO:0004252), heme binding (GO:0020037), transporter activity (GO:0005215), phosphopantetheine binding (GO:0031177) and hydrolase activity (GO:0016787) were among the significantly enriched molecular functions.

KEGG enrichment (FDR < 0.05) indicated several pathways that were significantly enriched in Aae.wMel$_{2017}$ mosquitoes, but not the other release years. Starch and sucrose metabolism (aag00500), other glycan degradation (aag00511), glyoxylate and dicarboxylate metabolism (aag00630), glycine, serine, and threonine metabolism (aag00260), oxidative phosphorylation (aag00190), glycolysis / gluconeogenesis (aag00010), galactose metabolism (aag00052), amino sugar and nucleotide sugar metabolism (aag00520) and lysosome (aag04142) were among the uniquely enriched pathways. Moreover, additional genes of KEGG pathways such as biosynthesis of amino acids (aag01230), carbon metabolism (aag01200) and metabolic pathways (aag01100) were also identified during KEGG pathway enrichment analysis.

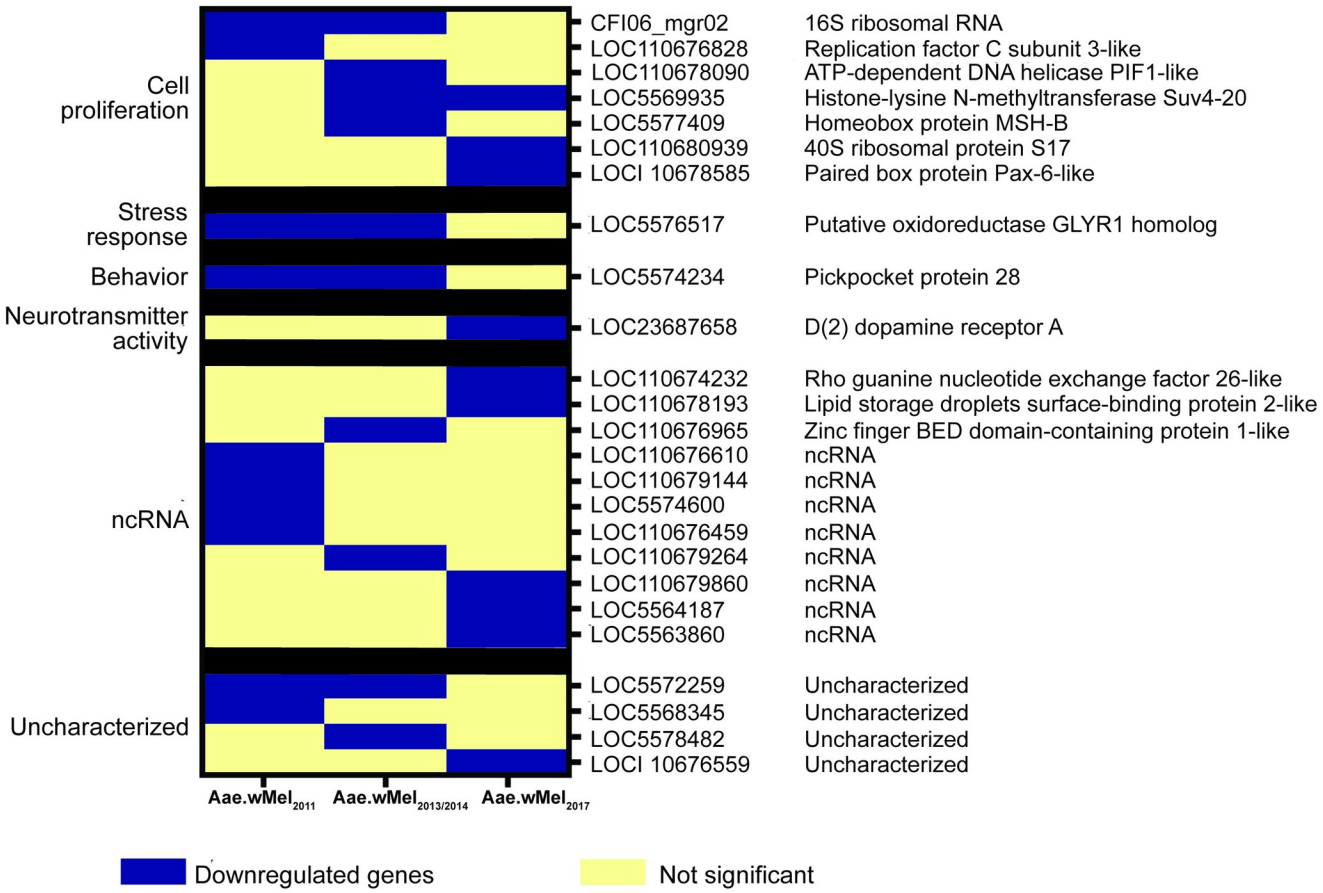

Fig 6. Topmost downregulated genes with highest fold difference between *Wolbachia*-infected and uninfected *Aedes aegypti*, according to release year. The type of downregulated gene (cell proliferation, stress response, behaviour, neurotransmitter activity, ncRNA, and uncharacterised) is shown.

https://doi.org/10.1371/journal.pntd.0011222.g006

Downregulated DEGs in Aae.wMel$_{2011}$ mosquitoes are related to DNA replication

Eighty-four downregulated DEGs were uploaded for GO analysis using the DAVID bioinformatics tool. Nucleosome assembly was the only GO term pertaining to biological processes that was significantly enriched (Fisher's exact $P < 0.05$). Two GO terms, Nucleus (GO:0005634) and nucleosome (GO:0000786), were the significantly enriched cellular locations at Fisher's exact $P < 0.05$. None of the GO terms classified as molecular functions (GO:0003677: DNA binding) were significantly enriched (Fisher exact $P > 0.05$). Three functional annotation clusters resulted from a DAVID cluster analysis, in which the first cluster had three GO terms (GO:0005634: nucleus, GO:0000786: nucleosome and GO:0003677: DNA binding), four UNIPROT keywords (DNA-binding, nucleosome core, chromosome, and nucleus) and an InterPro term (IPR009072: histone-fold). The second cluster included one GO term (GO:0008270: zinc ion binding) and two InterPro terms (IPR011011: Zinc finger, FYVE/PHD-type and IPR013083: Zinc finger, RING/FYVE/PHD-type). The third cluster was included with three UniProt keywords: transmembrane helix, transmembrane and membrane; one GO term: GO:0016021: integral component of membrane.

