MONASH University

ENDOSYMBIONT-HOST-PATHOGEN EVOLUTION:

THE IMPORTANCE OF IMMUNITY GENES IN HOW INSECT HOSTS INTERACT WITH ENDOSYMBIONTS AND PATHOGENS

Rosemarie Isabel Herbert

Bachelor of Biomedical Science, Bachelor of Science (Hons H1), Bachelor of Arts

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Abstract

Biodiversity is rapidly and dramatically changing due to urbanisation, population growth and climate change. These changes alter arthropod species' distribution and the spread of the pathogens they vector. Mosquitoes are key vectors of infectious diseases that pose a significant threat to human health, including diseases such as malaria and Dengue virus (DENV). Formerly, control methods were limited to insecticide spraying and instigating human behavioural changes such as use of bed netting. The current focus for developing a control strategy for DENV is to exploit the biological characteristics of the endosymbiont, *Wolbachia.*

Wolbachia is a maternally inherited endosymbiont that has a range of effects on its insect, nematode and other invertebrate hosts. *Wolbachia* infections alter pathogen infectivity, including halting DENV transmission by *Aedes aegypti*. This protective ability is linked to *Wolbachia* priming the immune system and several other processes. In nematode species, *Wolbachia* presence is essential for reproduction. Antibiotic treatment that targets *Wolbachia* treats lymphatic pathologies by killing the causative nematodes. However, there are nuances of *Wolbachia*-host interactions that are not yet well understood.

In this thesis, I have analysed transcription data from *Wolbachia*-infected and *Wolbachia*-free insects and nematodes to find commonalities in response between diverse organism types. I found that the removal of *Wolbachia* infection affected a range of genes and processes associated with chaperones, metabolic rate, ER/Golgi, cytoskeleton, chitin and lipids. The identified genes provide a guide for experimental work going forward. This thesis also specifically examined the early host response to novel *Wolbachia* infections in a range of insect cell lines. Creating novel cell infections proved to have a low success rate and was not achievable in all the cell lines of interest. In those cell lines that I could establish an infection, a focus on innate immune genes revealed that there was little consistency in responses. This has significant implications for those attempting to establish novel *Wolbachia* infections for the purposes of biocontrol as responses cannot necessarily be predicted from closely related species. Additionally, testing of antibiotics for controlling nematode pathologies relies on an insect cell line, and thus studies of transcriptional responses in this cell system may not be representative of what occurs within nematodes. Establishing cell lines that directly model the same species and *Wolbachia* strain of interest in both cases should be a top priority.

Although *Drosophila* is not a vector of human pathogens, its immune system has been extensively studied across its diverse species and much is known about its geographical distribution. I tested whether geographical restriction of species distribution has influenced the evolution of immune genes and thus refined immune pathways to target pathogens. Through this, I found that antiviral response genes experience greater constraint in *Drosophila* found in the

tropics. The tropics are home to a range of pathogens and thus changes in the ambient temperature may have a significant effect on disease incidence. This work can form a starting point for predicting other insects' responses to climate change.

This thesis has contributed to the body of knowledge about *Wolbachia's* interactions with its diverse hosts, highlighting both similarities and difference of transcriptional responses to the gain and loss of *Wolbachia* infections. In turn, this work will inform how *Wolbachia* can best be exploited as a control method and aid predictions of disease spread in response to temperature change.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature: 

Print Name: Rosemarie Herbert

10th November 2017

Publications during enrolment

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Thesis including published works declaration

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This thesis includes 1 original paper published in Symbiosis. The core theme of the thesis is *Wolbachia*-host-pathogen interactions. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of Biological Sciences under the supervision of Professor Elizabeth McGraw.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

Yi Dong informed cell maintenance and Emily Kerton assisted with DNA/RNA extractions in Chapter 3. Henry Ye and Alison Carrasco assisted with experimental design in Appendix 1.

In the case of Chapters 2-4, my contribution to the work involved the following:

Thesis Chapter	**Publication Title**	**Status**	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution**	**Co-author(s), Monash student**

2	Shared transcriptomic responses in arthropods and nematodes to the loss of *Wolbachia* infection	Under revision for resubmission to *Symbiosis*	80%. Concept and collecting data and writing manuscript	Elizabeth McGraw, input into manuscript 20%	No
3	The nature of the immune response in novel *Wolbachia*:host associations	Published in *Symbiosis* August 2017	80%. Concept and collecting data and writing manuscript	Elizabeth McGraw, input into manuscript 20%	No
4	Geographical distribution influences purifying selection on innate immunity genes in *Drosophila*	Under revision for submission to *PeerJ*	80%. Concept and collecting data and writing manuscript	Elizabeth McGraw, input into manuscript 20%	No

I have renumbered sections of published papers to generate a consistent presentation within the thesis.

Student signature: [redacted] Date: 10th November 2017

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: [redacted] Date: 10th November 2017

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"An investment in knowledge pays the best interest." - Benjamin Franklin

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Chapter 1

General Introduction

CHANGES TO THE BIODIVERSITY LANDSCAPE AND THEIR IMPLICATIONS

Arthropod species make up a large share of the planet's animal biodiversity. Biodiversity is composed primarily of specialist species defined by their particular climatic zones and/or feeding behaviours on particular organisms (Forister et al. 2012). While species distributions become narrower towards the tropics (Addo-Bediako et al. 2000), the greatest of biodiversity in free-living organisms, including vectors of infectious and parasitic diseases, is found in that area (Bonds et al. 2012). Insects have key roles in plant reproduction, and the regulation of pests and parasites. Thus, insect diversity can be predicted and estimated by use of a proxy of plant diversity (Stork et al. 2015). Insects depend on plants as a food source through herbivory and pollination, and for shelter and nesting (Novotny and Basset 2005). Hence the loss of plant diversity decreases insect diversity (Haddad et al. 2009).

Biodiversity is dramatically changing due to urbanisation and human population growth, in addition to global climate change (Cardinale et al. 2012). The biodiversity of free-living organisms such as insects will drop due to a lack of insect genetic variation (Merila et al. 2001) and changes to plant species diversity will change insect distribution (Haddad et al. 2009). The distribution of *Drosophila* and other insects is known to be heavily influenced by environmental variables (Chown et al. 2002). There are limits in *Drosophila's* ability to respond to temperature changes, where genetic variation in temperature tolerance is low in narrowly distributed tropical species, but higher in widely distributed species (Kellermann et al. 2009; Rodríguez-Trelles et al. 2013). The decimation of tropical species in the event of climate change (Addo-Bediako et al. 2000; Deutsch et al. 2008) may leave widely distributed species to become primary pathogen vectors as viruses can rapidly adapt to the newly limited insect numbers (Bonds et al. 2012). The pathogen burden is likely to increase, as these emerging vectors lack the necessary adaptation to keep the host-pathogen arms race in check (Bonds et al. 2012). Tropical areas already have the greatest pathogen burden of the world (Guernier et al. 2004), and this will only increase as climate extremes develop.

Climate change will increase the risks of vector establishment in new areas and thus influence pathogen transmission. Infectious diseases come to light when there is a resurgence of pre-existing endemic conditions or new microorganisms colonise an area (Randolph and Rogers 2010). Host-pathogen interactions limit the transmission of these diseases by physical and immunological processes, but extrinsic environmental factors also play a role in success. In particular, vector-borne pathogens appear to colonise new areas more efficiently than those transmitted by other means (Gubler 2008; Kilpatrick and Randolph 2012), likely because insect vectors are highly mobile due to human interactions and the greater transmission potential of vector-borne pathogens (Kilpatrick and Randolph 2012; Wilder-Smith et al. 2012). Arthropod vectors account for some of the greatest infectious disease burden in humans (Hill et al. 2005). These

diseases include a range of parasitic infections, most notably malaria, but also viral diseases whose disease burden is rapidly growing (World Health Organization 2015).

For example, an emergent vector of dengue virus (DENV) is the mosquito *Aedes albopictus.* Compared to *Aedes aegypti,* DENV's primary vector, *Ae. albopictus* can tolerate a wider range of environmental conditions and hence exists in both tropical and temperate regions (Hanson and Craig 1994). In concert with knowledge of climatic trends, its spread by trade and travel routes can be predicted, as there is more than 80 years' worth of information on its pervasive spread (Tatem et al. 2006). Despite its environmental adaptability*, Ae. albopictus* has previously not been noted as a successful vector of infectious diseases in the environment (Gratz 2004; Paupy et al. 2009), although it shows an ability to transmit 22 arboviruses under laboratory conditions (Moore and Mitchell 1997). Another vector predicted to spread is *Anopheles gambiae*, the primary vector of malaria in Africa (Onyango et al. 2016; White et al. 2011).

HOST IMMUNE RESPONSE

Insects are exposed to a wide range of pathogens, both the human pathogens they vector (Hill et al. 2005), and their own pathogens (Markow and O'Grady 2007; Webster et al. 2015). The ability of vectors to transmit pathogens and thus enable disease spread is dependent on the interactions between the vector and the pathogen, particularly the effect of the pathogen on immune response. Viruses must overcome or avoid the immune response of the vector to infect a range of tissues and progress to the point where the pathogen loads are sufficiently high and in the right tissues to allow transmission (Hardy et al. 1983). Viruses may extract a fitness cost on the vector: for example, Dengue in *Ae. aegypti* (Sylvestre et al. 2013), West Nile in *D. melanogaster* (Ciota et al. 2011) and Chikungunya in *Ae. aegypti* and *Ae. albopictus* (Reiskind et al. 2010) all extract fitness costs from their hosts. Thus insects must have a system to repel such invaders and minimise these costs. The initial barriers are physical, followed by physiological responses. Transcriptional response studies have provided a means to study closely how viruses activate their vectors' immune systems (Chotkowski et al. 2008; Paradkar et al. 2012). Insects have a well described innate immune system that can recognise conserved pathogen motifs (Buchon et al. 2014; Karlikow et al. 2014; Myllymaki and Ramet 2014; Xu and Cherry 2014). In addition, although it has long been thought that insects lack an adaptive immune system, *D. melanogaster* has recently been shown to have an adaptive RNAi-based response to viral pathogens (Tassetto et al. 2017) (see (Flemming 2017) for a review).

The first barrier against pathogen insults is formed of chitin. This cuticle layer in the exoskeleton of the insect prevents pathogen access to the hemolymph (Zhu et al. 2016). Additionally, chitin is found in the alimentary canal, the tracheal system and genital ducts of insects (Zhu et al. 2016). In the alimentary canal it forms the peritrophic matrix (Merzendorfer and Zimoch 2003). This porous lining shields the epithelia from infectious agents (Zhu et al. 2016) and serves as the primary first barrier against further bodily infection by viruses and parasites after a pathogen-laden meal. If a pathogen overcomes these physical barriers, invading the hemolymph, then the insect immune system is activated. The immune response to pathogens can be a general response to a wide range of invaders (Cellular Response), or the insect can respond specifically to different categories of pathogens (Humoral Response). A summary of the process of arbovirus transmission in a mosquito vector is outlined in Figure 1.

Cellular Response

The cellular immune response in insects is the second line of defense and is mediated by hemocytes (immune cells) that detect non-self molecules. Hemocytes can be divided into three cell classes: plasmatocytes, lamellocytes and crystal cells (Vlisidou and Wood 2015). The activation of these cells relies on recognition of conserved pathogen molecules such as lipopolysaccharides (LPS) and peptidoglycans (PGN) (Medzhitov and Janeway 1997). Recently, a study in *Cell* showed that hemocytes can also mediate an adaptive immune response in *Drosophila* for antiviral defense (Tassetto et al. 2017).

Mainly, plasmatocytes are responsible for the phagocytosis of smaller pathogens while lamellocytes target larger pathogens (Vlisidou and Wood 2015). Both cell types phagocytose or encapsulate the pathogen to destroy it. Lamellocytes also use melanisation (characterized by wound darkening) to kill the pathogen with toxic compounds that include phenoloxidase (PO) and cytotoxic reactive intermediates of oxygen (ROI) and nitrogen (RNI) (Nappi and Christensen 2005; Nappi and Ottaviani 2000; Nappi and Vass 1993). The action of crystal cells is also via melanisation but the goal is to restrict pathogens to a particular area to aid phagocytosis (Vlisidou and Wood 2015). The restriction of pathogen spread by the cellular response allows a timely humoral response to take place.

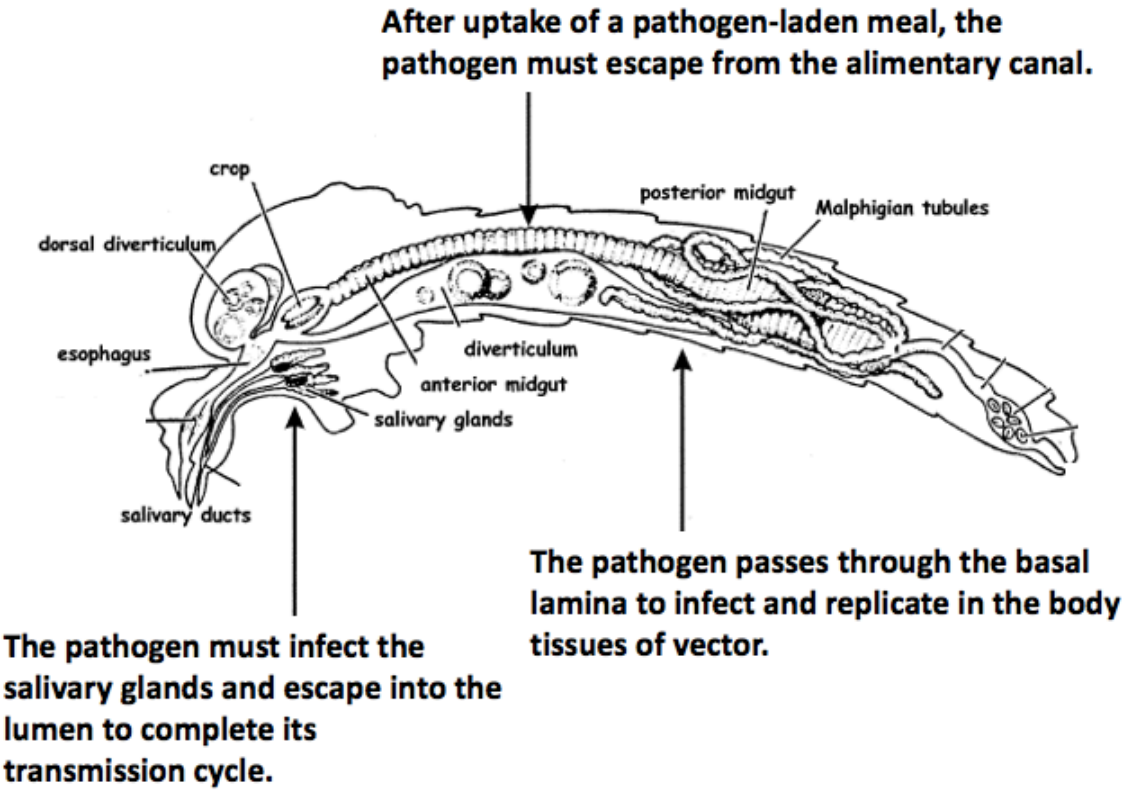

Figure 1 The process of arbovirus transmission in mosquitoes. After the insect vector's ingestion of a blood meal, arboviruses must escape from the alimentary canal into the body of the vector and infect the salivary glands before transmission can occur via the saliva. Figure adapted from Black et al. (2002).

Humoral Response

The humoral response is different from the cellular response in that it can target specific pathogens. It does this by recognition of conserved pathogen molecules and activation of pathogen specific pathways. There are four major inducible pathways in the humoral response, namely the TOLL and IMD pathways for bacteria (Buchon et al. 2014), and the JAK/STAT and RNAi pathways for viruses (Myllymaki and Ramet 2014; Xu and Cherry 2014) (Figure 1). The initial regulatory activation step is via proteins with κB response elements that activate downstream processes to produce antimicrobial or antiviral peptides (Engstrom et al. 1993; Kappler et al. 1993). Antimicrobial peptides (AMPs) are formed by the fat body of the insect; there are seven different classes: attacin, cecropin, defensin, diptericin, drosocin, drosomycin, and metchnikowin (Meister et al. 1997). The TOLL and IMD pathways control most of the known genes regulated by microbial infection; in addition these AMP genes are involved in nearly all known *Drosophila* innate immune reactions (De Gregorio et al. 2002).

The TOLL pathway is activated in response to Gram-positive bacteria and fungi and has known roles in development (Lemaitre et al. 1996a). Pathogen recognition activates Spätzle cleavage, then binds the Toll receptor that recruits the Tube/myd88 complex, followed by the Pelle kinase activation (Michel et al. 2001). Pelle kinase triggers an intracellular signaling cascade that activates translocation of Dif (a dorsal related gene) in the nucleus that then leads to transcription of AMP genes (Stoven et al. 2000). The TOLL pathway activates drosomycin, defensin and metchnikowin (Lemaitre et al. 1996b; Lowenberger et al. 1995). In addition, there appears to be cross talk, if not direct involvement, of TOLL in the antiviral response (Xi et al. 2008b), via interactions with the JAK/STAT and RNAi pathways.