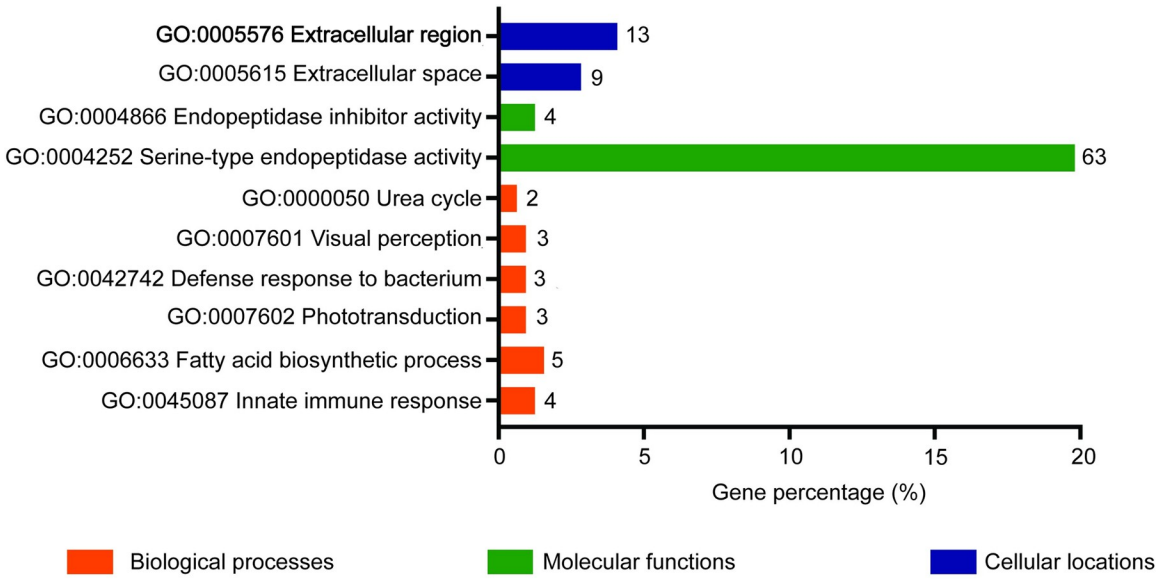

Fig 7. Gene ontology analysis of upregulated differentially expressed genes common to all *Wolbachia*-infected *Aedes aegypti* versus uninfected mosquitoes. The number next to each bar refers to the number of genes involved in the biological process, molecular function or cellular location.

https://doi.org/10.1371/journal.pntd.0011222.g007

A considerable number (36/84) of genes were unmapped to the DAVID cloud map gene IDs. Among these unmapped genes, 16 were non-coding RNA genes (S4 Table) and three were uncharacterized, while information for the rest is given in the S5 Table.

Downregulated DEGs in Aae.wMel$_{2013/2014}$ and Aae.wMel$_{2017}$ mosquitoes are related to multicellular organism development

When considering Aae.wMel$_{2013/2014,}$ there were 71 downregulated DEGs, among which 26 genes (36.6%) of the Gene IDs were not identified in the DAVID cloud map. There was a single biological process significantly enriched at Fisher's exact $P < 0.05$: multicellular organismal process (GO:0032501). None of the cellular locations or molecular processes were significantly enriched. DAVID pathway enrichment analysis identified four KEGG pathways that were enriched: notch signalling pathway (aag04330), lysine degradation (aag00310), dorso-ventral axis formation (aag04320) and Wnt signalling pathway (aag04310). However, none of these were enriched at Fisher's $P < 0.05$. There were three annotation clusters that were enriched at a score > 0.5 in DAVID functional annotation clustering. The first cluster included three UniProt keywords (Receptor, Transducer and G-protein coupled receptor) and two InterPro protein families (IPR000276: G protein-coupled receptor, rhodopsin-like, IPR017452: GPCR, rhodopsin-like, 7TM). The second cluster included one GO term (GO:0016021 integral component of membrane) and three UniProt key words (transmembrane helix, transmembrane and membrane). Two UniProt keywords (DNA-binding and nucleus) and one GO term (GO:0005634 nucleus) comprised the third cluster. Genes that were not mapped to DAVID cloud map were manually checked using an NCBI Gene search (S6 Table). Eleven out of 26 were ncRNA (S4 Table). Three genes were uncharacterized (LOC110674591, LOC110674325 and LOC110680306), while six out of remaining 12 genes were transcription factors (LOC110675182, LOC110678581, LOC110675146, LOC110678585, LOC110676930 and LOC110674313).

Among 509 DEGs observed in Aae.wMel$_{2017}$ mosquitoes, 405 downregulated DEGs were significantly enriched for 12 biological processes and 10 molecular functions in DAVID bioinformatics analysis (S1 Box). Moreover, cellular locations such as nucleus (GO:0005634), nucleosome (GO:0000786), MCM complex (GO:0042555) and origin recognition complex (GO:0000808) were significantly enriched at Fisher's exact $P < 0.05$. Genes that were not mapped to DAVID cloud map were manually checked using an NCBI Gene search (S7 Table). There were 104 downregulated DEGs that were not mapped to the DAVID cloud map, among which 34 were ncRNA (S4 Table) and 11 were uncharacterised (LOC110676333, LOC110678663, LOC110674783, LOC110678024, LOC110681172, LOC110680396, LOC110681054, LOC110681485, LOC110676281, LOC110676629, LOC110675455 and LOC110679465). The other DEGs were basically related to immune response, cell proliferation and development (S7 Table). KEGG mapper results identified 12 and 58 pathways connected to downregulated DEGs in Aae.wMel2013/2014 and Aae.wMel2017 mosquitoes.