The other pathway that specifically targets bacteria is the IMD pathway which responds to Gram-negative bacteria and viruses (Lemaitre and Hoffmann 2007). When bacterial peptidoglycans bind to cell surface peptidoglycan recognition proteins (PGRPs), this triggers the intracellular adaptor IMD (Choe et al. 2002). Signal transduction leads to Relish cleavage and the Rel domain translocates to the nucleus to result in production of antimicrobial peptides (Dushay et al. 1996; Rutschmann et al. 2000). The IMD pathway controls the transcription of diptericin, cecropin, attacin, drosocin, defensin and metchnikowin (Lemaitre et al. 1995).

The RNAi pathway specifically targets viruses by recognition of viral dsRNA molecules produced in virus-infected cells (Karlikow et al. 2014; Sabin et al. 2010). These long molecules are cleaved by Dcr-2 and its cofactor R2D2 into siRNA (Bernstein et al. 2001; Liu et al. 2003; Liu et al. 2006), which is then processed through the pre-RISC complex to form single strands (Elbashir et al. 2001). One of these single strands is retained within the Ago2-catalytic domain of the RISC complex, where it can then bind viral RNA in the cell to promote specific degradation of its target (Rand et al. 2004).

Thus although RNAi is a general response to viruses (Kemp et al. 2013), it can also independently sense and target specific viruses (Dostert et al. 2005). Recent research suggests that this phenomenon is due to hemocytes taking up dsRNA from infected cells and endogenously producing virus-derived complementary DNA (Tassetto et al. 2017). These form an immunological memory that can confer passive protection against specific viruses (Tassetto et al. 2017).

The JAK/STAT pathway responds to tissue damage and specific viral threats (Agaisse and Perrimon 2004; Kemp et al. 2013; Myllymaki and Ramet 2014). Unlike the RNAi pathway, it cannot identify viruses independently and requires separate activation by cytokines (Dostert et al. 2005). Upd1, Upd2 and Upd3 (unpaired proteins) are found associated with the extracellular matrix (ECM) and are the activator ligands of the JAK/STAT pathway (Agaisse and Perrimon 2004; Agaisse et al. 2003; Harrison et al. 1998; Hombria and Brown 2002). Upd binds to the transmembrane protein *dom*e (Brown et al. 2001; Chen et al. 2002), which in turn activates Hop and STAT92E (Binari and Perrimon 1994; Hou et al. 1996). STAT92E translocates into fat body cells (Agaisse et al. 2003) where it promotes expression of genes such as vir-1 that respond to viral infection (Agaisse and Perrimon 2004; Lemaitre and Hoffmann 2007). The magnitude of the response induced by STAT92E is affected by regulator proteins (Betz et al. 2001; Callus and Mathey-Prevot 2002; Karsten et al. 2002) and a positively acting protein signal-transducing adapter molecule (Mesilaty-Gross et al. 1999). These proteins include tep1 (Lagueux et al. 2000) and totA (Agaisse et al. 2003). The gene totA also requires Relish for activation and this creates cross-talk between the IMD and JAK/STAT pathways (Agaisse et al. 2003). To further complicate the picture, the JAK/STAT pathway also has a role in embryonic segmentation (Binari and Perrimon 1994).

These four inducible pathways have important roles to protect hosts from pathogens that have already invaded past physical barriers into host cells, and cross-talk between the pathways allows for effective responses to a range of pathogens despite the lack of an adaptive immune system. Endosymbiotic bacteria also reside within cells, and thus the innate immune system is also involved in endosymbiont-host interactions.

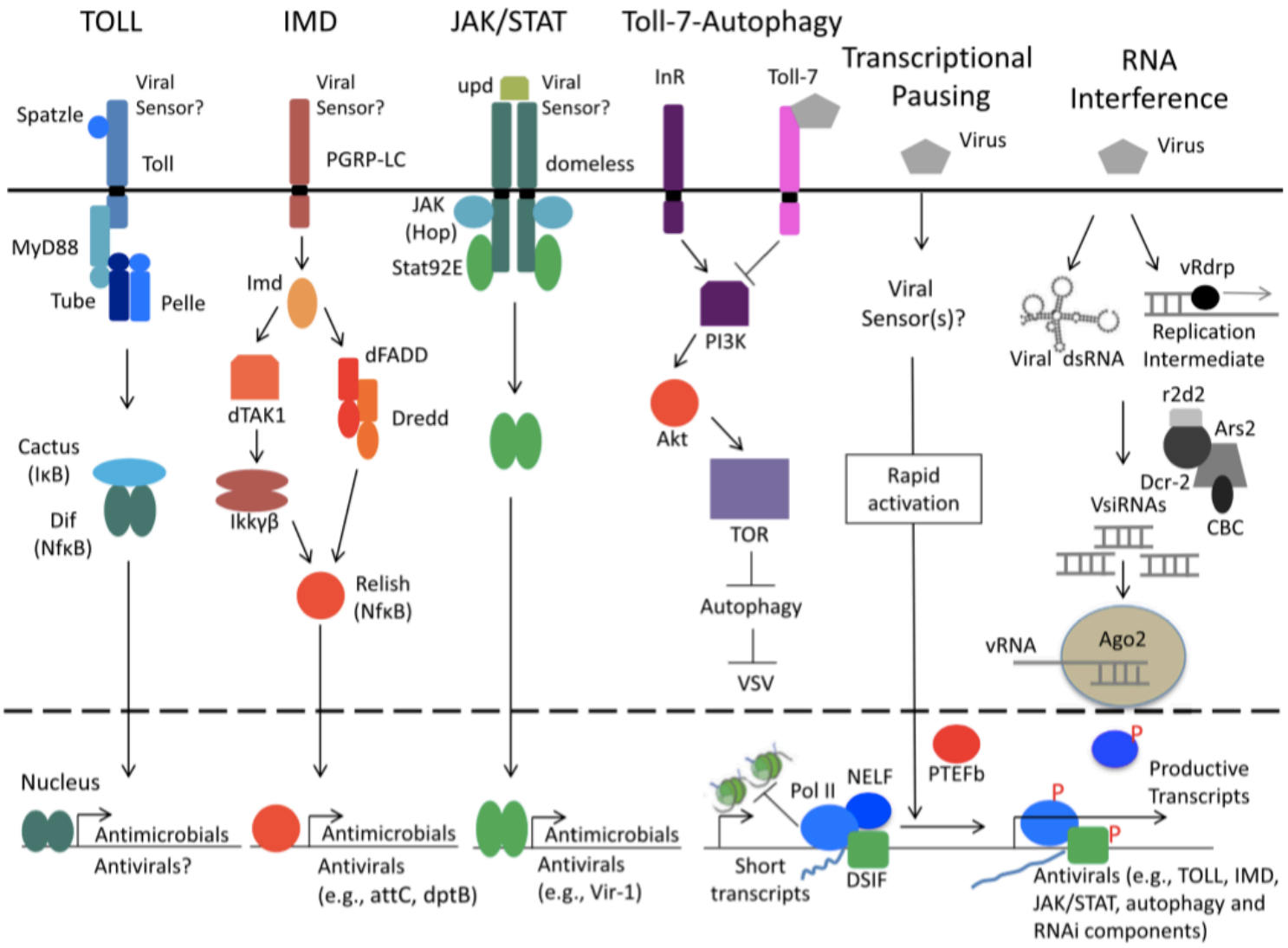

Figure 2 Innate immune pathways in *Drosophila*: TOLL, IMD, JAK/STAT, autophagy and RNAi. Not shown: melanization. The solid horizontal black line indicates the cell surface, while the dotted horizontal black line indicates the nucleus-cytoplasm boundary. Figure taken from Xu and

ENDOSYMBIONT-HOST INTERACTIONS

Wolbachia is a Gram-negative maternally inherited endosymbiotic bacterium that is present in around 40% of known insect species (Zug and Hammerstein 2012; Zug et al. 2012). *Wolbachia* is an α-Proteobacterium from the Rickettsiales order (Werren et al. 2008) yet it is very different from the other genera in Rickettsiales as it does not routinely infect vertebrates. Rather, genetically distinct *Wolbachia* strains are found in a highly diverse host range that includes arthropods (insects, mites (Breeuwer and Jacobs 1996), spiders (Oh et al. 2000), crustaceans (Cordaux et al. 2001; Cordaux et al. 2012)) and nematodes (Sironi et al. 1995).

Wolbachia is best known for its ability to induce in its hosts diverse reproductive abnormalities that result in its spread through invertebrate host populations (Werren et al. 2008). Several strategies have evolved, including male killing, parthenogenesis, feminisation and cytoplasmic incompatibility (CI). CI occurs most frequently of these. Although it has now been linked to two prophage WO genes in *Drosophila* species (Beckmann and Fallon 2013; LePage et al. 2017) and a *Wolbachia* deubiquitylating enzyme (Beckmann et al. 2017), the mechanisms are still poorly understood (Bossan et al. 2011; Poinsot et al. 2003). It is likely that these mechanisms are partially dependant on the length of the endosymbiont-host relationship and whether it is novel or native. Host jumps between invertebrates provide unique opportunities to study the genes that underlie these mechanisms.

The extensive host jumps performed by some *Wolbachia* strains are identified by the little concordance between host and *Wolbachia* phylogeny (Cordaux et al. 2001; Werren et al. 1995). The range of possible means for transmission of symbionts between species include, but are not limited to: wounding by parasitoid wasps (Ahmed et al. 2015; Gehrer and Vorburger 2012) and mites (Jaenike et al. 2007), cross-infection from infected to uninfected hosts during copulation (Moran and Dunbar 2006) and cofeeding (Caspi-Fluger et al. 2012) as well as direct introduction from the environment (Kikuchi et al. 2007). These host jumps may account for why *Wolbachia* strains are found in a highly diverse host range.

Experimental studies into *Wolbachia*'s interactions with its hosts are limited as *Wolbachia* does not survive more than a week outside cells (Rasgon et al. 2006) and no transformation system is available. In addition, studies into *Wolbachia*-nematode interactions are limited because separating the two requires antibiotic treatment. Such treatment may create transcriptomic changes not related to the specific endosymbiont-host interaction, but rather related to antibiotic toxicity. Several genes with a role in arthropod *Wolbachia*-host interactions have been identified. These include ankyrin domains (Wu et al. 2004), wsp surface proteins (Baldo et al. 2010; Pinto et al. 2012) and molecular motors dynein and kinesin (Ferree et al. 2005). Nematodes produce Lipid A, the usual component of proteobacterial membranes, because *Wolbachia* lacks the membrane biogenesis genes required to synthesise these molecules (Foster et al. 2005; Henrichfreise et al. 2009). This provides a point of contact between the two organisms that may mediate their

mutualistic relationship (Foster et al. 2005; Henrichfreise et al. 2009). In addition, *Wolbachia* genomes lack the minimal genome content and stability that is associated with other obligate endosymbionts (Tamas et al. 2002): they have a high number of mobile and repetitive elements and there are many genes with hypothetical protein functions due to duplications of short open reading frames (Wu et al. 2004). Examination of the interactions of *Wolbachia* with its hosts can be approached without reliance on what is known about *Wolbachia* genomes.

In **Chapter 2**, I examined available transcriptome data from *Wolbachia* and *Wolbachia*-free hosts (summarized in Table 1) as the range of hosts could provide a broad picture of common interactions without knowledge of the *Wolbachia* genome. Many of the effects of *Wolbachia* are strain- and host- specific (Serbus et al. 2008), yet my findings are that *Wolbachia* does affect several key areas of host gene expression across diverse hosts. I identified a range of novel biological functions and a list of candidate genes of further interest for experimental validation. Some functions of interest included: chitin processing, metabolism, lipids and the immune system. Looking at commonalities of the host response to *Wolbachia* is important to our understanding of how removal of *Wolbachia* can protect against filarial infections, yet its artificial introduction to arthropods can protect against a diverse range of pathogens.

Table 1 Summary of transcriptional studies encompassing both nematodes and arthropods infected with *Wolbachia*

Host species	Tissue type	*Wolbachia* infection/strain	Antibiotic Treatment	Reference
D. melanogaster	Testes	Native	Tetracycline	Zheng et al. (2011b)
A. vulgare	Ovaries	Native	N/A	Chevalier et al. (2012)
A. tabida	Ovaries	Native	Rifampicin	Kremer et al. (2012)
B. malayi	Whole body	Native	Doxycycline	Rao et al. (2012)
B. malayi	Whole body	Native	Tetracycline	Ghedin et al. (2009)
L. sigmodontis	Whole body	Native	Tetracycline	Strubing et al. (2010)
T. urticae	Whole body	Native	Tetracycline	Zhang et al. (2015)
S. oryzae	Whole body	Native	N/A	Vigneron et al. (2012)
Ae. aegypti	Whole body	Non-native, *w*AlbB	N/A	Pan et al. (2012)
An. gambiae	Cells	Non-native, *w*AlbB	N/A	Hughes et al. (2011b)
D. melanogaster	Cells	Non-native, *w*Ri	Tetracycline	Xi et al. (2008a)
An. gambiae	Cells	Non-native, *w*Ri	N/A	Hughes et al. (2011b)
B. mori	Cells	Non-native, *w*Sr	N/A	Nakamura et al. (2011)

ENDOSYMBIONT-HOST-PATHOGEN INTERACTIONS

As previously discussed, insects' main insect defence mechanism against pathogens is their innate immune system. The presence of *Wolbachia* in mosquitoes (Rances et al. 2012; Ye et al. 2013) and flies (Hoffmann et al. 2011; Teixeira et al. 2008) activates the insect's immune response, in a process referred to as 'immune priming'. *Wolbachia* increases gene expression of antimicrobial peptides such as defensins and cecropins, antiviral pathways, TOLL pathway genes, and genes involved in melanisation (Bian et al. 2010; Moreira et al. 2009a; Pan et al. 2012; Rances et al. 2012). Elevated basal host immunity makes the host resistant to subsequent pathogen invasion. *Wolbachia* infection in mosquitoes has been shown to interfere with the replication of a broad range of pathogens: viruses, filarial nematodes, bacteria and the malaria parasite (Hughes et al. 2011a; Kambris et al. 2010; Kambris et al. 2009; Moreira et al. 2009a; Ye et al. 2013). *Wolbachia*-based pathogen blocking has been partly attributed to competition between the pathogen and *Wolbachia* for limited host resources such as intracellular space (Moreira et al. 2009a) and lipids (Caragata et al. 2013). This is unsurprising as viruses are known to depend on host lipids for replication (Lu et al. 1999; Mackenzie et al. 2007), and *Wolbachia* does not have a complete gene set for lipid synthesis (Wu et al. 2004). Such properties can be exploited to use *Wolbachia* as a biocontrol agent against diseases found in the tropical regions of the world.

Treatment of Nematode Lymphatic Pathologies

Lymphatic pathologies and onchocerciasis are caused by nematodes, affecting 150 million people worldwide with 1.5 billion people at risk (Michael et al. 1996). Traditionally, treatment for these infections has relied on a limited number of drugs (Hoerauf 2006), but drug resistance is emerging (Osei-Atweneboana et al. 2007; Schwab et al. 2005). Thus new drugs must be identified, targeting either the nematodes (Kumar et al. 2007) or their mutualistic *Wolbachia* endosymbionts (Taylor et al. 2013).

The majority of nematodes that cause human disease depend on *Wolbachia* for reproductive success, and thus have co-evolutionary histories intertwined with *Wolbachia* (Taylor et al. 2005). However, not all nematodes are reliant on *Wolbachia* (Ferri et al. 2011). The genome sequencing and subsequent analysis of some mutualistic *Wolbachia* strains and their hosts has identified some areas of direct interaction (Darby et al. 2012; Foster et al. 2005; Ghedin et al. 2007), for example the synthesis of metabolites such as haem (Foster et al. 2005; Ghedin et al. 2007). Additional information about other strains however shows that the provision of vitamins or cofactors is not universal to all endosymbiont and nematode interactions (Darby et al. 2012).

Despite the development of some useful drugs to eliminate *Wolbachia* and kill the nematode host, not all treatments are equally effective (Hoerauf et al. 1999; Slatko et al. 2010; Taylor et al. 2005; Taylor et al. 2010; Taylor et al. 2014;

Taylor et al. 2013). The anti-*Wolbachia* consortium (A-WOL) aims to identify existing FDA approved drugs that can eliminate nematode infections by specifically targeting *Wolbachia* (see www.a-wol.com). However, the identification pipeline is heavily reliant on a single *Wolbachia*-infected arthropod cell line of natively infected *Ae. albopictus* C6/36 Wp cells (Taylor et al. 2014); therefore specific *Wolbachia*-nematode relationships have been neglected. Additionally lateral gene transfer may incorporate *Wolbachia* DNA into the nematode genome (Dunning Hotopp et al. 2007; McNulty et al. 2010) and halt the ability to target *Wolbachia* to remove nematode infections. Without further knowledge of the underlying *Wolbachia*-nematode relationship there is no clear path to develop new drugs or compensate for the emergence of drug resistance (Esterre et al. 2001; Osei-Atweneboana et al. 2007).