Discussion

Our transcriptome analysis has identified DEGs in *w*Mel-infected *Ae*. *aegypti* descended from mosquitoes released in Cairns, Australia, in 2011, 2013–14 and 2017. We found that there was a significantly higher number of DEGs in Aae.wMel$_{2017}$ mosquitoes compared with Aae.wMel$_{2011}$ and Aae.wMel$_{2013/2014}$ mosquitoes. There are several potential explanations for the difference between years. The results of the quantitative PCR analysis suggest that it is likely not associated with overall *Wolbachia* density within mosquitoes. It could be due to intrinsic differences between sets of populations, but it is also plausible that expression in subsets of genes has become attenuated as the mosquito undergoes evolutionary changes over time in response to *Wolbachia* infection [25]. Previous studies have demonstrated the attenuation of *Wolbachia*-mediated phenotypes (particularly CI, fecundity and fitness effects) in *Drosophila* spp. infected with the virulent "popcorn" strain of *Wolbachia* [44,45], although most traits associated with *w*Mel appear to be stable at the level of the phenotype [26]. Our data do not allow us to distinguish between competing hypotheses to explain the difference in gene expression between Aae.wMel$_{2017}$ mosquitoes and those from earlier releases. However, if co-evolution of *Wolbachia* and mosquito selects for attenuated gene expression over time, we might predict a similar pattern will be observed in the 2017 release populations in Australia over the next few years, and in other releases globally in the future.

The DEGs that we have identified may give insight into how *Wolbachia* infection is maintained in the mosquito, and potentially the virus-blocking response so useful for arbovirus biocontrol. If the virus blocking phenotype is underpinned by the expression of a gene, or set of genes, rather than structural modifications to host cells by *Wolbachia*, this is more likely to be differentially expressed in all release groups (i.e. the common DEGs) than are other genes. Although we were unable to directly test virus blocking in the release populations studied here, we focus on characterising the DEGs common to all the release groups as they may reveal clues to this important phenotype. As Aae.wMel$_{2017}$ mosquitoes had significantly higher numbers of both up- and down-regulated DEGs compared with the other release years, the gene expression changes in that group will also be discussed to help understand this observation which may be related to ongoing host evolution of *Wolbachia* mediated effects [27].

Broadly, DEGs common to all release years were categorized into immune responses, metabolic changes, and cell proliferation gene-function groups. Innate immune priming is one possible mechanism of viral blocking by *Wolbachia* infected mosquitoes [32], with protection provided to the insect by pre-activation or upregulation of antimicrobial encoding genes [30]. There are four types of genes that are involved in immune responses in mosquitoes: pattern

recognition receptors (PRRs), activation of immune signalling, immune effector mechanisms and immune modulation by the regulation of mosquito homeostasis [46]. In this study, the genes responsible for pathogen recognition, such as CTLGA8, alpha-2-macroglobulin and leucine-rich repeat-containing protein 40-like were among the top 10 differentially expressed genes with highest fold change. Similar findings were previously identified in a study on gene expression in *Anopheles gambiae* cells during *Wolbachia* infection [47]. We also identified antimicrobial peptides such as defensin-C (S3 Table), two PRRs such as gram-negative bacteria-binding protein 1(GNBP1), peptidoglycan-recognition protein 2 (PGRP1) and one other gene (uncharacterized protein LOC5577955 and isoform X1) that are related to Toll and IMD pathway to be significantly upregulated in all *w*Mel-infected *Ae*. *aegypti* [10,32].

We also found the iron binding protein transferrin 1, which is suggested to have functions in iron metabolism and immune function [48], was the upregulated DEG with either highest or second highest fold change in the mosquitoes from all release histories. Transferrin-1 gene upregulation in response to *Wolbachia* infection in mosquitoes has been previously reported [32,49], while iron dependence of *Wolbachia* on different host species has also been identified [50–52]. This iron sequestration from the host has also been suggested as a pathogen blocking mechanism via alteration of iron binding during DENV and Zika virus infection of *Ae*. *aegypti* [53].

The upregulation of lysozyme genes that degrade pathogens was also evident in our study. It has previously been shown that DENV infection triggers autophagy which initiates lysosomal degradation of the virus [54]. Autophagy is a conserved mechanism that degrades cellular components to maintain tissue homeostasis [55], and has been reported in other dipterans [56–58]. Altogether, our study indicates that many immune components including PRRs, signalling pathways and immune effectors are significantly altered in gene expression in mosquitoes infected with *w*Mel, and this response is conserved at least 8 years after initial invasion of the endosymbiont into natural populations.

Competition for host cellular resources has been suggested as another mechanism of virus blocking induced by *Wolbachia* infection [30,37,38], as *Wolbachia* is dependent on the mosquito cell for lipid and amino acid biosynthesis [29]. In our study, we also observed the upregulation of genes related to fatty acid metabolism including fatty acid synthase, elongation of very long chain fatty acids protein 4 and 7, and acyl-CoA Delta (11) desaturase isoform X2 as well as myeloid differentiation 2-related lipid recognition protein. When considering DEGs of amino acid metabolism, our study revealed that all *w*Mel-infected *Ae*. *aegypti* had significantly upregulated glycine, serine and threonine metabolism (aag00260), biosynthesis of amino acids (aag01230), arginine biosynthesis (aag00220), tyrosine metabolism (aag00350), arginine and proline metabolism (aag00330). It has been previously demonstrated that cysteine, glutamate, glutamine, proline, serine and threonine are used as energy sources by *w*Mel [59], and *Ae*. *aegypti* fecundity and egg viability was affected by competition with *Wolbachia* for amino acids [37]. In another study, leucine, tryptophan, methionine, valine, histidine, lysine, phenylalanine, arginine, asparagine and threonine were found to be essential for successful egg production while cysteine, glycine and isoleucine were considered semi-essential for egg production [60]. Overall, upregulation of the genes related to amino acid metabolism in all *w*Mel-infected *Ae*. *aegypti* in our study supports previous studies which have shown the effects of *Wolbachia* infection on insect host physiology and metabolism, particularly in *Ae*. *aegypti* transinfected with the *w*MelPop strain [37].