Biocontrol of Arthropod-Borne Diseases

Arthropod vectors are responsible for some of the most onerous infectious disease burdens in humans and are the cause of death for 1.5 million individuals a year (Hill et al. 2005). These diseases include a range of parasitic infections, most notably malaria, but also viral diseases the disease burden of which is rapidly growing (World Health Organization 2015). DENV is the most prevalent arthropod-borne virus that affects humans today (Gubler 1998; Gubler 2002; Kyle and Harris 2008). Dengue fever is a re-emerging tropical disease that is endemic in over 100 countries with an estimated 390 million infections reported annually (Bhatt et al. 2013; Gubler 1998; Gubler 2002; Wilder-Smith et al. 2012). The disease is severely debilitating (Bhatt et al. 2013; Gubler 2002; Shepard et al. 2011; Shepard et al. 2013) and the more severe forms of dengue fever, namely dengue haemorrhagic fever and dengue shock syndrome, are potentially fatal (Sabchareon et al. 2012). DENV is transmitted between humans by *Aedes* mosquitoes, with *Ae. aegypti* the principal vector and *Ae. albopictus* playing a minor role. Conventional vector control strategies such as source reduction and insecticide have become less effective due to increased insecticide resistance in mosquito populations (McGraw and O'Neill 2013; Sabchareon et al. 2012). Increased rates of human travel and urbanisation, as well as the widening geographic distribution of *Ae. aegypti*, mean that the incidence of dengue fever is on the rise (Wilder-Smith et al. 2012).

DENV transmission can be controlled by the pathogen-blocking abilities of *Wolbachia* (Iturbe-Ormaetxe et al. 2011). *Wolbachia*'s maternal transmission ensures introduced strains can rapidly invade and replace wild populations (Walker et al. 2011b; Xi et al. 2005b). While present in 28% of mosquitoes such as *Ae. albopictus, Culex pipiens* and *C. quinquefasciatus, Wolbachia* is not naturally present in *Anopheles* species or *Ae. aegypti* (Kittayapong et al. 2000). A range of *Wolbachia* strains has been introduced to date into *Ae. aegypti*: *w*Mel and *w*MelPop from *D. melanogaster* (McMeniman et al. 2009; Walker et al. 2011a), *w*AlbB from *Ae. albopictus* (Xi et al. 2005b) and a superinfection of *w*Mel/*w*AlbB (Joubert et al. 2016). wMel forms stably inherited infections in *Ae. aegypti* and impairs DENV transmission (McMeniman et al. 2009; Walker et al. 2011a) (Iturbe-Ormaetxe et al. 2011). The antiviral activity of *Wolbachia* remains

stable in field releases (Frentiu et al. 2014) and early releases of *Wolbachia* into the field have spread to fixation in populations (Hoffmann et al. 2011). *Wolbachia*-infected *Ae. aegypti* mosquitoes are currently being released into the wild in countries that include Vietnam, Brazil and Indonesia to test the ability of *Wolbachia* to interrupt endemic DENV and Zika virus transmission (Ritchie 2014) (see www.eliminatedengue.com).

Pathways that target bacteria (TOLL and IMD) are usually assayed to quantify the effect of *Wolbachia* on its insect host. Most studies look for expression change in genes that encode the AMPs diptericin, cecropin and defensin (Table 2. In cell lines, native *Wolbachia* infections do not induce a heightened immune response in comparison to uninfected *Drosophila* (Bourtzis et al. 2000; Rances et al. 2012) or *Aedes* species (Caragata et al. 2017). The opposite is true in novel *Wolbachia* infections, where introduction of *Drosophila* derived *Wolbachia* into a different line of *Drosophila* cells (Xi et al. 2008a) or *Wolbachia* into non-native *Aedes* species (Bian et al. 2010; Kambris et al. 2009; Pan et al. 2012; Rances et al. 2012), activates the immune signalling pathway. This is despite the fact that previous studies found that some *Wolbachia* infections cannot persist in their novel hosts (Voronin et al. 2010; Xi et al. 2008a). Evidence thus far therefore suggests that *Wolbachia* infections affect the expression of immune pathways in native hosts less than in novel hosts.

Increases in innate immune gene expression correlate with higher densities of *Wolbachia* and also better virus blocking (Rances et al. 2012; Ye et al. 2013). In *Ae. aegypti* mosquitoes, *w*Mel exhibits higher symbiont loads, broader tissue distributions and a greater fitness cost than in its native fly host (Ross et al. 2014; Turley et al. 2013; Voronin et al. 2010; Walker et al. 2011b). Interestingly, *w*AlbB exhibits low levels of infection in its native mosquito host (Walker et al. 2011b; Xi et al. 2005b) as well as after transinfection into the naturally uninfected *Ae. aegypti* (Axford et al. 2016; Xi et al. 2005b). *w*AlbB shows reduced tissue density compared to *w*Mel, causes fewer fitness consequences, and induces weaker pathogen blocking (Axford et al. 2016; Lu et al. 2012; Pan et al. 2012; Walker et al. 2011b; Xi et al. 2005a; Xi et al. 2005b). When coinfected the two strains co-localise, and the *w*AlbB strain exhibits a similar density to a single infection in *Ae. aegypti*, while *w*Mel exhibits a higher density (Joubert et al. 2016).

It is thought that the reason *Wolbachia* is so effective at blocking DENV in *Ae. aegypti* is because this is a novel host pairing. While the induced immune response may play a part in *Wolbachia*'s anti pathogen effects (Bian et al. 2010; Pan et al. 2012; Rances et al. 2012) it cannot entirely explain the effect (Caragata et al. 2013; Moreira et al. 2009a). Given the lower response of native hosts to *Wolbachia*, the hyperactivated immune response is likely to reduce with coevolution. This may offer a potential path by which resistance emerges and this likely will prompt the development of another novel *Wolbachia* mosquito line.

While *in vivo* experiments provide valuable information as to how and when *Wolbachia* acts on its hosts, the complexities of the host and bacterial interactions are difficult to isolate, and there are problems with very low transinfection success rates. Instead, as described in **Chapter 3** I used a range of arthropod cell lines to examine the effect of transient *Wolbachia* infection on immune gene expression. I created novel infections in diverse species, introducing two *Wolbachia* strains (*w*Mel, *w*AlbB). Newly *w*AlbB-infected lines included three mosquitos (*Ae. aegypti, Ae. albopictus*, *An. gambiae*) and a moth (*Spodoptera frugiperda*). *w*AlbB infections continued to be stable over 10 subsequent passages post-infection. My second approach was to infect cell lines with *w*Mel, but this proved to be more difficult due to biological incompatibilities. I could establish novel *Wolbachia* infections in *Ae. albopictus*, *D. melanogaster* and *S. frugiperda* but interestingly, it proved impossible to insert *w*Mel into a naïve Aag2 line. Cell-line adaptation of *Wolbachia* (*w*Mel) in mosquito cells can cause it to lose its ability to effectively infect its original host, *D. melanogaster* (McMeniman et al. 2008). In the three *w*Mel infected cell lines we created, *Wolbachia* had dropped out by four passages. I then more closely examined the nature of the immune response immediately after infection and across several passages. *D. melanogaster* cells infected with *w*Mel suppressed expression of TOLL and IMD pathway genes, while the mosquito and moth lines showed an activation of the immune response. This comparative approach determined that there are few commonalities in the early host immune response across these species, and further that there is no attenuation of gene expression change in the first several passages despite *Wolbachia* dropping out of the novel host cell lines.

Table 2 A summary of previous studies that examined the effect of *Wolbachia* infections on immune gene expression in *Aedes* and *Drosophila* species. Blank cells indicate that the study did not test the listed gene.

	Host organism	***Wolbachia* strain**	**Immune Genes**				**Reference**
			Relish	Defensin	Cecropin	Diptericin	
Aedes species	*Ae. albopictus*	*w*AlbB		Unaffected			Bourtzis et al. (2000)
	Ae. aegypti	*w*AlbB	Upregulated	Upregulated	Upregulated	Upregulated	Bian et al. (2010)
	Ae. aegypti	*w*AlbB	Upregulated	Upregulated	Upregulated	Upregulated	Pan et al. (2012)
	Ae. aegypti	*w*Mel		Upregulated	Upregulated	Upregulated	Rances et al. (2012)
	Ae. aegypti	*w*MelPop	Upregulated	Upregulated	Upregulated		Kambris et al. (2009)
	Ae. aegypti	*w*MelPop		Upregulated	Upregulated	Upregulated	Rances et al. (2012)
	Ae. fluviatilis	*w*Flu	Unaffected	Unaffected	Unaffected	Unaffected	Caragata et al. (2017)
Drosophila species	*D. melanogaster*	*w*MelCS		Unaffected		Unaffected	Wong et al. (2011)
	D. melanogaster	*w*Mel		Unaffected	Unaffected	Unaffected	Rances et al. (2012)
	D. melanogaster	*w*MelPop		Unaffected	Upregulated	Unaffected	Rances et al. (2012)
	D. melanogaster (S2 cell line)	*w*Ri	Upregulated	Unaffected	Unaffected	Upregulated	Xi et al. (2008a)
	D. simulans	*w*Ri		Unaffected	Unaffected	Unaffected	Bourtzis et al. (2000)

HOST-PATHOGEN EVOLUTION

The immune system of *Drosophila* has been well-studied (Drosophila 12 Genomes et al. 2007; Tzou et al. 2002). It has high rates of pathogen exposure (Markow and O'Grady 2007) , thus making it a valuable tool for the study of host-pathogen evolution. One way to look at this evolution is to examine adaptation through comparison of the rates of synonymous (d_S) and nonsynonymous (d_N) nucleotide substitutions in homologous protein-coding gene sequences as a ratio (ω) (Nei and Kumar 2000). Adaptive evolution is also known as 'positive selection': some codon sites within a gene evolve differently along specific lineages in response to evolutionary adaptation. $\omega > 1$ is considered evidence for positive selection, while $\omega < 1$ indicates purifying selection, and $\omega = 1$ indicates neutral evolution.

Positive selection is difficult to detect, as it tends to act on a few select sites within a short evolutionary time-period. Three different methods to detect this selection have been developed: (1) site-specific models; (2) branch-specific models; and (3) branch-site models. The first of these methods, site-specific models, are best to detect significant changes in a single species within a phylogenetic tree, but can return a high rate of false positives (Nielsen and Yang 1998; Yang et al. 2000). The second, branch-specific models, tend to have high false negative rates as differences must be detected in most of the sequence before they become apparent in the ω value (Yang 1998; Yu and Irwin 1996; Zhang et al. 1997). The last of these, branch-site models, are the most discerning method since it allows for the use of an *a priori* model where previous knowledge about the history of the species in question can be used to refine the selection tests (Yang and Nielsen 2002; Zhang et al. 2005).

Thus, when 12 *Drosophila* species' genomes were sequenced in 2007 (Drosophila 12 Genomes et al. 2007), this provided a wealth of information to form *a priori* models to examine positive selection in this powerful model organism. Initial conservative examinations could detect positive selection in a range of immunity-related genes (Drosophila 12 Genomes et al. 2007). In fact, immunity related gene sequences are fixed by adaptive evolution at nearly double the rate of non-immune genes (Obbard et al. 2009; Sackton et al. 2007; Schlenke and Begun 2003). Early studies in this area identified dorsal, Dredd, Imd, Relish, Spn43Ac, Tehao, and Toll as genes that undergo an accelerated rate of adaptive evolution (Schlenke and Begun 2003). Many studies identify Relish as a particularly important gene that is under positive selective pressure (Begun and Whitley 2000; Jiggins and Kim 2007; Levine and Begun 2007; Obbard et al. 2009; Sackton et al. 2007). As previously discussed, Relish is part of the IMD signalling pathway. Other genes in this pathway that show adaptive evolution include ird5, key, Dredd (Sackton et al. 2007) and dFADD (Jiggins and Kim 2007). As these genes interact, this suggests that they may be evolving as a gene cassette in reaction to pressure from a taxon-specific host-pathogen interaction (Sackton et al. 2007).

Similarly, genes in the antiviral RNAi pathway evolve rapidly (Obbard et al. 2009). However, there are lower rates of adaptive evolution in the TOLL pathway compared to other pathways (Obbard et al. 2009). There are still genes under selection, including serine proteases upregulated by the TOLL pathway (Jiggins and Kim 2007), but the observed limited evolution in this pathway is probably due to constraint with its other functions in development. Additionally, there are 14 pathogen detection genes under significant positive selective pressure, of which the majority are related to phagocytosis in some way (TEPs: *TepI*, *TepIV*; nimrods: *NimB1*, *NimB4*; CD36 homologs: *crq*, CG31217, *emp)* (Sackton et al. 2007). The authors hypothesise that this significant pressure arises from the coevolutionary arms race because phagocytosis targets are likely to have variable structures (Sackton et al. 2007). The additional genes identified included PGRP-LC and PGRC-LB, but most PGRP and Gram-negative Bacterial-Binding Protein (GNBP) receptors are not positively adapted (Obbard et al. 2009; Sackton et al. 2007). This is probably because they bind highly conserved pathogen molecules (Jiggins and Kim 2006).

Detection of positive selection as a measure of adaptive evolution is dependent on the accuracy of nucleotide alignments, which are inherently less reliable at high synonymous divergence. The more genetically diverse the species of interest, the more difficult it is to detect positive selection because sequences have likely diverged substantially and this results in sequence saturation. This same detection problem arises in short genes because there is a smaller number of synonymous sites. As an alternative approach, it is possible to identify patterns of purifying selection by looking at sequences that have not diverged.

Chapter 4 reports a study in which I identified purifying selection by geographical range for genes that represent various aspects of the immune response in *Drosophila* species. I found an overabundance of purifying selection for genes that control viral infection in flies whose ranges are restricted to the tropics. This pattern could result from differential exposure to viruses in the tropics that may include greater viral prevalence, diversity or load. These alternate hypotheses cannot be differentiated without a better understanding of the native viruses of these fly species relative to those documented for *D. melanogaster* and *D. simulans* (Webster et al. 2015). As tropical regions expand due to shifts in climate, so too will the distributions of insects, including a number of vectors (Régnière et al. 2012). Understanding how geographic region shapes the immune response to pathogens may lead to different short and long-term predictions about insect susceptibility to pathogens. In addition, I have identified specific genes that potentially underpin the co-evolutionary response of flies to viral pathogens. Future comparative work may reveal whether these patterns are generalizable for insects in the tropics, and if there are consistent gene types that experience heightened selective constraints. Lastly, I identified likely anti-pathogen candidate genes for genetic modification in insect vectors, but the

capacity for such genes to harbor and maintain genetic variation through time and over broad geographic landscapes must be further explored.

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Chapter 2

Shared Transcriptional Responses of Arthropods and Nematodes to the Loss of *Wolbachia* Infection

ABSTRACT

Wolbachia is a maternally inherited endosymbiont found in insects, nematodes and other invertebrates. It is responsible for a wide range of effects, including roles in reproduction and immune system priming. Currently, novel introduction of *Wolbachia* into insects is used for biocontrol while antibiotic treatment against *Wolbachia* in nematodes treats lymphatic pathologies. However, the molecular mechanisms underlying the *Wolbachia/*host interactions are poorly understood. We used publically available transcriptome datasets to re-examine differentially expressed patterns associated with *Wolbachia* infection, using Gene Ontology and the associated differentially expressed gene lists to identify potential fundamental avenues of symbiont x host interaction. We found that the removal of *Wolbachia* infection in arthropods and nematodes affects expression of genes representing 47 GO terms common to three or more host organisms. We identified the categories of chaperones, metabolic rate, ER/Golgi, cytoskeleton, chitin and lipids as potentially the main underlying components to understanding fitness effects, pathogen blocking and potential drug targets. Our study has produced a set of candidate genes and processes in hosts to be investigated experimentally.

Key Words: *Wolbachia*, endosymbiont, transcriptome, insect, nematode

INTRODUCTION

Wolbachia is a Gram-negative, maternally inherited endosymbiotic α-Proteobacterium from the Rickettsiales order (Werren et al. 2008). *Wolbachia* is different from other genera in this group in that it does not routinely infect vertebrates and because of its diverse host range. Genetically distinct *Wolbachia* strains are found in arthropods (insects, mites, spiders, crustaceans), and nematodes (Werren et al. 2008). *Wolbachia* is best known for its ability to induce reproductive abnormalities that have the effect of increasing its spread through host populations (Werren et al. 2008). More recently, *Wolbachia* has been shown to limit infection with viruses, bacteria and parasites (Moreira et al. 2009a; van den Hurk et al. 2012).

Arthropod-borne viral diseases have a significant impact on public health (Mayer et al. 2017), dengue virus alone infects more than 390 million people annually (Bhatt et al. 2013). *Wolbachia* limits replication of dengue (Moreira et al. 2009a), Zika (Dutra et al. 2016), yellow fever (van den Hurk et al. 2012) and Chikungunya (Moreira et al. 2009a; van den Hurk et al. 2012) in the bodies of mosquito vectors. After *Wolbachia* had been shown to spread into wild populations of mosquitoes following field release (Hoffmann et al. 2011), global trials were begun to examine whether the symbiont could reduce dengue virus replication in mosquitoes sufficiently to lower incidence of dengue fever in humans (McGraw and O'Neill 2013). Despite widespread field testing (McGraw and O'Neill 2013), we still have a very poor understanding of the fundamental ways in which the symbiont interacts with its hosts and how pathogen blocking is mediated (Caragata et al. 2013; Moreira et al. 2009a; Pan et al. 2012; Rainey et al. 2016; Rances et al. 2012; White et al. 2017). Planning for the evolution of resistance against the *Wolbachia*-mediated pathogen blocking in both virus and vectors requires an understanding of how *Wolbachia* limits pathogen replication.

Wolbachia is also being investigated in another arena of human health, namely its mutualistic relationship with the nematodes that cause lymphatic pathologies and onchocerciasis in 150 million people worldwide (Michael et al. 1996). In field trials, antibiotics have successfully been used to treat human nematode infections by targeting the worm's *Wolbachia* (Johnston et al. 2014; Taylor et al. 2010; Townson et al. 2000) – a welcome new strategy as drug resistance to standard nematode-specific drugs is emerging (Osei-Atweneboana et al. 2007; Schwab et al. 2005). Inevitably, drug resistance will occur against these antibiotics as well, and new targets will need to be identified (Clare et al. 2015). Examining the extensive genetic information about nematodes and their *Wolbachia* endosymbionts is essential to this process.

The use of experimental approaches to study *Wolbachia*:host interactions has been limited as *Wolbachia* does not survive more than a week outside of cells (Rasgon et al. 2006) and genetic transformation has not yet been achieved.

Regardless, a number of genes have been identified that appear to mediate endosymbiont-host interactions including ankyrin domains (Wu et al. 2004), wsp surface proteins (Baldo et al. 2010) and molecular motors dynein and kinesin (Ferree et al. 2005). *Wolbachia* genomes lack the core minimal genome content and stability associated with other obligate endosymbionts (Tamas et al. 2002) and so they are clearly heavily dependent on the host cell for a range of resources. Further, because *Wolbachia* reside within host cell membranes (Cho et al. 2011; Voronin et al. 2004) they can secrete effector molecules to exert direct action on host processes and physiologies.

With the availability of a growing number of transcriptional studies there is an opportunity to compare genome wide patterns of expression of diverse infected hosts and to begin to explore fundamental pathways by which *Wolbachia* may operate. This may shed light on how *Wolbachia* manipulates host reproductive biology, affects host fitness and limits the replication of co-infecting pathogens. A better understanding of these phenomena may have practical implications when promoting *Wolbachia* as a biocontrol agent against mosquito borne pathogens and also when targeting *Wolbachia* as a means of limiting filarial infections in humans and farm animals. Many of the effects of *Wolbachia* are strain- and host- specific, yet our findings demonstrate that *Wolbachia* affects several key areas of host gene expression across diverse hosts.

METHODS

Data were mined from 12 studies in which enriched transcriptomic responses were characterised in a range of *Wolbachia*-infected organisms relative to *Wolbachia*-free controls (Table 1). The studies differed in how they obtained *Wolbachia*-free individuals for comparison: (1) hosts that were naturally *Wolbachia*-infected but had been antibiotic treated to completely remove the *Wolbachia* infection, (2) hosts that occurred naturally as both *Wolbachia*-infected and *Wolbachia*-free, and (3) hosts that were novelly infected with *Wolbachia,* then antibiotic treated to remove the infection.

Host sources span eight arthropods and two nematodes and include studies on cells, tissues and whole animals. For six studies, differentially expressed gene lists were available and we converted these to Entrez gene ID format using DAVID (Huang da et al. 2009). Gene Ontology (GO) mappings for each organism were selected from the UniProtKB database (http://www.uniprot.org/uniprot). REVIGO (Supek et al. 2011) was then used to reduce the number of redundant GO notations (setting 0.5 - small), followed by identification of biological groupings based on different GO classification levels using GOView (http://www.webgestalt.org/GOView/) (Online Resource 1A[1]). Significance cutoffs were retained as per the original studies (Table 1). Because cutoffs vary slightly across studies this will have introduced some bias. For example, when comparing two studies with a high stringency cut off and a low cutoff, we are likely to have more false negatives than positives. The other six studies did not provide gene lists, and so to continue analysis we extracted the GO terms provided at the most detailed hierarchy.

We then compared GO terms (and associated genes where possible) across all the available data sets and reported only GO terms that were shared by three or more organisms, to gain a sense of common responses. Using Venny 2.0, Venn diagrams of biological groupings were produced of GO Terms shared between: (A) nematode samples, (B) native tetracycline treated samples, (C) non-native Diptera samples, (D) native arthropods, excluding Diptera, (E) native gametes.

[1] **Online Resource 1A** Excel file containing the complete GO term lists generated from the differentially expressed gene lists given for six studies, and the given GO terms from the other studies.

Table 1 Summary of the 12 studies with transcriptional data available for analysis.

Host species	Tissue type	*Wolbachia* infection type and strain	Antibiotic Treatment	Study design	Significance Cut-off point	All data obtained from suppl. materials
D. melanogaster	testes	Native	tetracycline	GeneChip 15K *Drosophila* Genome Array, tested for *Wolbachia* presence by wsp PCR	1.5 fold change	Zheng et al. (2011b)
A. vulgare	ovaries	Native	N/A	EST library, laboratory lineages maintained with known *Wolbachia* infection status	randomisation procedure, hyper-geometrical test, 2.5% false discovery rate	Chevalier et al. (2012)
A. tabida	ovaries	Native	rifampicin	EST library, tested for *Wolbachia* presence by PCR	hyper-geometrical test, functional enrichment analysis	Kremer et al. (2012)
B. malayi	whole body	Native	doxycycline	Version-2 Filarial Microarray, *Wolbachia* genes assayed on microarray	2 fold change	Rao et al. (2012)
B. malayi	whole body	Native	tetracycline	*B. malayi* microarray BmV2array, *Wolbachia* genes assayed on microarray	2 fold change	Ghedin et al. (2009)
L. sigmodontis	whole body	Native	tetracycline	*B. malayi* microarray BmV2array, *Wolbachia* genes assayed on microarra	2 fold change	Strubing et al. (2010)
T. urticae	whole	Native	tetracycline	transcriptome sequencing, tested for *Wolbachia*	>1 fold change, followed by GO term enrichment of p <	Zhang et al. (2015)

	body			presence by PCR	0.05	
S. oryzae	whole body	Native	N/A	EST library, laboratory lineages maintained with known *Wolbachia* infection status	hyper-geometrical test, 5.5% false discovery rate	Vigneron et al. (2012)
Ae. aegypti	whole body	Non-native, *w*AlbB	N/A	microarray based on the V1.2 version annotation of the *Ae. aegypti* full genome sequence, laboratory lineages maintained with known *Wolbachia* infection status	>1 fold change	Pan et al. (2012)
An. gambiae	cells	Non-native, *w*AlbB	N/A	Affymetrix GeneChip microarrays, laboratory lineages maintained with known *Wolbachia* infection status	2 fold change, 5% false discovery rate	Hughes et al. (2011b)
D. melanogaster	cells	Non-native, *w*Ri	tetracycline	GeneChip microarray, tested for *Wolbachia* presence by PCR	1.2 fold change, 5% false discovery rate	Xi et al. (2008a)
An. gambiae	cells	Non-native, *w*Ri	N/A	Affymetrix GeneChip microarray*s*, laboratory lineages maintained with known *Wolbachia* infection status	2 fold change, 5% false discovery rate	Hughes et al. (2011b)
B. mori	cells	Non-native, *w*Sr	N/A	Silkworm 22K oligo-microarray, tested for *Wolbachia* presence by PCR	1.5 fold change	Nakamura et al. (2011)

RESULTS & DISCUSSION

It has been known for some time that *Wolbachia* influences fundamental aspects of host biology, but this is the first instance where transcriptional profiles from multiple taxa have been compared. Profiles were taken from studies that compared the transcriptomic responses of *Wolbachia*-free and *Wolbachia*-infected hosts to find genes that were expressed differentially between the hosts. We grouped GO Terms that these studies described as enriched, and found that they represented a broad range of shared biological functions. We identified 47 GO terms that were present in three or more organisms and delineated the relevant genes of interest representing these categories (Online Resource 1B[2]). The reasoning behind this cut off was to ensure that: (1) the analysis was not reliant on two samples from the same organism (eg. two *B. malayi* samples), or from the same study (eg. *An. gambiae* Hughes et al. (2011b)), and (2) the analysis compared samples outside of Diptera to compensate for the saturation of previously examined data in Diptera. We then stepped up a level in the structural hierarchy of GO Terms, reducing the number of terms to 26 (Figure 1), and then searched for the key terms of 'lipids' and 'chitin' to draw out any additional genes related to these functions. Below we have summarised the potential role of each of these GO terms considering the literature.

Chaperones. When *Wolbachia*-infected mosquitoes are released into a wild population, cytoplasmic incompatibility (CI) allows *Wolbachia* to efficiently infect the population (Werren et al. 2008). The exact molecular mechanisms underlying CI are still unknown but it is suggested that in *Drosophila* chaperones may underlie the CI phenotype, particularly through impact on spermatogenesis (Zheng et al. 2011a). Our evidence recapitulates the idea of interaction between *Wolbachia* and chaperones. We identified two arthropod species with the corresponding GO term, GO:0006457, (*A. tabida* and *D. melanogaster* (*w*Ri)), in addition to several chaperone associated genes in the remaining arthropod samples (Online Resource 1B). In the absence of *Wolbachia,* the *D. melanogaster* testis sample showed downregulated chaperone binding, while in *Ae. aegypti* there was downregulation of genes that promote folding, in addition to a shared gene between the two mosquito samples. *An. gambiae* also upregulates a protein with a similar function, yet downregulates a heat shock protein chaperone. We infer that the CI phenotype may relate to *Wolbachia's* effect on chaperone genes. It is also possible that as chaperones are responsible for cell stress responses, the downregulation of their binding partners promotes chaperone activity to respond to the disturbed cellular homeostasis in the wake of *Wolbachia's* removal.

[2] **Online Resource 1B** Excel file including GO Term lists from 3 nematode and 11 arthropod samples. To be included in this table, GO Terms must be represented in > 3 organisms. Where possible, upregulated genes are shown in yellow, while downregulated genes are shown in blue.

Metabolic rate. *Wolbachia* perturbs the activity and metabolic rate of its arthropod hosts (Evans et al. 2009) and can starve its host by preferentially increasing its own energy supply under low nutrition conditions (Ross et al. 2016). When *Wolbachia* is antibiotic treated in arthropods, it increases its use of the electron transport chain to retain adequate levels of energy production (Darby et al. 2014). It is therefore unsurprising we noticed that ATP synthesis coupled electron transport (GO:0042773) was shared by arthropods and nematodes including *L. sigmodontis* (Nad4), *D. melanogaster, Ae. aegypti* and *An. Gambiae* (Online Resource 1B). The NADH dehydrogenase genes identified under this GO term are important in energy production; their function is dependent on iron-sulphur clusters (D'Elia et al. 2006; Tripoli et al. 2005). To limit oxidative stress during treatment with doxycycline, *Wolbachia* downregulates its own oxidative stress causing genes and alters expression of host genes responsible for iron-sulfur cluster production (Darby et al. 2014). However, *Wolbachia* may nevertheless contribute to oxidative stress by generating reactive oxygen species (ROS) via iron-dependent aerobic metabolism (Gill et al. 2014). It is likely that these underlying genes are affecting host fitness and are possibly independent of the specific strain of *Wolbachia.*

Iron. Inhibiting heme synthesis in *Wolbachia* is currently of great interest as a filiaral control agent (Slatko et al. 2010). *Wolbachia* regulates the expression of iron-related genes in response to external stressors (Darby et al. 2014; Kremer et al. 2009). For example, in *Drosophila, Wolbachia* can enhance the fecundity of flies that are reared on an iron-deficient diet (Brownlie et al. 2009). For many nematodes, heme synthesis by *Wolbachia* is essential for development because the host genomes lack genes in the heme biosynthetic pathway (Pfarr and Hoerauf 2005). Inhibitiion of heme synthesis in *Wolbachia* affects nematode viability (Wu et al. 2009), making it difficult to discern whether *Wolbachia* provides heme directly to the nematode or whether it contributes to processing of heme from endogenous sources such as the vertebrate host (Gill et al. 2014). We found that after loss of *Wolbachia* many genes that bind heme are differentially regulated in *An. aegypti* AAEL014614 (Limbach and Wu 1985), *D. melanogaster* (*w*Ri) and *L. sigmodontis* (upregulated Bm1_50430) (Online Resource 1B). In *An. gambiae* a gene responsible for heme a biosynthesis is downregulated (AGAP001744) (Zhang et al. 2016). This evidence supports the current hypothesis to account for the variation in direction of effect on gene expression: that *Wolbachia* must give different advantages to its various hosts depending on the nature of their diverse symbiotic relationships.

Lipids. *Wolbachia* affects multiple GO terms that relate to lipids including lipid transport, phospholipid biosynthesis and fatty acid metabolism. Taking genes across this broad grouping met our requirement of 'three organisms with gene lists' for inclusion (Online Resource 1B). In the presence of *Wolbachia,* cellular lipid profiles are depleted (Molloy et al. 2016). It has already been posited that cholesterol modulation by *Wolbachia* plays a functional role in DENV infection in *w*Mel-infected *D. melanogaster* (Caragata et al. 2013). In the absence of *Wolbachia, Anopheles* genes associated with lipid

particles were downregulated while AAEL001194 (FASN1) was downregulated in *Ae. aegypti* with fatty acid synthase activity as well as a role in the triglyceride biosynthetic process (Garrido et al. 2015). When we searched the other samples for genes related to lipids (not listed under the GO term), we found that *Drosophila melanogaster* possesses genes with lipase activity (CG18641, CG6113, CG11619) that were affected. For the two nematode samples with genes, the *L. sigmodontis* gene was downregulated, while a fatty-acid binding protein in *B. malayi* was upregulated (Bm1_44400). Thus, our results consolidate the observed pattern of *Wolbachia* influencing lipids and indicate several genes of interest that may specifically influence pathogen blocking.

ER/Golgi. In nematodes, *Wolbachia* is found in close association with the endoplasmic reticulum and the nucleus, which likely promotes energy and nutrient sharing between them (Chagas-Moutinho et al. 2015). Similarly in *Drosophila*, *Wolbachia*-enclosing membranes have continuity with the endoplasmic reticulum (Voronin et al. 2004) and *Wolbachia* are found in golgi-related vesicles near membrane biogenesis (Cho et al. 2011). Here we identify several specific genes corresponding to these functions (Online Resource 1B). Following *Wolbachia* loss, *An. gambiae* upregulates AGAP012717 - responsible for regulation of ER proliferation and expansion and Golgi morphology in mutant larvae fat body cells (Zhao et al. 2015). In *Ae. aegypti* AAEL014177 is upregulated to promote endoplasmic reticulum membrane and Golgi organization (Lee et al. 2011). *D. melanogaster* (CG6488) and *Ae. aegypti* (AAEL006945, AAEL01376) both have downregulated genes relating to Golgi transport (Rosa-Ferreira et al. 2015; Rosenbaum et al. 2014). The loss of *Wolbachia* would tend to decrease the level of lipids and cholesterol needed by the cell, and thus transport of these cellular components is decreased (Caragata et al. 2013).

Cytoskeleton. *Wolbachia* is known to utilize the host actin cytoskeleton during oogenesis and spermatogenesis although the actin and bacteria do not necessarily directly interact (Newton et al. 2015). However, *Wolbachia*-infected flies lacking profilin and villin show a drop in *Wolbachia* titers in the initial generation, followed by a recovery of *Wolbachia* in subsequent generations (Newton et al. 2015). The capability of *Wolbachia* to migrate from the abdomen of *Drosophila* to the germline can only be achieved through altering the actin cytoskeleton (Frydman et al. 2006). When *Wolbachia* is lost there is upregulation of genes in the structural constituent of cytoskeleton (AGAP010510, AAEL011478) (Hoyle et al. 2000) and genes with roles in actin filament organisation (AAEL014845, AAEL011704, AAEL014843, AAEL011708) (Kiger et al. 2003) (Online Resource 1B). In contrast, *B. malayi* (Bm1_47805) and *Ae. aegypti* (AAEL009287, AAEL014335) downregulate genes that organise the actin cytoskeleton (Ojelade et al. 2015). *Wolbachia* could adapt in response to different host actin levels and types and this could explain why despite sharing the GO terms of Protein folding and Microtubule based processes, no consistent direction of expression was identified in the underlying genes.