KEGG BRITE hierarchical clustering of DEGs upregulated in *w*Mel infected mosquitoes from all years indicated that some genes responsible for membrane trafficking (S3 Table) were upregulated. Those genes were low density lipoprotein receptor adapter protein 1-A which is involved in clathrin-mediated endocytosis, perlucin-like protein a C-lectin receptor which is involved in phagocytosis, and glutamyl aminopeptidase, an endoplasmic reticulum (ER)-Golgi

intermediate compartment (ERGIC) protein which is involved in ER-Golgi transport forward pathways. These changes suggest a dysregulation of membrane proteins due to *Wolbachia*, which could contribute to impaired viral entry and replication [61,62]

Our analysis identified that some downregulated genes were related to cell proliferation including transcription and translation and, additionally, organism development. We identified that there were some commonly affected pathways in the Aae.wMel$_{2013/2014}$ and Aae.wMel$_{2017}$ mosquitoes. Those included Notch signalling, Wnt signalling and mTOR pathways (S1 Box). Notably, there was a significantly higher number of genes downregulated in Aae.wMel$_{2017}$ than Aae.wMel$_{2011}$ and Aae.wMel$_{2013/2014}$ mosquitoes. Specifically, Aae.wMel$_{2017}$ mosquitoes showed downregulation of pathways that control cell replication. These observations may be tied to *Wolbachia*'s reliance on the host cell, for example its manipulation of the cytoskeleton, to achieve successful replication [29]. A previous study has also observed the involvement of cell replication pathways and Notch signalling in *w*Mel-infected mosquitoes when subjected to selection on dengue virus-blocking [63].

Several non-coding RNAs (ncRNAs) appeared among the top-most up- and down-regulated DEGs. It is increasingly appreciated that long ncRNAs are important in various biological processes including, but not limited to, cell differentiation, epigenetic and non-epigenetic based gene regulation, involvement in the defence system, responses to stimuli and stress response, viral replication and antiviral defence [64–70]. Our study also identified ncRNA loc110674601, which is significantly aligned with Arginine-glutamic acid dipeptide repeats protein (blastn alignment not shown). This protein plays a role as a transcriptional repressor during mouse development and in the control of cell survival [71]. Loc110673988 (AAEL022454) was among the top 10 upregulated DEG in Aae.wMel2017. A previous study has indicated that this gene is involved in mosquito cellular immunity [72]. There were eight ncRNA among the topmost downregulated genes. Importantly, the downregulated genes with highest fold change in Aae.wMel$_{2011}$ (LOC110676610) and Aae.wMel$_{2017}$ (LOC110679860: ncRNA) were ncRNAs. Our data suggest that long ncRNAs may play hitherto unappreciated roles in the ability of *Wolbachia* to successfully colonise the mosquito host.

It important to note that whilst every effort was made to sample as many locations as possible in our study, only approximately half of the traps contained sufficient numbers of *Ae. aegypti* to include in our analysis. This limited our ability to compare gene expression differences between mosquitoes in geographically separate suburbs for a given release date. Furthermore, although we show different gene expression profiles between different release dates, future sampling should be conducted in other global release locations, such as Yogyakarta [16] or Kuala Lumpur [9], to examine whether geographically separate local release sites show geographical and temporal differences in gene expression.

Conclusions

There is a general decrease in the number of DEGs as a result of *w*Mel *Wolbachia* infection with time post-release. However, *w*Mel infection is characterized by a prolonged transcriptomic signature with respect to upregulated genes (up to 8 years) while downregulated gene signatures were partially fixed until 5–6 years. Upregulated genes and pathways in the host associated with *w*Mel infection were mainly related to immunity and metabolism (especially amino acid and lipid metabolism), while downregulated genes were related to reproduction and organism development. This fixed gene signature comprises transcriptomic alterations in immunity, stress response, behavior and metabolic changes. There were also effects on genes associated with host reproduction which were strongest in the most recent releases but not evident after 8 years.

Materials and methods

Sample collection, RNA extraction and cDNA library preparation

Aedes aegypti eggs were collected from the Cairns suburbs of Caravonica, Gordonvale, Yorkeys Knob, Edge Hill, Parramatta Park, Bungalow and Cairns North in April 2019 (Fig 1 and Table 1). At the time of sampling, Caravonica was one of the few remaining locations in the Cairns region with *Wolbachia*-free *Ae. aegypti*.

Field-collected eggs were reared under standard insectary conditions as described by Huang et al. [24]. Post emergence, adult mosquitoes were maintained on 15% honey water as a nutrient source. On day 4 post emergence, they were anaesthetised on wet ice and sorted by species and sex. Females were washed in absolute EtOH before being placed in RNALater (Qiagen) and stored at -80˚C.

RNA was extracted from pools of 5 females using an RNeasy Mini kit (Qiagen) by first homogenizing them using a plastic pestle in 600 μL of lysis buffer in a 1.5 mL microfuge tube. RNA was then extracted following the manufacturer's recommended method and the presence of *Wolbachia* in the pool tested using previously outlined methods [24]. The RNA was analysed on a TapeStation and quality assessed by determination of the RNA integrity number (RIN). Samples with RIN scores less than 7.8 were excluded from further analysis. A polyadenylated fraction was purified from the total RNA (1 μg) using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). This fraction was used to construct cDNA library using a previously described method [73]. Briefly, poly(A) RNA (2–5 ng) was converted to cDNA using the Protoscript II kit (New England Biolabs) and a supplied mix of random hexamer and $d(T)_{23}VN$ primers, followed by conversion to double-stranded cDNA using a cocktail of RNase H, DNA ligase and DNA polymerase I (New England Biolabs). The product was used to construct a barcoded cDNA library using the Nextera XT system (Illumina) which was sequenced on NovaSeq 6000 at the Australian Genome Research Facility (AGRF), generating paired 2x 150 nt reads. A total of approximately 60 million reads was obtained for each sample. Reads are available from the NCBI Short Read Archive under Accession Number PRJNA867516.

Bioinformatic analysis

Raw transcriptomic data (616.51Gb) was uploaded to Galaxy [74] Australia cloud and subjected to quality control using fastqc tool [75]. Reads from four lanes were merged using concatenate tail-to-head (cat) as per R1 and R2 and then trimmed and adaptor sequences were removed. Reads with quality Phread score < 30 and read length < 50 were excluded using the Trim Galore tool. Next, the *Ae. aegypti* reference genome GCF_002204515.2 was downloaded from NCBI and mapped to the trimmed sequence pairs using Hisat2. Gene expression was quantified using the feature counts tool. Differentially expressed genes were then identified using DESeq2 (FDR < 0.05 and absolute fold change ± 2) by comparing gene expression of *w*Mel *Ae. aegypti* released at different times (2011, 2013–14 and 2017) against wild type *Ae. aegypti*. Downstream analysis of upregulated genes was performed after comparing gene lists using Venny 2.1.0- BioinfoGP. Gene ontology (GO) analysis was performed using the DAVID bioinformatics tool, with GO terms identified to be significantly enriched using Fisher's exact *P*-value < 0.05 [43].