Chitin. A single GO term relating to chitin biosynthesis (GO:0006030) was shared across five taxa. (Online Resource 1B) This category is interesting as chitin is utilized not only in the cuticle but also to line the alimentary canal, the tracheal system and genital ducts in both insects and nematodes (Zhu et al. 2016). In the alimentary canal it forms the peritrophic matrix (Merzendorfer and Zimoch 2003). This porous lining shields the epithelia from infectious agents (Zhu et al. 2016) and following a blood meal, serves as the primary first barrier against further bodily infection by viruses and parasites. In nematodes, chitin genes have been identified as a potential drug target against *B. malayi* (Kumar et al. 2007). Because of these important functions, we searched our datasets for genes that related to chitin. In addition to those genes listed in Online Resource 1, we found downregulated AAEL002959 in *Ae. aegypti* – knockdown *Drosophila* mutants of the homologous gene have thinned chitin-ECM (Pesch et al. 2016), and an *An. gambiae* upregulated gene (AGAP001093) responsible for chitin-containing cuticle pigmentation, which occurs prior to molting and eclosion (Dembeck et al. 2015). *Wolbachia* is not found near the cuticle during or immediately after moulting (Fischer et al. 2011). However, we noted that genes with matrix metalloprotease functions, which degrade old cuticle at moults, were differentially regulated after the loss of *Wolbachia* (Glasheen et al. 2010). It is likely that a careful balance of these genes is occurring, as both *Ae. aegypti* (AAEL012110, AAEL012217) and *An. gambiae* (AGAP012745/AGAP003696, AGAP006904) had genes that were downregulated and upregulated respectively. We also observe an upregulated gene in *B. malayi,* Bm1_07750. In the absence of a *Wolbachia* infection chitin is continually synthesized and degraded (Merzendorfer and Zimoch 2003). We suggest that *Wolbachia* may perturb this balance, and in so doing influence the pathogen blocking phenotypes associated with infections.

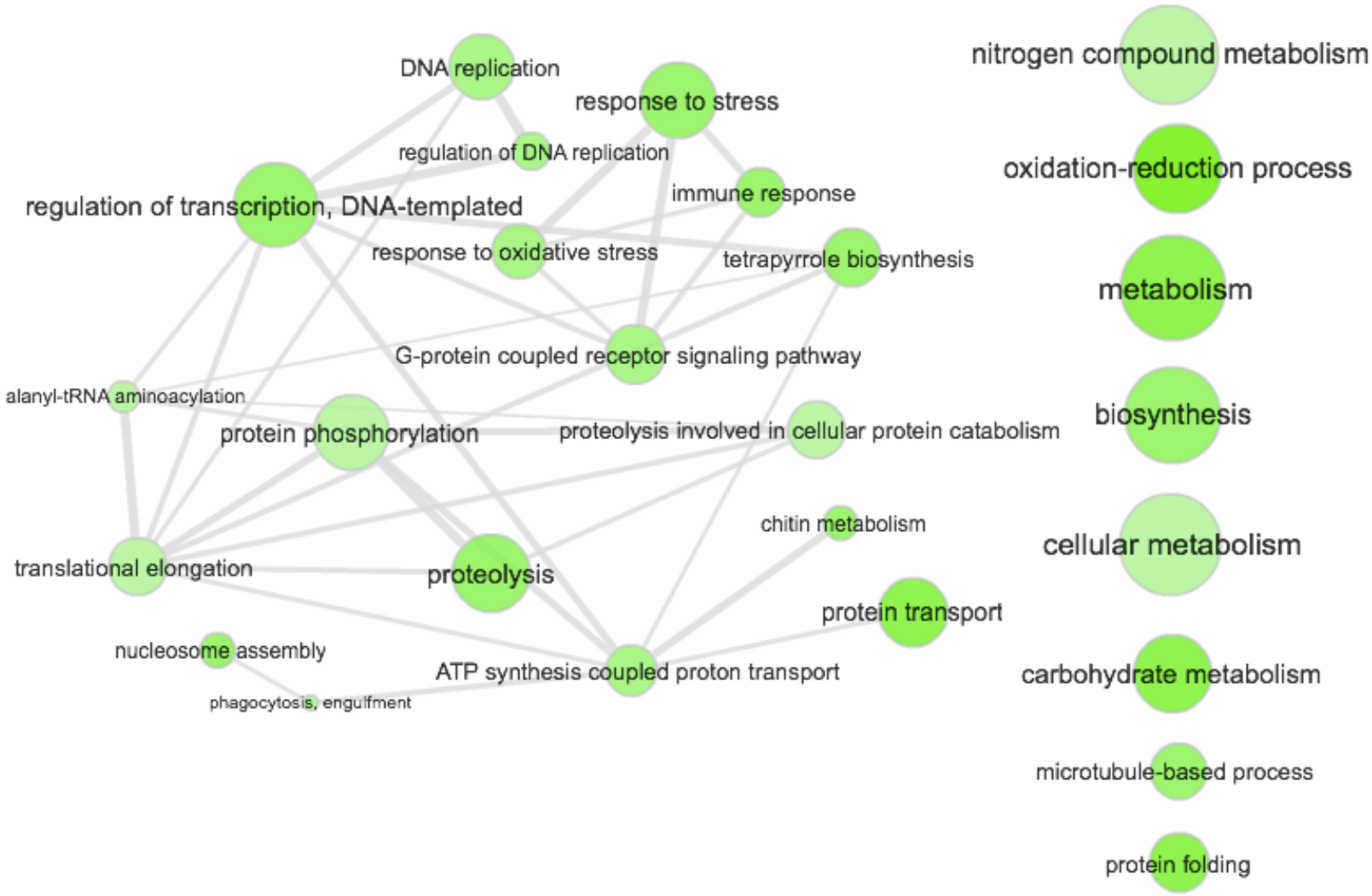


Figure 1 A biological term network of the 26 broad category GO Terms found to be represented in three or more organisms. The size of the nodes corresponds to the number of organisms that share the GO Terms, while edges indicate overarching interactions of the terms.

It occurred to us that the cut off of 3 organisms might have excluded some comparisons of interest. Thus, we then grouped by a number of other interesting categories that were not met by our criteria, particularly the nematode samples because they automatically didn't mean the three organism cut off (Figure 2).

Comparison of the two *B. malayi* datasets reveal that transcriptome responses to *Wolbachia* removal could be sensitive to the type of antibiotic used to clear the infection (Figure 2A). The use of doxycycline results in 97 differentially expressed GO terms (Rao et al., 2012), while tetracycline affects 93 GO terms (Ghedin et al., 2009). The five terms shared by the tetracycline treated nematode samples included the energy-related GO:0005743 and GO:0000166. As previously discussed (see *Metabolic Rate*) It is known that tetracycline treatment can have effects on the mitochondria (Ballard & Melvin, 2007) and other research suggests that mitochondria are not affected by *Wolbachia* (Mouton et al., 2009). To explore this, we then asked whether there any other shared responses to tetracycline treatment across both nematodes and arthropods (Figure 1B). This did not illuminate new insights, as the single shared GO Term (GO:0042302) had previously passed the cutoff of three organisms (see *Chitin*).

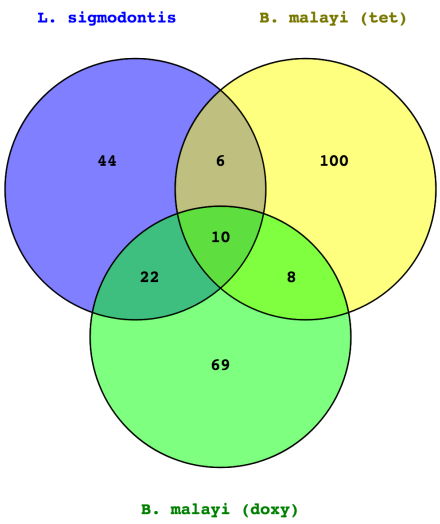
We then compared the shared terms of non-native Diptera samples (Figure 1C). As this included more than 3 organisms, most of the terms had already been investigated. However, previous research had revealed differences between strains of *Wolbachia* (Hughes et al., 2011). The *w*AlbB infected *A. aegypti* and *A. gambiae* shared metabolism-related GO terms which could be specifically reacting to *w*AlbB (GO:0006596, GO:0006270). *w*AlbB has less powerful effects on its host compared to other *Wolbachia* strains, due to its lower density of infection (Walker et al., 2011b, Xi et al., 2005). These qualities have made the *w*Mel and *w*AlbB strains preferred options for biocontrol, despite *w*AlbB in the naturally infected *Ae. albopictus* not blocking DENV effectively (Lu et al., 2012). The 10 GO Terms shared by the *w*Ri infected *D. melanogaster* and *An. gambiae* again reflect the altered metabolism of *Wolbachia*-infected hosts. Unsurprisingly, the examination of data from native arthropods when excluding Diptera (Figure 1D), did not reveal an overlap, likely because these samples are from a wide range of organisms including arachnids and crustaceans.

To examine potential specific effects *Wolbachia* could be having on the reproductive tissue of its hosts, we compared samples derived from ovaries (*A. tabida, A. vulgare*) and testes (*D. melanogaster*). The single shared GO term between the ovary derived samples of *A. tabida* and A. vulgare, GO:0007272, has a role in neuron ensheathment, connecting it to *Lipids.* A single GO term (GO: 0009055) was down-regulated in *D. melanogaster* and *A. tabida* in the absence of a *Wolbachia* infection. The underlying *D. melanogaster* gene (CG12262) is associated with lipids, metabolism and the immune response (Handu et al., 2015). Half the GO terms shared by *D. melanogaster* and *A. vulgare* were upregulated: sulfate assimilation (GO:0000103, CG13473), chitin metabolic process (GO:0006030, CG3348) and hexose metabolic process (GO:0019318, CG4988), recapitulating our earlier discussion of *Lipids* and *Metabolic Rate.* The majority of genes

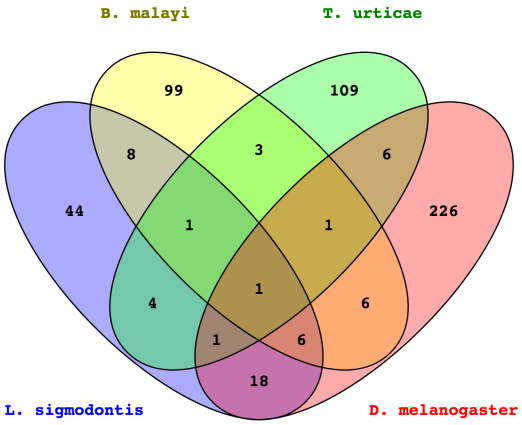
(98%) did not show similar responses across the three tissues. This is unsurprising because the characteristics of arthropods are highly varied, and tissue expression patterns of *Wolbachia* are dependent on what affect *Wolbachia* might be having on its host. The *A. vulgare* dataset consisted of only six differentially expressed genes, and of these, the three downregulated genes could not be explicitly linked with GO terms.

It should be noted that our analysis examines only transcriptomic data and does not consider the poor correlation between mRNA and protein expression levels (Haider and Pal 2013). This can be due to several factors, including mRNA half-lives, post-translation machinery, and the purification/quantification methods (Haider and Pal 2013). Although transcriptomic and proteomic data may not always show a direct match between the gene and its corresponding protein, a broader analysis at the functional level often reveals a shared context. For example, the correspondence of reads and abundance in an RML12 cell line (*Ae. albopictus*) and the *Wolbachia* strain *w*Mel-Pop-CLA is low, but when examined at a functional level, the two show a convincing upregulation of cell wall, membrane and envelope biogenesis (Darby et al. 2014). Thus, we may be relatively sure that our results are identifying true areas of interest yet the level of differential expression may not reflect the whole picture. Additional caveats include the problems of using transcriptomic arrays to assay expression: these are limited by the exclusion of some genes from the array, either due to using an organism unspecific probe (eg. the *L. sigmodontis* study included in our analysis uses a *B. malayi* probe set), or bias of selected gene sets (eg. a probe set that focuses on immune gene expression).

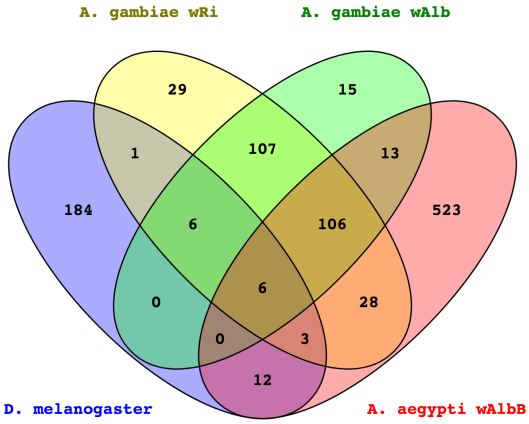
A


L. sigmodontis
B. malayi (tet)
44
6
100
10
22
8
69
B. malayi (doxy)

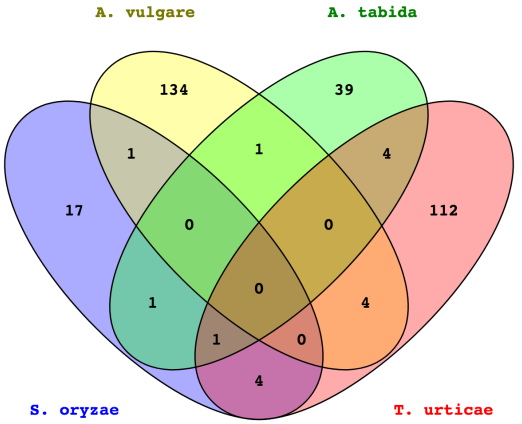
B


B. malayi
T. urticae
99
109
8
3
6
44
1
1
226
4
1
6
1
6
18
L. sigmodontis
D. melanogaster

C


A. gambiae wRi
A. gambiae wAlb
29
15
1
107
13
184
6
106
523
6
0
28
0
3
12
D. melanogaster
A. aegypti wAlbB

D


A. vulgare
A. tabida
134
39
1
1
4
17
0
0
112
0
1
4
1
0
4
S. oryzae
T. urticae

E

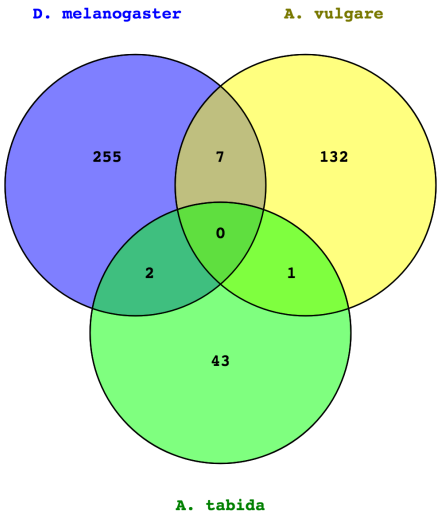

D. melanogaster
A. vulgare
255
7
132
0
2
1
43
A. tabida

Figure 2 Shared GO Terms at the lowest classification level possible. tet indicates tetracycline treated samples, while doxy indicates doxycycline treated samples.
A. GO Terms shared between the three nematode samples.
B. GO Terms shared by native tetracycline treated samples
C. GO terms shared by non-native Diptera samples
D. GO Terms shared by native arthropods, excluding Diptera
E. GO Terms shared by native gametes, where the *D. melanogaster* sample is from testis and *A. tabida* and *A. vulgare* are from ovaries

CONCLUSION

This study has summarized transcriptome data from *Wolbachia*-infected hosts relative to non-infected hosts. We have identified genes and biological functions common to diverse host/*Wolbachia* pairings. This approach, given the depth and diversity of transcriptomic studies available, has produced a set of candidate genes and processes in hosts that are significantly altered by the presence/absence of *Wolbachia,* producing a range of phenotypic effects. These genes and processes may be explored in mosquitoes and filarial nematodes with a view to understanding fitness effects, pathogen blocking and potential drug targets. Our study is limited by the number of specific genes that could be extracted from data sets. It is therefore likely to represent a conservative sample. Additionally, the examination of proteomic datasets in a similar manner would add weight to our findings. Researchers should be encouraged to assay these two functional measurements at the same time, particularly in those interested in disease control. As the number of available transcriptome datasets increases, we expect there will be more scope for identifying key shared genes that may explain *Wolbachia's* effects on hosts, in turn providing further information that could be exploited for medical research.

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Chapter 3

The nature of the immune response in novel *Wolbachia*-host associations

ABSTRACT

Wolbachia is an obligate, intracellular symbiont that is commonly found in insects and causes a diverse array of reproductive manipulations. Though it is normally transmitted vertically, the occasional horizontal host species jump can be seen in the lack of concordance between *Wolbachia* and host phylogenies. In the laboratory, the symbiont can be artificially introduced into novel hosts then selected to produce persistent infections. In the case of the vector of dengue virus, *Aedes aegypti*, the symbiont was successfully introduced with the aim of developing the bacterium for biocontrol. In this insect and others, *Wolbachia* limits co-infection with pathogens including viruses, bacteria and parasites. Here we novelly infected cell lines derived from diverse insect species with *Wolbachia,* in an attempt to determine if there are commonalities in the early host response to the symbiont. We then monitored the expression of genes in the antibacterial TOLL and IMD pathways in the first several passages. We focused on immunity gene expression as it underpins the bulk of the transcriptional response to *Wolbachia* and because it may play a role in the pathogen blocking effect. We found that successful cell infections of *Wolbachia* were difficult to achieve and often required repeated rounds of reinfection. We saw significant variation in the nature of the transcriptional changes across cell lines and no attenuation of gene expression changes in the first several passages. These results suggest that insect species are likely to exhibit distinct responses to *Wolbachia* infection. They also reveal that any evolution of an attenuated transcriptional response, as predicted by long-standing *Wolbachia* x host associations, is not likely to occur rapidly. The findings will have implications for biocontrol programs that rely on the novel infection of naïve hosts.

KEYWORDS

evolution, innate immunity, *Wolbachia*, Dengue, endosymbiont, insects

INTRODUCTION

Wolbachia is a gram-negative, maternally inherited endosymbiotic bacterium that is present in 40-60% of known insect species (Zug and Hammerstein 2012). *Wolbachia* is also known to infect non-insect arthropods including mites (Breeuwer and Jacobs 1996), spiders (Oh et al. 2000), crustaceans (Cordaux et al. 2001; Cordaux et al. 2012) and nematodes (Sironi et al. 1995). Numerous *Wolbachia* strains have undergone extensive host jumps between invertebrates, primarily identified by the lack of strong concordance between host and *Wolbachia* phylogeny (Werren et al. 1995; Zug et al. 2012). The range of possible means for transmission of symbionts between species include, but are not limited to: wounding by parasitoid wasps (Ahmed et al. 2015; Gehrer and Vorburger 2012) and mites (Jaenike et al. 2007), cross-infection from infected to uninfected hosts during copulation (Moran and Dunbar 2006) and cofeeding (Caspi-Fluger et al. 2012), and directly from the environment (Kikuchi et al. 2007). While these transfer events appear common on phylogenetic timescales, they are rare in real time.

While the history of successful host jumps can be seen in the phylogeny, many more cross species infection events must occur that are unsuccessful. Researchers that attempt to create artificially transinfected host species in the laboratory, are fully aware of the difficulty of transitioning *Wolbachia* between species (Bian et al. 2013a; McMeniman et al. 2009; Xi et al. 2005a). Often many thousands of embryos must be infected to obtain a single female that harbors *Wolbachia* in the germline and then transmits the symbiont to her offspring. Frequently these newly infected insects fail to produce offspring. Such transitions work best when the donor and recipient host are closely related. The assumption is that long associated *Wolbachia* and host species have coadapted with time. This notion is supported by improved transinfection rates in insects following periods of co-culture of *Wolbachia* in cells of the target species (McMeniman et al. 2008).

Wolbachia infection in insects has been shown to interfere with the replication of a broad range of co-infecting pathogens including: viruses, filarial nematodes, bacteria and the malaria parasite (Hedges et al. 2008; Kambris et al. 2010; Kambris et al. 2009; Moreira et al. 2009a; Teixeira et al. 2008; Ye et al. 2013). *Wolbachia* was artificially transinfected into the mosquito vector of dengue virus (DENV), *Aedes aegypti* in a bid to develop the symbiont as a form of biocontrol. A range of *Wolbachia* strains have been introduced to date: *w*Mel and *w*MelPop from *Drosophila melanogaster* (McMeniman et al. 2009; Walker et al. 2011a), *w*AlbB from *Aedes albopictus* (Xi et al. 2005b) and a superinfection of *w*Mel/*w*AlbB (Joubert et al. 2016). In the novelly infected *Ae. aegypti, Wolbachia* appears to evoke a more substantial immune response than in the donor hosts as measured by the number of genes with changed expression and the magnitude of that expression change (Pan et al. 2012; Rances et al. 2012; Ye et al. 2013) (Table 1). While this induced immune response may play a part in *Wolbachia*-mediated pathogen blocking (Bian et al. 2010; Pan et al. 2012; Rances et al. 2012) it is not sufficient to explain the effect (Caragata et al. 2013; Moreira et al. 2009a). Given

the limited immune response of native hosts to *Wolbachia*, the hyperactivated response in novel hosts is expected to decline with time, via coevolution (McGraw et al. 2002). As native hosts tend to harbor lower *Wolbachia* densities and contracted tissue distributions as a result of coadaptation (Bian et al. 2013b; Osborne et al. 2012; Pan et al. 2012) this may offer a potential path by which reduced activation of the immune response emerges.

The main insect defense mechanism against pathogens is the innate immune system. There are four major inducible pathways, namely the TOLL and IMD pathways for bacteria (Buchon et al. 2014), and the JAK/STAT and RNAi pathways for viruses (Myllymaki and Ramet 2014; Xu and Cherry 2014). Some of these pathways may participate in the production of antipathogen effects. In addition, some of these pathways, particularly the pathways that target bacteria – TOLL and IMD, may directly effect *Wolbachia* loads in the host. The activity of these pathways is commonly measured by expression change in genes encoding the antimicrobial peptides diptericin, cecropin and defensin. Native *Wolbachia* infections in *Drosophila* have no effect on the transcription of these antimicrobial peptide genes (Bourtzis et al. 2000; Rances et al. 2012; Wong et al. 2011), but a strain of *Wolbachia* introduced from *Drosophila simulans* into *Drosophila melanogaster* lead to increased expression of diptericin expression (Xi et al. 2008a) (Table 1). *Ae. albopictus* and *Ae. aegypti* mosquitoes upregulate expression of these key peptide genes, particularly in response to novel *Wolbachia* infections (Bian et al. 2010; Kambris et al. 2009; Pan et al. 2012; Rances et al. 2012). However, in another *Aedes* species that is naturally *Wolbachia* infected*, Ae. fluviatilis,* TOLL and IMD immune gene transcription is unaffected (Caragata et al. 2017). Evidence thus far therefore suggests that *Wolbachia* infections affect the expression of immune pathways in their native hosts less than in their novel hosts.

While *in vivo* experiments provide valuable information about adaptation, very low transinfection rates of insects render them virtually intractable for study of repeated introductions of *Wolbachia*. Here we have examined the nature of the early host response to *Wolbachia*, by infecting a range of cell lines from diverse species with two *Wolbachia* strains, *w*Mel from supergroup A and *w*AlbB from supergroup B. In the newly infected lines represented by two mosquito species (*Ae. albopictus*, *Anopheles gambiae*), a moth (*Spodoptera frugiperda*) and *D. melanogaster*, we have examined the nature of the immune response immediately following infection and across several passages. We have sought to determine whether there are commonalities in the nature of the early host immune response across these species and if they exhibit rapid evolution.

Table 1 Summary of previous studies' results on innate immune genes in *Wolbachia* infections of *Aedes* and *Drosophila* species. Blank cells indicate that gene expression for that sample was not tested.

	Host organism	***Wolbachia* strain**	**Immune Genes**				**Reference**
			Relish	Defensin	Cecropin	Diptericin	
Aedes species	*Ae. albopictus*	*w*AlbB		Unaffected			Bourtzis et al. (2000)
	Ae. aegypti	*w*AlbB	Upregulated	Upregulated	Upregulated	Upregulated	Bian et al. (2010)
	Ae. aegypti	*w*AlbB	Upregulated	Upregulated	Upregulated	Upregulated	Pan et al. (2012)
	Ae. aegypti	*w*Mel		Upregulated	Upregulated	Upregulated	Rances et al. (2012)
	Ae. aegypti	*w*MelPop	Upregulated	Upregulated	Upregulated		Kambris et al. (2009)
	Ae. aegypti	*w*MelPop		Upregulated	Upregulated	Upregulated	Rances et al. (2012)
	Ae. fluviatilis	*w*Flu	Unaffected	Unaffected	Unaffected	Unaffected	Caragata et al. (2017)
Drosophila species	*D. melanogaster*	*w*MelCS		Unaffected		Unaffected	Wong et al. (2011)
	D. melanogaster	*w*Mel		Unaffected	Unaffected	Unaffected	Rances et al. (2012)
	D. melanogaster	*w*MelPop		Unaffected	Upregulated	Unaffected	Rances et al. (2012)
	D. melanogaster (S2 cell line)	*w*Ri	Upregulated	Unaffected	Unaffected	Upregulated	Xi et al. (2008a)
	D. simulans	*w*Ri		Unaffected	Unaffected	Unaffected	Bourtzis et al. (2000)

MATERIALS & METHODS

Cell culture. The following recipient cell lines are naturally *Wolbachia* free: Aag2 (Peleg 1968), RML12 (Kuno 1983), Mos55 (Pudney et al. 1972), S2 (Schneider 1972) and SF9 (Vaughn et al. 1977). We also used the Ae23T cell line (O'Neill et al. 1997) and the Aeg2*w*Mel.tet cell line (Terradas et al. 2017), both of which had been previously tetracycline treated to remove *Wolbachia* infections. Aag2 cells with *w*Mel (Terradas et al. 2017) or Ae23 cells naturally infected with *w*AlbB (O'Neill et al. 1997) served as donors of *Wolbachia*. The latter cell line was established from eggs of *Ae. albopictus* that were naturally infected with both *w*AlbA and *w*AlbB, but only one of these co-infecting *Wolbachia* strains was present in the resulting cell line (O'Neill et al. 1997). Further details for the cell lines used can be found in Table 2.

While maintaining cell lines, several methods to best encourage *Wolbachia* infection levels were piloted, varying the percentage of FBS used to supplement cells (5%-25%), the length of passaging times (3-14 days), degree of cell detachment, flask size when expanding cell numbers (12 well plate - 75-cm^2 flask) and amount of introduced media at the point of passage. All uninfected cell lines were cultured in 25-cm^2 cell culture flasks at 26°C in 5 ml of growth medium, supplemented with 10% (v/v) of heat-inactivated foetal bovine serum and penicillin/streptomycin (50U⁄50 µg ml^{-1}). Aag2, Ae23 and RML12 cell lines were maintained in medium consisting of equal volumes of Mitsuhashi&Maramorosh and Schneider's insect medium (Sigma, France) (O'Neill et al. 1997). Aa23T cells were maintained in the same medium, with a 20% FBS concentration to improve their replication rate. S2 and Mos55 cells were maintained in Schneider's insect medium, and SF9 cells were cultured in SF9 medium. Cells newly infected with *w*Mel were maintained for only five passages with the *Wolbachia* infection dropping out by passage six. In contrast, cells newly infected with *w*AlbB exhibited greater stability and were maintained for 10 passages without any loss of *Wolbachia*. *Wolbachia* was assessed at each passage by FISH.

Table 3 Characteristics of the cell lines used in this experiment, including *Wolbachia* donor sources.

Cell line abbreviation	Source organism of cell line	Source material	Reference
Aag2 + *w*Mel	*Ae. aegypti* *Wolbachia* strain from *D. melanogaster* (*w*Mel)	Cell line: embryos *Wolbachia* source: ovaries	Terradas et al. (2017)
Ae23 + *w*AlbB	*Ae. albopictus* *Wolbachia* strain from *Ae. albopictus* (*w*AlbB)	Cell line: Embryos *Wolbachia* source: naturally infected	O'Neill et al. (1997)
Aag2	*Ae. aegypti*	Cell line: Embryos Naturally *Wolbachia* free	Peleg (1968)
RML12	*Ae. albopictus*	Cell line: Larvae Naturally *Wolbachia* free	Kuno (1983)
Mos55	*An. gambiae*	Cell line: Larvae Naturally *Wolbachia* free	Pudney et al. (1972)
S2	*D. melanogaster*	Cell line: Embryos Naturally *Wolbachia* free	Schneider (1972)
SF9	*S. frugiperda*	Cell line: Ovaries Naturally *Wolbachia* free	Vaughn et al. (1977)
Ae23T	*Ae. albopictus*	Cell line: embryos Native *Wolbachia* removed by tetracycline	O'Neill et al. (1997)
Aag2*w*Mel.tet	*Ae. aegypti* *Wolbachia* strain from *D. melanogaster*	Cell line: embryos *Wolbachia* source: ovaries *Wolbachia* removed by tetracycline	Terradas et al. (2017)

Infection experiments. Infection of cell lines with *D. melanogaster*-derived *Wolbachia* was carried out using the shell vial technique, as previously described (McMeniman et al. 2008; Terradas et al. 2017; Voronin et al. 2010).

Infection of cell lines with *Wolbachia* sourced from established cell lines was carried out using a blend of the protocols found in Dobson et al. (2002) and Lu et al. (2012). *Wolbachia* was sourced from Aag2 cells hosting *w*Mel or Ae23 cells hosting *w*AlbB then introduced into *Wolbachia* uninfected cell lines (Figure 1). In brief, source *Wolbachia* cells were cultured in 75-cm^2 flasks containing 12 ml of medium until they reached 95% confluency. Cells were collected by shaking flasks vigorously, then centrifuged at 1000 RPM at 4°C before discarding the supernatant and resuspending the cells in 10 ml of SPG buffer (218 mM sucrose, 3.8 mM KH_2PO_4, 7.2 mM MK_2HPO_4, 4.9 mM L-glutamate, pH 7.5) per 12 ml original media (Lu et al. 2012). Two washes were performed by centrifugation at 1000 RPM at 4°C followed by resuspension in 10 ml of SPG buffer per 50 ml of original cell culture. Cells were sonicated on ice for 2 bursts of 10 seconds (18-23% power), and then cell debris was pelleted at 1000 RPM at 4°C. The resultant supernatant was filtered successively through a 5 µm filter and 2.7 µm filter and *Wolbachia* cells were resuspended in SPG buffer at a ratio of 100 µl per 50 ml original source media, then 50 µl of this was immediately overlaid on an 80% confluent monolayer (2 ml media) of each of the cell lines of interest in a single well of a 12-well plate (Corning Life Sciences). The cells were transferred into a 25-cm^2 flask with 3 ml of new medium 3 days later when cells approached 95% confluency. When cells started detaching from the flask, 2 ml was spun down at 400 RPM at 4°C and resuspended in 200 µl of TRIzol® (Life Technologies, Carlsbad, California, USA). The remaining cell-containing medium (~2-3 ml) was transferred into 75-cm^2 flasks with 10 ml of fresh medium.

Fluorescence in situ hybridisation (FISH). FISH was carried out as per (Moreira et al. 2009a) using the *Wolbachia* probes [50-ACCAGATAGACGCCTTCGGCC-30] and [50-CTTCTGTGAGTACCGTCATTATC-30] (Xi et al. 2005a). For negative controls of FISH, pre-infection cell lines were used.

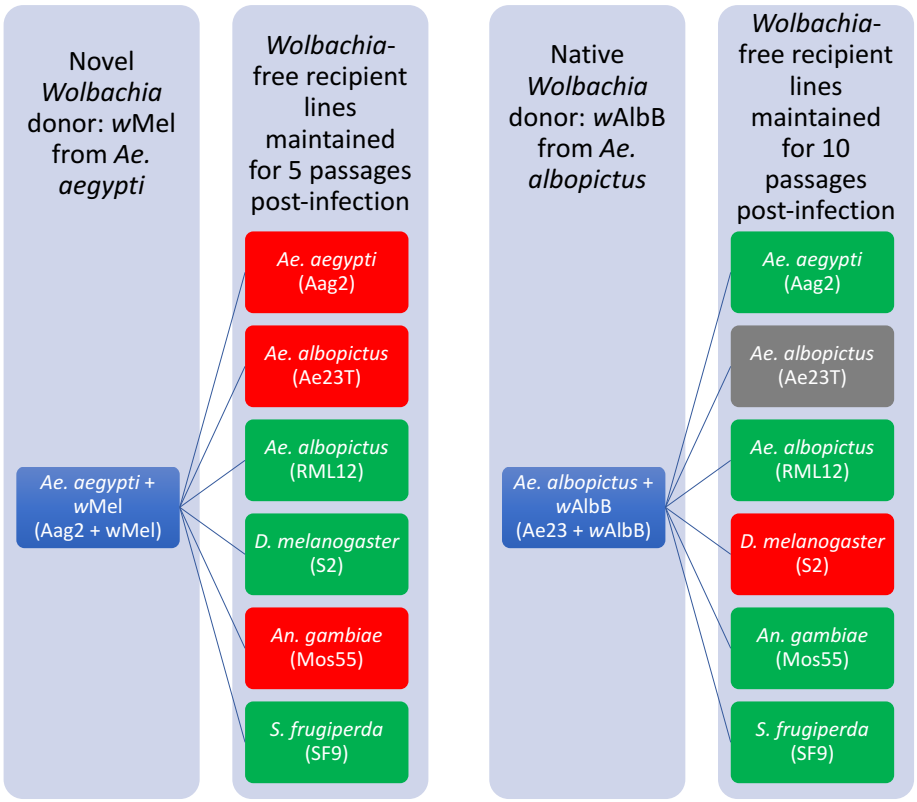

Figure 1 Flow chart illustrating the two *Wolbachia* source cell lines (blue), the novel cell line infections established and the number of passages for which the novel cells lines were maintained. Colours of novel cells indicate successful infection (green), unsuccessful infection (red) and not attempted (dark grey).