Upregulated DEGs that were common to all *w*Mel *Ae. aegypti* populations were input into KEGG mapper (https://www.genome.jp/kegg/tool/map_pathway2.html) to identify altered gene expression pathways. Downstream analysis of downregulated genes was performed according to the mosquito release year with KEGG mapper and DAVID bioinformatics tool.

Any DEG that was not identified by the DAVID bioinformatics cloud map was characterised manually by searching either NCBI or VectorBase gene search, or protein/nucleotide blast.

Principal Components Analysis (PCA) was performed using PCAGO [76], an R-based interactive tool using DESeq2-rlog normalised counts to visualise clustering of samples. Normality of gene expression values were assessed by the Shapiro-Wilks test, as implemented in SPSS software [77]. As expected, gene expression values (counts per million-CPM) were not normally distributed. Thus, the non-parametric Kruskal-Wallis test was used to check relationships between gene expression and timepoint of release, as implemented in SPSS using a *P*-value of < 0.05 to determine statistical significance.

Quantification of *Wolbachia* in *Ae. aegypti*

To ensure that the differences observed above were due to gene regulation to attenuate a costly immune and/or metabolic detoxoxification response and not due to changes in *Wolbachia* density, the *w*Mel in *Ae. aegypti* collected from 2013–14 and 2017 release locations was quantified using the quantitative PCR developed by Lee et al. [42]. The *Wolbachia* density was analysed using a Mann-Whitney U test in GraphPad Prism Version 9.1.0 [78].

Supporting information

S1 Fig. Density of *Wolbachia* in *Aedes aegypti* descended from mosquitoes released in the Cairns region of northern Australia in 2013–14 and 2017. Each dot is an individual mosquito, and bars and whiskers are medians and 95% confidence intervals, respectively. There was no significant difference ($P > 0.05$; Mann-Whitney U test) in *Wolbachia* density between the years.
(PDF)

S1 Table. Mapping statistics.
(PDF)

S2 Table. Pairwise comparison of median gene expression (CPM) according to release history.
(PDF)

S3 Table. KEGG pathway enrichment analysis of 357 commonly upregulated DEGs across all time points.
(PDF)

S4 Table. Unmapped downregulated DEGs which were non-coding RNA from *Aedes aegypti* with different release years.
(PDF)

S5 Table. Unmapped downregulated DEGs in Aae.wMel$_{2011}$ mosquitoes which are not ncRNA.
(PDF)

S6 Table. Unmapped downregulated DEGs from Aae.wMel$_{2013/2014}$ mosquitoes.
(PDF)

S7 Table. Unmapped downregulated DEGs in Aae.wMel$_{2017}$ mosquitoes.
(PDF)

S1 Box. Significantly enriched GO terms pertaining to biological processes and molecular functions in Aae.wMel$_{2017}$ mosquitoes.
(PDF)

Acknowledgments

We wish to thank Tom Schmidt (University of Melbourne) and Michael Townsend (Australian Institute of Tropical Health and Medicine, James Cook University) for collecting the mosquito eggs used in this study. Thanks also to the Queensland University of Technology's Faculty of Health Research Methods Group, particularly Dimitrios Vagenas, for advice on aspects of the data analysis.

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References

1. Powell JR. Mosquito-borne human viral diseases: why *Aedes aegypti*? Am J Trop Med Hyg. 2018; 98 (6):1563–5. https://doi.org/10.4269/ajtmh.17-0866 PMID: 29557341
2. Scott TW, Takken W. Feeding strategies of anthropophilic mosquitoes result in increased risk of pathogen transmission. Trends Parasitol. 2012; 28(3):114–21. https://doi.org/10.1016/j.pt.2012.01.001 PMID: 22300806

3. Gubler DJ. Dengue, urbanization and globalization: the unholy Trinity of the 21(st) Century. Trop Med Health. 2011; 39(4 Suppl):3–11. https://doi.org/10.2149/tmh.2011-S05 PMID: 22500131
4. Achee NL, Gould F, Perkins TA, Reiner RC Jr., Morriso AC, Ritchie SA, et al. A critical assessment of vector control for dengue prevention. PLoS Negl Trop Dis. 2015; 9(5):e0003655. https://doi.org/10.1371/journal.pntd.0003655 PMID: 25951103
5. Flores HA, O'Neill SL. Controlling vector-borne diseases by releasing modified mosquitoes. Nat Rev Microbiol. 2018; 16(8):508–18. https://doi.org/10.1038/s41579-018-0025-0 PMID: 29777177
6. Wang GH, Gamez S, Raban RR, Marshall JM, Alphey L, Li M, et al. Combating mosquito-borne diseases using genetic control technologies. Nat Commun. 2021; 12(1):4388. https://doi.org/10.1038/s41467-021-24654-z PMID: 34282149
7. Caragata EP, Dutra HL, Moreira LA. Exploiting intimate relationships: controlling mosquito-transmitted disease with *Wolbachia*. Trends Parasitol. 2016; 32(3):207–18. https://doi.org/10.1016/j.pt.2015.10.011 PMID: 26776329
8. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F, et al. Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. Nature. 2011; 476 (7361):454–7. https://doi.org/10.1038/nature10356 PMID: 21866160
9. Nazni WA, Hoffmann AA, NoorAfizah A, Cheong YL, Mancini MV, Golding N, et al. Establishment of *Wolbachia* strain *w*AlbB in malaysian populations of *Aedes aegypti* for dengue control. Curr Biol. 2019; 29(24):4241–8 e5. https://doi.org/10.1016/j.cub.2019.11.007 PMID: 31761702
10. Bian G, Xu Y, Lu P, Xie Y, Xi Z. The endosymbiotic bacterium *Wolbachia* induces resistance to dengue virus in *Aedes aegypti*. PLoS Pathog. 2010; 6(4):e1000833. https://doi.org/10.1371/journal.ppat.1000833 PMID: 20368968
11. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, et al. A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and *Plasmodium*. Cell. 2009; 139(7):1268–78. https://doi.org/10.1016/j.cell.2009.11.042 PMID: 20064373
12. Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, McMeniman CJ, et al. The *w*Mel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. Nature. 2011; 476 (7361):450–3. https://doi.org/10.1038/nature10355 PMID: 21866159
13. O'Neill SL, Ryan PA, Turley AP, Wilson G, Retzki K, Iturbe-Ormaetxe I, et al. Scaled deployment of *Wolbachia* to protect the community from dengue and other *Aedes* transmitted arboviruses. Gates Open Res. 2018; 2:36. https://doi.org/10.12688/gatesopenres.12844.3 PMID: 30596205
14. Ryan PA, Turley AP, Wilson G, Hurst TP, Retzki K, Brown-Kenyon J, et al. Establishment of *w*Mel Wolbachia in *Aedes aegypti* mosquitoes and reduction of local dengue transmission in Cairns and surrounding locations in northern Queensland, Australia. Gates Open Res. 2019; 3:1547. https://doi.org/10.12688/gatesopenres.13061.2 PMID: 31667465
15. World Mosquito Program. Global Progress 2021 [cited 2021 29/3/2021]. Available from: https://www.worldmosquitoprogram.org/.
16. Utarini A, Indriani C, Ahmad RA, Tantowijoyo W, Arguni E, Ansari MR, et al. Efficacy of *Wolbachia*-infected mosquito deployments for the control of dengue. N Engl J Med. 2021; 384(23):2177–86. https://doi.org/10.1056/NEJMoa2030243 PMID: 34107180
17. Ross PA, Axford JK, Yang Q, Staunton KM, Ritchie SA, Richardson KM, et al. Heatwaves cause fluctuations in *w*Mel *Wolbachia* densities and frequencies in *Aedes aegypti*. PLoS Negl Trop Dis. 2020; 14(1): e0007958. https://doi.org/10.1371/journal.pntd.0007958 PMID: 31971938
18. Ross PA, Wiwatanaratanabutr I, Axford JK, White VL, Endersby-Harshman NM, Hoffmann AA. *Wolbachia* infections in *Aedes aegypti* differ markedly in their response to cyclical heat stress. PLoS Pathog. 2017; 13(1):e1006006. https://doi.org/10.1371/journal.ppat.1006006 PMID: 28056065
19. Ulrich JN, Beier JC, Devine GJ, Hugo LE. Heat sensitivity of *w*Mel *Wolbachia* during *Aedes aegypti* development. PLoS Negl Trop Dis. 2016; 10(7):e0004873. https://doi.org/10.1371/journal.pntd.0004873 PMID: 27459519
20. Garcia GA, Hoffmann AA, Maciel-de-Freitas R, Villela DAM. *Aedes aegypti* insecticide resistance underlies the success (and failure) of *Wolbachia* population replacement. Sci Rep. 2020; 10(1):63. https://doi.org/10.1038/s41598-019-56766-4 PMID: 31919396
21. Nguyen TH, Nguyen HL, Nguyen TY, Vu SN, Tran ND, Le TN, et al. Field evaluation of the establishment potential of *w*MelPop *Wolbachia* in Australia and Vietnam for dengue control. Parasit Vectors. 2015; 8:563. https://doi.org/10.1186/s13071-015-1174-x PMID: 26510523
22. Edenborough KM, Flores HA, Simmons CP, Fraser JE. Using *Wolbachia* to eliminate dengue: will the virus fight back? J Virol. 2021; 95(13):e0220320. https://doi.org/10.1128/JVI.02203-20 PMID: 33853965

23. Dainty KR, Hawkey J, Judd LM, Pacidonio EC, Duyvestyn JM, Goncalves DS, et al. *w*Mel *Wolbachia* genome remains stable after 7 years in Australian *Aedes aegypti* field populations. Microb Genom. 2021; 7(9). https://doi.org/10.1099/mgen.0.000641 PMID: 34468309

24. Huang B, Yang Q, Hoffmann AA, Ritchie SA, van den Hurk AF, Warrilow D. *Wolbachia* genome stability and mtDNA variants in *Aedes aegypti* field populations eight years after release. iScience. 2020; 23 (10):101572. https://doi.org/10.1016/j.isci.2020.101572 PMID: 33083739

25. Lau MJ, Schmidt TL, Yang Q, Chung J, Sankey L, Ross PA, et al. Genetic stability of *Aedes aegypti* populations following invasion by *w*Mel *Wolbachia*. BMC Genomics. 2021; 22(1):894. https://doi.org/10.1186/s12864-021-08200-1 PMID: 34906084

26. Ross PA, Robinson KL, Yang Q, Callahan AG, Schmidt TL, Axford JK, et al. A decade of stability for *w*Mel *Wolbachia* in natural *Aedes aegypti* populations. PLoS Pathog. 2022; 18(2):e1010256. https://doi.org/10.1371/journal.ppat.1010256 PMID: 35196357

27. Ross PA, Hoffmann AA. Local adaptation of *Aedes aegypti* mosquitoes to *Wolbachia*-induced fitness costs. BioRxiv. 2022. https://doi.org/10.1101/2022.05.06.490959

28. Haslun JA, Hauff-Salas B, Strychar KB, Cervino JM, Ostrom NE. Variation in immune-related gene expression provides evidence of local adaptation in *Porites astreoides* (Lamarck, 1816) between inshore and offshore meta-populations inhabiting the lower Florida Reef Tract, USA. Water. 2021; 13 (15):2107. https://doi.org/10.3390/w13152107

29. Lindsey ARI, Bhattacharya T, Newton ILG, Hardy RW. Conflict in the intracellular lives of endosymbionts and viruses: a mechanistic look at *Wolbachia*-mediated pathogen-blocking. Viruses. 2018; 10(4). https://doi.org/10.3390/v10040141 PMID: 29561780

30. Terradas G, McGraw EA. *Wolbachia*-mediated virus blocking in the mosquito vector *Aedes aegypti*. Curr Opin Insect Sci. 2017; 22:37–44. https://doi.org/10.1016/j.cois.2017.05.005 PMID: 28805637

31. Chrostek E, Marialva MS, Yamada R, O'Neill SL, Teixeira L. High anti-viral protection without immune upregulation after interspecies *Wolbachia* transfer. PLoS One. 2014; 9(6):e99025. https://doi.org/10.1371/journal.pone.0099025 PMID: 24911519

32. Rances E, Ye YH, Woolfit M, McGraw EA, O'Neill SL. The relative importance of innate immune priming in *Wolbachia*-mediated dengue interference. PLoS Pathog. 2012; 8(2):e1002548. https://doi.org/10.1371/journal.ppat.1002548 PMID: 22383881