Quantitative PCR. The TRIzol® method from Invitrogen (Life Technologies, Carlsbad, California, USA) was used to extract total RNA from cell samples. RNA quality and quantity was checked with a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Inc.). Synthesis of cDNA was performed with Superscript II Reverse Transcriptase (Invitrogen) as per the manufacturer's instructions. The genes assayed were: attacin, cactus, cecropin, defensin, dFADD, dorsal, IMD, MyD88, relish and TAB2 (Table 3). Amplification was carried out using the LightCycler LC480 system (Roche, Meylan, France) with a Platinum SYBR Green qPCR superMix (Invitrogen). qRT-PCR reactions were conducted using a 2 minute step at 50°C, 2 minute step at 95°C and 40 cycles of 15 seconds at 95°C and 30 seconds at 56°C. A fluorescence measurement was made at the end of each cycle. A melting curve analysis was performed at the end of the amplification program to examine for primer-dimers or nonspecific amplification. Duplicate qRT-PCR reactions were performed for each sample. Relative expression of genes was calibrated against the reference gene using the $\Delta\Delta C_T$ calculation method (Livak and Schmittgen 2001).

Statistical Analysis. Multiple infection experiments were carried out (Table 4). In most cases only a subset of the recipients became infected. The successful experiment for each donor line was defined by achieving successful infections in parallel in all of the recipients. Statistical analysis was carried out on the final successful study in each of its parts, on the single sample available. To determine whether there was an association between gene expression and cell line in this experiment we performed a general linear model on log transformed expression data. Posthoc comparisons were then carried out and multiple corrections accounted for using a Bonferroni correction (Bonferroni 1935). Pearson chi-squared test was used to assess whether there was a significant difference in the direction (suppression or increase) of gene expression by insect compared to the original uninfected cell line, with a minimum of 1.5 fold change. We then tested for the effect of passage number on expression for all insects and genes, individually and as a group (ANOVA). All analyses were carried out in SPSS (IMB Statistics for Windows, Version 20.0 Armonk, NY).

Table 3 qPCR primers used to amplify innate immune genes. * indicate that the gene was a housekeeping gene used to account for differing cell numbers in samples.

	Gene target	PRIMER	SEQUENCE
Ae. aegypti	Rps17*	forward	TCCGTGGTATCTCCATCAAGCT
		reverse	CACTTCCGGCACGTAGTTGTC
	Relish	forward	CATATTGCCGTAGAGAGCGA
		reverse	GTGTAACCAGCATTGTTCGG
	Cactus	forward	TCGGACAATCTGTTCAGCTC
		reverse	GTTGTTTCTGGCTACCGGAT
	Attacin	forward	TTGGCAGGCACGGAATGTCTTG
		reverse	TGTTGTCGGGACCGGGAAGTG
	Defensin-B	forward	GGCCATTACTAGTGCCTACCC
		reverse	GTTTCAGGCGGAAGTTCTCC
	Dredd	forward	GTGGCTGTTATGCGAGAAGA
		reverse	AGCGTAGTTCTGCCTGAGGT
	MYD88	forward	GCTGATTTCTGAGCGGTGCC
		reverse	GGCATCTTCCAGCTTGTCCC
	IMD	forward	GTCGGACACAATTCGGCAGA
		reverse	CCATTTTGCCGAGCGTTGGT
	Cecropin A	forward	GCTTTAGCCCCAGCTACAAC
		reverse	TCACAAAGTTATTTCTCCTGATCG
Ae. albopictus	β-actin*	forward	CCTGGGTATGGAAGCCTGCGGTATC
		reverse	GGCAATGATCTTGATCTTCATGGTGGATGG
	Relish	forward	CGGTCGAAGTTCTGATGCAG
		reverse	CATCGGTGGTATCATGGGGA
	Cactus	forward	CCTCTGCTTCTCGACCTCAA
		reverse	ACGGGTTGGGATTGGATGAT
	Attacin	forward	CGATCATCAGCTGTGCAACA
		reverse	ATGCTGGGTTCGGATTGAGT
	Defensin-A	forward	CGTCCCTACTGTGATTTGTTTCC
		reverse	AAGTTCTCCACGGCAGCTT
	Defensin-C	forward	GACGAACTGCCCGAGGAAA
		reverse	CAAGCACTATCCCCAACACC
	IMD	forward	ATCCCGGCATCTGGGTTACG
		reverse	ACCTAGCGTTGGTTCGTCGT
	MYD88	forward	ACGAAAGATGACACACGGCAC
		reverse	ACGTAACACCAGCCAGCAGA
	dFADD	forward	GATCCGAGACCACCCAGCAG
		reverse	TCCCTTTCGGCCGATTCCAA
	TAB2	forward	TCCCAAGAAGCCGCCAGAAA
		reverse	AGGTCCGACACGACATGGTC
	Cecropin	forward	CGCAATCGTTCTACTAGCCGC
		reverse	CCGGAGAAAACCACATCGCG
	Dorsal	forward	ACACCCCAGAACACATGCCA

		reverse	TGGGGTTGTGTTGCCGTAGT
D. melanogaster	Actin*	forward	GCGTCGGTCAATTCAATCTT
		reverse	AAGCTGCAACCTCTTCGTCA
	Relish	forward	TCCTTAATGGAGTGCCAACC
		reverse	TGCCATGTGGAGTG ATTAT
	Cactus	forward	CTCACTAGCCACTAGCGGTAA
		reverse	CCCGAATCACTGGTTTCGTTT
	Attacin	forward	GGCCCATGCCAATTTATTCA
		reverse	CATTGCGCTGGAACTCGAA
	MYD88	forward	TACCCACCGCCATCCCAATC
		reverse	TCGGATCGCAGCACCTTCTT
	IMD	forward	CCGGGCACTCTCAGTAACGT
		reverse	CGACTCCTCTGCGGTACTGG
	dFADD	forward	GAGATTGGTTCGCGACGCAG
		reverse	TCGGCCAATCGGAGTTCCTG
	TAB2	forward	TCCCAACCCCAGTCCAAACC
		reverse	CGGGTATCGTGGGGAACTCC
	Dorsal	forward	ATCCGTGTGGATCCGTTTAA
		reverse	ATCCGTGTGGATCCGTTTAA
	Defensin	forward	GCCAGAAGCGAGCCACAT
		reverse	CGGTGTGGTTCCAGTTCCA
	Cecropin-A	forward	TCTTCGTTTTCGTCGCTCTC
		reverse	CTTGTTGAGCGATTCCCAGT
S. frugiperda	EDC*	forward	GCCGTATCCAAAGCATGACA
		reverse	TGGTGACGGCCAAAGGAA
	Relish	forward	TTCTCCGCACAGTTCTCCAT
		reverse	CCCACCATCTCGCTCACATA
	Cactus	forward	GTCGTTGGCCAAGTCCAAAA
		reverse	AGCAGAAGAACATCCCACCA
	Attacin	forward	GTGTTCAGTGCCATTGGAGG
		reverse	TGTGGGATGGTAGGCATGTT
	Defensin	forward	CCGCTTCTGTTGACCTTTCA
		reverse	CTCTGCTCCTCCACAACAGA
	IMD	forward	AGCAAGGACAGCAGCAAGGA
		reverse	TGGTGGCTTAGGACTGTGAGC
	MYD88	forward	CATTCATGCCTCGGGTTGCC
		reverse	AGGCCCAACTAGCAACACTCA
	TAB2	forward	GCCTCCCTAACGCATGCCTA
		reverse	CGCTTTCTGAGGGCCATCAC
	Dorsal	forward	CAATGACGCGGGCACTTACG
		reverse	GCAGCTTTACGGACGCGTTT
	dFADD	forward	CAAACGAAAGGCACAGCGAA
		reverse	AGAGTTCGGCAGTTTCATAGCA
	Cecropin-A	forward	AGCAGCTTCATCATCGTTATCA
		reverse	CTTTGCTCAACAGTGGGCA

Table 4 Summary of novel cell lines infected with *Wolbachia* in this experiment. Blank cells indicate that *Wolbachia* infections for that cell line could not be established.

Cell line	Number of attempts at *w*Mel infection	Number of successful *w*Mel infections established	Number of attempts at *w*AlbB infection	Number of successful *w*AlbB infections established
Aag2	7		5	1
Ae23T	7	1		
RML12	7		5	1
S2	7	1	3	
Mos55	7		5	1
SF9	7	1	5	1

RESULTS

Cell Culture**.** To optimize conditions for *Wolbachia*-infected cells, several cell passaging procedures were trialled. We found that using 20% FBS promoted *Wolbachia* growth, as did extending the length of passaging times to 14 days and allowing at least 50% of cells to detach prior to passaging. Upon each passage, 60% of the new flask's volume was composed of the previous flask's detached cell-containing media and the remainder was fresh media. This encouraged healthy cell growth in all cell lines, and prevented the early loss of cell lines in the first passage after *Wolbachia* infection.

Establishing persistent *Wolbachia* infections. We aimed to infect novel cell lines with *w*Mel (*D. melanogaster* origin) as it is the current strain of *Wolbachia* being released into the field as a biocontrol agent. Using *D. melanogaster* ovaries, we attempted to infect all the *Wolbachia*-negative cell lines at our disposal five times in parallel. However, in every case the cultured cells experienced bacterial contamination from the raw ovary preparation. Instead, by using a Aag2 cell line previously infected with *w*Mel (Terradas et al. 2017) it was possible to produce a single *w*Mel infected line (n = 1) at ~85% infection frequency (Figure 2) for each of Ae23T, S2 and SF9 but not Aag2, RML12 or Moss55. This level of success required seven independent attempts (Table 3) and by passage 4 the *Wolbachia* had largely disappeared from the lines. In contrast, more of the recipient cell lines were successfully infected with the *w*AlbB strain. It took five independent infection attempts to achieve persistent high density *w*AlbB infections in Aag2, RML12, Mos55 and SF9 cell lines (Figure 3) but not S2 or Ae23Tcells (Table 3). The successful *w*AlbB infections were maintained for > 10 cell passages, and the cell infection frequency approached 90% in some cell lines. These findings confirm the role of the *Wolbachia* genome, not just the host genome in dictating the success of partnerships.

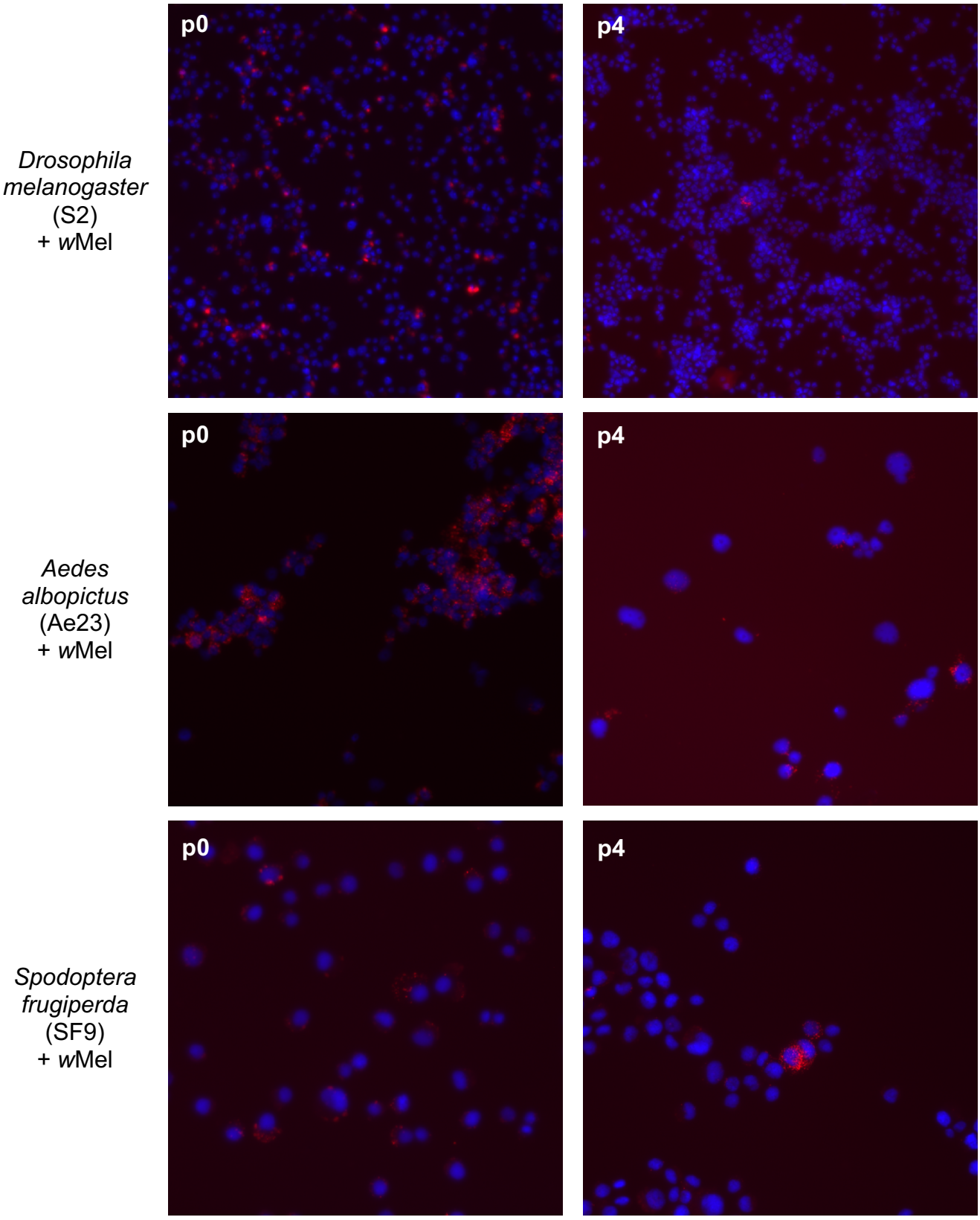

Figure 2 FISH for *w*Mel at x100 magnification in three cell lines at two time points: passages 0 and 4 post-infection. Host nuclei stained in blue (DAPI) and *Wolbachia* 16S rRNA stained in red (specific probe labelled with rhodamine). The number of cells imaged reflects the number of cells fixed onto the FISH slide. Infections wane by passage 4.