33. Thomas S, Verma J, Woolfit M, O'Neill SL. *Wolbachia*-mediated virus blocking in mosquito cells is dependent on XRN1-mediated viral RNA degradation and influenced by viral replication rate. PLoS Pathog. 2018; 14(3):e1006879. https://doi.org/10.1371/journal.ppat.1006879 PMID: 29494679

34. Terradas G, Joubert DA, McGraw EA. The RNAi pathway plays a small part in *Wolbachia*-mediated blocking of dengue virus in mosquito cells. Sci Rep. 2017; 7:43847. https://doi.org/10.1038/srep43847 PMID: 28262718

35. Johnson KN. The impact of *Wolbachia* on virus infection in mosquitoes. Viruses. 2015; 7(11):5705–17. https://doi.org/10.3390/v7112903 PMID: 26556361

36. Lu P, Bian G, Pan X, Xi Z. *Wolbachia* induces density-dependent inhibition to dengue virus in mosquito cells. PLoS Negl Trop Dis. 2012; 6(7):e1754. https://doi.org/10.1371/journal.pntd.0001754 PMID: 22848774

37. Caragata EP, Rances E, O'Neill SL, McGraw EA. Competition for amino acids between Wolbachia and the mosquito host, *Aedes aegypti*. Microb Ecol. 2014; 67(1):205–18. https://doi.org/10.1007/s00248-013-0339-4 PMID: 24337107

38. Caragata EP, Rances E, Hedges LM, Gofton AW, Johnson KN, O'Neill SL, et al. Dietary cholesterol modulates pathogen blocking by *Wolbachia*. PLoS Pathog. 2013; 9(6):e1003459. https://doi.org/10.1371/journal.ppat.1003459 PMID: 23825950

39. Molloy JC, Sommer U, Viant MR, Sinkins SP. *Wolbachia* modulates lipid metabolism in *Aedes albopictus* mosquito cells. Appl Environ Microbiol. 2016; 82(10):3109–20. https://doi.org/10.1128/AEM.00275-16 PMID: 26994075

40. Lee CJ, Lin HR, Liao CL, Lin YL. Cholesterol effectively blocks entry of flavivirus. J Virol. 2008; 82 (13):6470–80. https://doi.org/10.1128/JVI.00117-08 PMID: 18448543

41. White PM, Serbus LR, Debec A, Codina A, Bray W, Guichet A, et al. Reliance of *Wolbachia* on high rates of host proteolysis revealed by a genome-wide RNAi screen of *Drosophila* cells. Genetics. 2017; 205(4):1473–88. https://doi.org/10.1534/genetics.116.198903 PMID: 28159754

42. Lee SF, White VL, Weeks AR, Hoffmann AA, Endersby NM. High-throughput PCR assays to monitor *Wolbachia* infection in the dengue mosquito (*Aedes aegypti*) and *Drosophila simulans*. Appl Environ Microbiol. 2012; 78(13):4740–3. https://doi.org/10.1128/AEM.00069-12 PMID: 22522691

43. Dennis G Jr., Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol. 2003; 4(5):P3. PMID: 12734009

44. Carrington LB, Hoffmann AA, Weeks AR. Monitoring long-term evolutionary changes following *Wolbachia* introduction into a novel host: the *Wolbachia* popcorn infection in *Drosophila simulans*. Proc Biol Sci. 2010; 277(1690):2059–68. https://doi.org/10.1098/rspb.2010.0166 PMID: 20219740

45. McGraw EA, Merritt DJ, Droller JN, O'Neill SL. *Wolbachia* density and virulence attenuation after transfer into a novel host. Proc Natl Acad Sci U S A. 2002; 99(5):2918–23. https://doi.org/10.1073/pnas.052466499 PMID: 11880639

46. Hillyer JF. Insect immunology and hematopoiesis. Dev Comp Immunol. 2016; 58:102–18. https://doi.org/10.1016/j.dci.2015.12.006 PMID: 26695127

47. Hughes GL, Ren X, Ramirez JL, Sakamoto JM, Bailey JA, Jedlicka AE, et al. *Wolbachia* infections in *Anopheles gambiae* cells: transcriptomic characterization of a novel host-symbiont interaction. PLoS Pathog. 2011; 7(2):e1001296. https://doi.org/10.1371/journal.ppat.1001296 PMID: 21379333

48. Yoshiga T, Hernandez VP, Fallon AM, Law JH. Mosquito transferrin, an acute-phase protein that is up-regulated upon infection. Proc Natl Acad Sci U S A. 1997; 94(23):12337–42. https://doi.org/10.1073/pnas.94.23.12337 PMID: 9356450

49. Kambris Z, Cook PE, Phuc HK, Sinkins SP. Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. Science. 2009; 326(5949):134–6. https://doi.org/10.1126/science.1177531 PMID: 19797660

50. Duchemin JB, Paradkar PN. Iron availability affects West Nile virus infection in its mosquito vector. Virol J. 2017; 14(1):103. https://doi.org/10.1186/s12985-017-0770-0 PMID: 28583206

51. Juneja P, Ariani CV, Ho YS, Akorli J, Palmer WJ, Pain A, et al. Exome and transcriptome sequencing of *Aedes aegypti* identifies a locus that confers resistance to *Brugia malayi* and alters the immune response. PLoS Pathog. 2015; 11(3):e1004765. https://doi.org/10.1371/journal.ppat.1004765 PMID: 25815506

52. Zhou G, Velasquez LS, Geiser DL, Mayo JJ, Winzerling JJ. Differential regulation of transferrin 1 and 2 in *Aedes aegypti*. Insect Biochem Mol Biol. 2009; 39(3):234–44. https://doi.org/10.1016/j.ibmb.2008.12.004 PMID: 19166934

53. Caragata EP, Rocha MN, Pereira TN, Mansur SB, Dutra HLC, Moreira LA. Pathogen blocking in *Wolbachia*-infected *Aedes aegypti* is not affected by Zika and dengue virus co-infection. PLoS Negl Trop Dis. 2019; 13(5):e0007443. https://doi.org/10.1371/journal.pntd.0007443 PMID: 31107912

54. Datan E, Roy SG, Germain G, Zali N, McLean JE, Golshan G, et al. Dengue-induced autophagy, virus replication and protection from cell death require ER stress (PERK) pathway activation. Cell Death Dis. 2016; 7:e2127. https://doi.org/10.1038/cddis.2015.409 PMID: 26938301

55. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature. 2008; 451(7182):1069–75. https://doi.org/10.1038/nature06639 PMID: 18305538

56. Moy RH, Gold B, Molleston JM, Schad V, Yanger K, Salzano MV, et al. Antiviral autophagy restricts Rift Valley fever virus infection and is conserved from flies to mammals. Immunity. 2014; 40(1):51–65. https://doi.org/10.1016/j.immuni.2013.10.020 PMID: 24374193

57. Nakamoto M, Moy RH, Xu J, Bambina S, Yasunaga A, Shelly SS, et al. Virus recognition by Toll-7 activates antiviral autophagy in *Drosophila*. Immunity. 2012; 36(4):658–67. https://doi.org/10.1016/j.immuni.2012.03.003 PMID: 22464169

58. Weiss-Sadan T, Maimoun D, Oelschlagel D, Kaschani F, Misiak D, Gaikwad H, et al. Cathepsins drive anti-inflammatory activity by regulating autophagy and mitochondrial dynamics in macrophage foam cells. Cell Physiol Biochem. 2019; 53(3):550–72. https://doi.org/10.33594/000000157 PMID: 31529928

59. Wu M, Sun LV, Vamathevan J, Riegler M, Deboy R, Brownlie JC, et al. Phylogenomics of the reproductive parasite *Wolbachia pipientis wMel*: a streamlined genome overrun by mobile genetic elements. PLoS Biol. 2004; 2(3):E69. https://doi.org/10.1371/journal.pbio.0020069 PMID: 15024419

60. Allman MJ, Fraser JE, Ritchie SA, Joubert DA, Simmons CP, Flores HA. *Wolbachia's* deleterious impact on *Aedes aegypti* egg development: the potential role of nutritional parasitism. Insects. 2020; 11(11). https://doi.org/10.3390/insects11110735 PMID: 33120915

61. Geoghegan V, Stainton K, Rainey SM, Ant TH, Dowle AA, Larson T, et al. Perturbed cholesterol and vesicular trafficking associated with dengue blocking in *Wolbachia*-infected *Aedes aegypti* cells. Nat Commun. 2017; 8(1):526. https://doi.org/10.1038/s41467-017-00610-8 PMID: 28904344

62. Jupatanakul N, Sim S, Dimopoulos G. *Aedes aegypti* ML and Niemann-Pick type C family members are agonists of dengue virus infection. Dev Comp Immunol. 2014; 43(1):1–9. https://doi.org/10.1016/j.dci.2013.10.002 PMID: 24135719

63. Ford SA, Albert I, Allen SL, Chenoweth SF, Jones M, Koh C, et al. Artificial selection finds new hypotheses for the mechanism of *Wolbachia*-mediated dengue blocking in mosquitoes. Front Microbiol. 2020; 11:1456. https://doi.org/10.3389/fmicb.2020.01456 PMID: 32733407

64. Bonasio R, Shiekhattar R. Regulation of transcription by long noncoding RNAs. Annu Rev Genet. 2014; 48:433–55. https://doi.org/10.1146/annurev-genet-120213-092323 PMID: 25251851

65. Etebari K, Furlong MJ, Asgari S. Genome wide discovery of long intergenic non-coding RNAs in Diamondback moth (*Plutella xylostella*) and their expression in insecticide resistant strains. Sci Rep. 2015; 5:14642. https://doi.org/10.1038/srep14642 PMID: 26411386

66. Fitzgerald KA, Caffrey DR. Long noncoding RNAs in innate and adaptive immunity. Curr Opin Immunol. 2014; 26:140–6. https://doi.org/10.1016/j.coi.2013.12.001 PMID: 24556411

67. Lakhotia SC. Long non-coding RNAs coordinate cellular responses to stress. Wiley Interdiscip Rev RNA. 2012; 3(6):779–96. https://doi.org/10.1002/wrna.1135 PMID: 22976942.

68. Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet. 2009; 10(3):155–9. Epub 2009/02/04. https://doi.org/10.1038/nrg2521 PMID: 19188922

69. Mizutani R, Wakamatsu A, Tanaka N, Yoshida H, Tochigi N, Suzuki Y, et al. Identification and characterization of novel genotoxic stress-inducible nuclear long noncoding RNAs in mammalian cells. PLoS One. 2012; 7(4):e34949. https://doi.org/10.1371/journal.pone.0034949 PMID: 22532836

70. Winterling C, Koch M, Koeppel M, Garcia-Alcalde F, Karlas A, Meyer TF. Evidence for a crucial role of a host non-coding RNA in influenza A virus replication. RNA Biol. 2014; 11(1):66–75. https://doi.org/10.4161/rna.27504 PMID: 24440876

71. The UniProt Consortium. UniProt: the universal protein knowledgebase in 2021. Nucleic Acids Research. 2021; 49:D480–D9. https://doi.org/10.1093/nar/gkaa1100 PMID: 33237286

72. Raddi G, Barletta ABF, Efremova M, Ramirez JL, Cantera R, Teichmann SA, et al. Mosquito cellular immunity at single-cell resolution. Science. 2020; 369(6507):1128–32. https://doi.org/10.1126/science.abc0322 PMID: 32855340

73. Huang B, Pyke AT, McMahon J, Warrilow D. Complete coding sequence of a case of chikungunya virus imported into Australia. Genome Announc. 2017; 5(19). https://doi.org/10.1128/genomeA.00310-17 PMID: 28495775

74. Afgan E, Baker D, Batut B, van den Beek M, Bouvier D, Cech M, et al. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. Nucleic Acids Res. 2018; 46 (W1):W537–W44. https://doi.org/10.1093/nar/gky379 PMID: 29790989

75. Bioinformatics Babraham. FastQC: a quality control tool for high throughput sequence data. Cambridge: Babraham Institute; 2010.

76. Gerst R, Holzer M. PCAGO: An interactive tool to analyze RNA-Seq data with principal component analysis. BioRxiv. 2019. https://doi.org/10.1101/433078

77. IBM Corp. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.; 2017.

78. GraphPad Software. GraphPad Prism version 9.0.1 for Windows. 9.1.0 ed. San Diego: GraphPad Software; 2021.