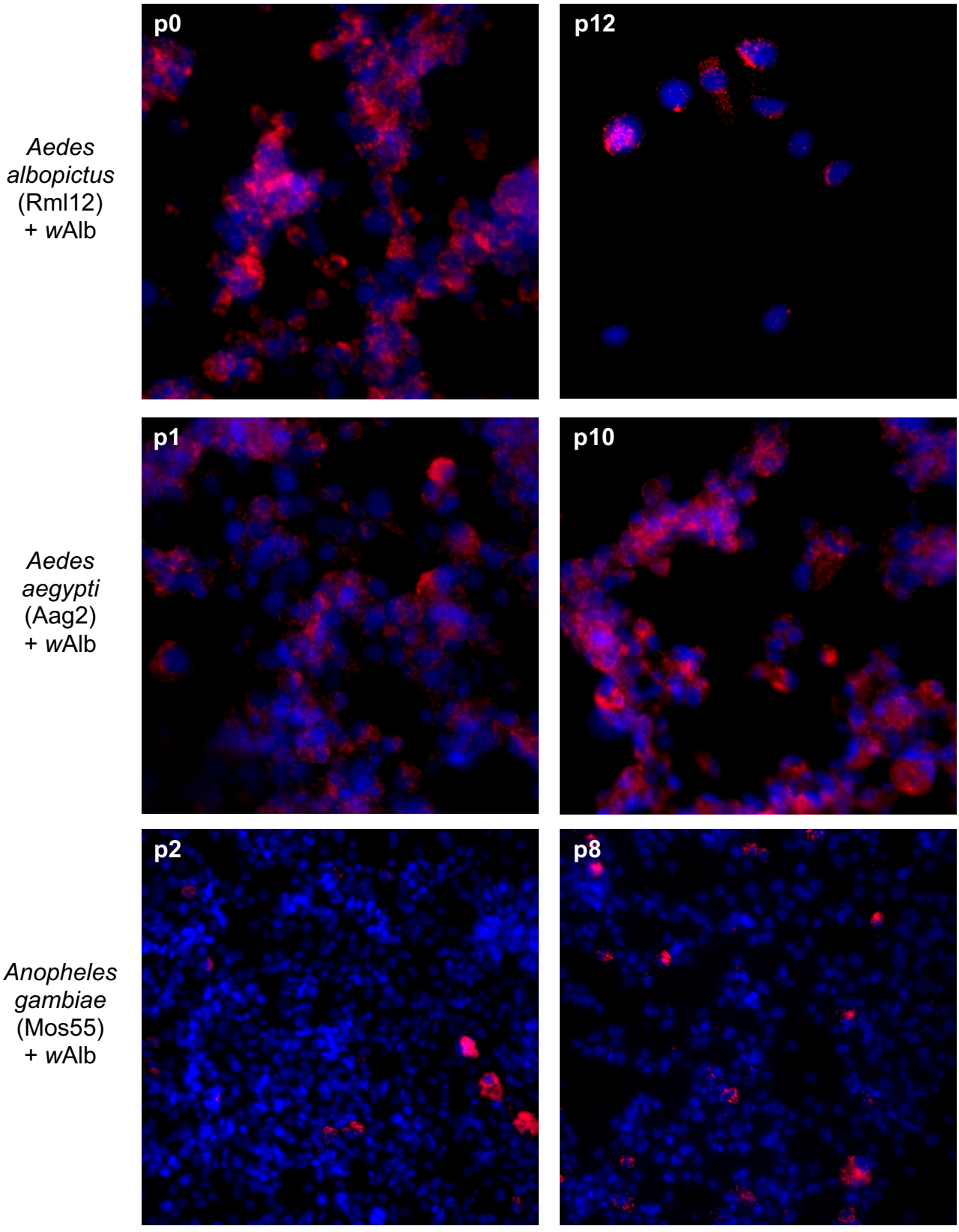

Figure 3 FISH for *w*AlbB at x200 magnification in three cell lines at early and late time points following infection. Host nuclei stained in blue (DAPI) and *Wolbachia* 16S rRNA stained in red (specific probe labelled with rhodamine). Infections remain stable over time.

Innate Immune Gene Expression. Eight different immunity genes were tested for changes in expression through time in *D. melanogaster*, *S. frugiperda* and *Ae. albopictus* cells following *Wolbachia* introduction (comparative to the uninfected cell line). A single sample was examined in each case. First, we examined the magnitude of expression change across cell lines, grouping all passage results together. We observed a significant effect ($F = 22.489$, $df = 2$, $p<0.001$) of cell line, marked by suppression of immune gene expression in *D. melanogaster* cells and increased expression of genes in *S. frugiperda* and *Ae. albopictus* ($X^2 = 37.363$, $df = 2$, $p<0.001$). We then compared each pair of insects. We detected significant differences between the fly and each of the other two insect samples, with the fly suppressing immune genes while mosquito and moth samples increased gene expression (fly:mosquito $X^2 = 23.587$, $df = 1$, $p<0.001$, fly:moth $X^2 = 28.390$, $df = 1$, $p<0.001$). No significant difference was observed between the moth and mosquito samples ($X^2 = 0.313$, $df = 1$, $p>0.05$). This may be because both the moth and mosquito are naturally *Wolbachia*-uninfected, while the fly is the original source of the *Wolbachia* strain.

These results were recapitulated when examining the fold change in expression for each species individually. *D. melanogaster* cells infected with *w*Mel suppressed expression of TOLL and IMD pathway genes in addition to 2 out of 3 other antimicrobial genes tested (Figure 4). In contrast, the *Ae. albopictus* cell line showed an overall trend of innate immune activation following *w*Mel infection (4 promoted and 2 suppressed). Relish was not expressed in *Wolbachia*-free cells. Finally, in *S. frugiperda* cells, the majority of genes were unaffected, with only Cactus, Dorsal and Attacin showing greater than 1.5 fold changes in expression levels compared to the original *Wolbachia*-free line. This range of responses suggests that there is no one gene or pathway defines the novel host response.

We then compared the direction of regulatory change of genes by cell line. Overall there was a significant effect of cell line ($X^2 = 37.363$, $df = 2$, $p<0.001$). Post hoc comparisons revealed that fly vs mosquito cell lines ($X^2 = 23.587$, $df = 1$, $p<0.001$) and fly vs moth cell lines ($X^2 = 28.390$, $df = 1$, $p<0.001$) differed from one another. The fly sample suppressed the majority of immune genes tested, while the mosquito and moth samples increased gene expression. There was no difference in expression between the mosquito and moth cell lines ($X^2 = 0.313$, $df = 1$, $p>0.05$).

We then tested for the effect of passage number on expression and found that there was no significant difference in magnitude of expression by cell line (fly $F = 0.613$, $df = 4$, $p>0.05$, mosquito $F = 0.475$, $df = 3$, $p>0.05$, moth $F = 2.005$, $df = 4$, $p>0.05$) or individual genes (attacin $F = 0.057$, $df = 4$, $p>0.05$, cactus $F = 0.273$, $df = 4$, $p>0.05$, cecropin $F = 0.379$, $df = 4$, $p>0.05$, defensin $F = 0.312$, $df = 4$, $p>0.05$, dFADD $F = 0.151$, $df = 4$, $p>0.05$, dorsal $F = 0.254$, $df = 4$, $p>0.05$, IMD $F = 0.828$, $df = 4$, $p>0.05$, MyD88 $F = 0.066$, $df = 4$, $p>0.05$, relish $F = 0.384$, $df = 4$, $p>0.05$, TAB2 $F = 0.160$, $df = 4$, $p>0.05$). Given the drop in *Wolbachia* infection levels (Figure 2) we had predicted we would see corresponding declines in

immune activation (Figure 4).

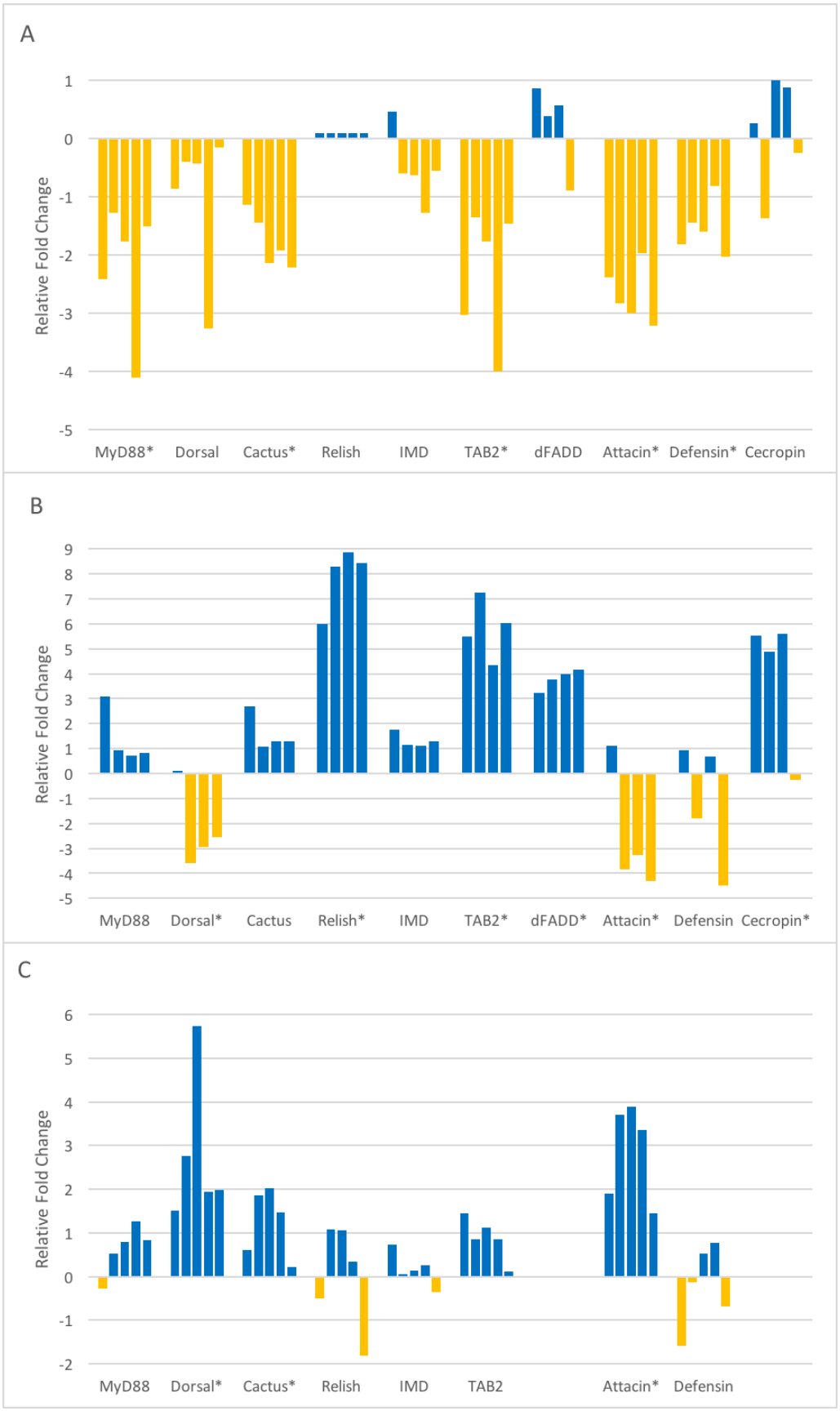

Figure 4 Fold change of TOLL, IMD and antimicrobial peptide genes in *Wolbachia* (*w*Mel) infected samples, relative to uninfected cell line. A: *Drosophila melanogaster* (S2). B: *Aedes albopictus* (Ae23). C: *Spodoptera frugiperda* (SF9). For each gene, the bars represent passages 0-4. *denotes >1.5 fold change in the majority of passages. Colors denote upregulation (blue) and down (yellow).

DISCUSSION

A specific analysis of potential immune genes important in the *Wolbachia*-host interaction has been conducted in vitro using insect cell lines of mosquito, fly and moth origin. We assessed the transcription of 2 major immunity gene pathways (Toll, IMD), and found that in *Drosophila* these pathways were suppressed, while the opposite was true in the mosquito and moth samples. We also examined whether this response changed over several cell passages, but saw no significant differences.

Establishing persistent *Wolbachia* infections. To replicate the biological conditions when establishing *Wolbachia* infections for biocontrol (McMeniman et al. 2009; Walker et al. 2011a), we attempted to use *D. melanogaster* ovaries to infect the *Wolbachia*-negative cell lines. Although we could establish a *w*Mel infection in naïve Aag2 cells, this infection was at very low levels (less than 10% of cells). We were unable to establish additional cell lines for comparative studies. Instead, we used a previously developed cell line of Aag2 (Terradas et al. 2017) as a source of *w*Mel as the *Wolbachia* was cell line adapted. In all *w*Mel infected cell lines *Wolbachia* infection was lost by five passages post-infection. Previous studies have also found that *w*Mel does not persist in S2 and Ae23 cell lines (Voronin et al. 2010; Xi et al. 2008a). The earlier loss of *w*Mel in our case may be explained by our method, since we infected all cells once in parallel instead of multiple sequential times. In the *Ae. albopictus* cell line, loss may also have been assisted by the upregulated innate immune response (Blagrove et al. 2012; Xi et al. 2008a). In *Ae. aegypti* mosquitoes, *w*Mel exhibits higher symbiont loads, broader tissue distributions and a greater fitness cost than in its native fly host (Ross et al. 2014; Turley et al. 2013; Voronin et al. 2010; Walker et al. 2011b). These greater fitness costs of *w*Mel likely explain our inability to establish *w*Mel infections in other cell lines as well, particularly the naïve Aag2 line. Cell-line adaptation of *Wolbachia* in mosquito cells caused it to lose its ability to infect its original host *D. melanogaster* (McMeniman et al. 2008). The Aag2 + *w*Mel line was separately established more than 5 years ago. We suggest that *w*Mel from Aag2 is cell-line adapted but that there has been a lack of selection to maintain diversity in the genome of *w*Mel *Wolbachia* which prevents it successfully re-establishing itself in a new host.

The high density *w*AlbB infections we created were stable over 10 subsequent passages. However, the loss of *w*AlbB infection in our donor cell line prevented us from assaying the changes in immune gene reactions in the earliest passages following infection. Interestingly, *w*AlbB exhibits low levels of infection in its native mosquito host (Walker et al. 2011b; Xi et al. 2005b) and following transinfection into the novel mosquito host, *Ae. aegypti* (Axford et al. 2016; Xi et al. 2005b). The tetracycline treated *Ae. albopictus* cell line (Ae23T) has previously been re-infected with a persistent *w*AlbB infection and with a wide range of other *Wolbachia* strains (Dobson et al. 2002), suggesting that native hosts are more

readily receptive to new *Wolbachia* infections than *Wolbachia*-naïve hosts. Likewise, novel *Wolbachia* infections using *w*Ri from *Drosophila simulans* were previously established in Ae23T, S2, C6/36 and SF9 cell lines using the shell vial technique (Dobson et al. 2002). The infection levels varied based on cell type (10% of the S2 cells and >90% of the SF9 cells) but were always less than the 100% infection rate found in the original host. In whole insects, *w*Ri transferred by microinjection from *D. simulans* to *D. melanogaster* also occurred at lower densities in the new host than in the native (Boyle et al. 1993). These studies suggest that the behavior of individual *Wolbachia*-host pairings may be affected by their history/lack of history of adaptation as well as by *Wolbachia*-strain specific characteristics.

The differences seen in infection success in the cell lines for *w*AlbB and *w*Mel are mirrored in *Ae. aegypti*, where recently a double infection of the two strains was created (Joubert et al. 2016). The former strain when alone has a reduced tissue density compared to *w*Mel, causes fewer fitness consequences and induces weaker pathogen blocking (Axford et al. 2016; Lu et al. 2012; Pan et al. 2012; Walker et al. 2011b; Xi et al. 2005a; Xi et al. 2005b). In the double infection, the two *Wolbachia* strains co-localise. The *w*AlbB strain exhibits a similar density to when it is alone in *Ae. aegypti*, while *w*Mel exhibits a higher density. The behaviour of *w*AlbB reflects that of its native host and a history of coadaptation in a closely related mosquito. The *w*Mel strain, in contrast, with a history of adaptation in both species and potentially greater selection for stronger growth during passaging in mosquito cells, responds differently to coinfection.

Innate Immune Gene Expression. Relish is critical in IMD signaling in response to challenge with Gram-negative bacteria in *Drosophila* (Dushay et al. 1996). Interestingly, we found that *w*Mel suppressed expression of TOLL and IMD pathway effectors but did not express Relish either basally or after *Wolbachia* infection. Thus we hypothesize that *Wolbachia* does not activate the immune response of *Drosophila* in a typical IMD signalling manner. Novel *w*Ri infected *Drosophila* cell lines have multiple genes in the TOLL and IMD immune signalling pathways that respond with higher expression (Xi et al. 2008a). In contrast, native host fly samples infected with *w*Mel, *w*MelCS or *w*MelPop-CLA in *D. melanogaster* and *w*Ri in *D. simulans* show no changes in antimicrobial expression (Bourtzis et al. 2000; Rances et al. 2012; Wong et al. 2011). The difference between the results of Bourtzis et al. (2000); Rances et al. (2012) and Xi et al. (2008a) are likely due to native vs non-native strains of *Wolbachia* used for the respective infections. We suggest that our finding, that *w*Mel affects gene expression in its native *D. melanogaster,* could be due to the long history of *w*Mel adaptation in *Ae. aegypti* cells. Alternatively, our results may differ as we captured the time-period directly following infection, while the previous study on S2 cells (Xi et al. 2008a) took snapshots of expression after multiple shell vial infection procedures.

Defensin is the main antibacterial peptide produced in *Ae. aegypti* in response to both Gram-positive and Gram-negative bacteria (Lowenberger et al. 1995). Novel infections of *w*AlbB, *w*Mel and *w*MelPop-CLA in *Aedes* mosquitoes induce

transcription of defensin, cecropin and diptericin (Bian et al. 2010; Kambris et al. 2009; Pan et al. 2012; Rances et al. 2012), possibly due to the upregulation of Relish and Cactus (Bian et al. 2010; Kambris et al. 2009). However the two mosquito native symbioses, *Ae. albopictus* and the closely related species *Ae. fluviatilis,* do not show changes in TOLL and IMD pathways dependent on *Wolbachia* presence/absence (Bourtzis et al. 2000; Caragata et al. 2017). Ae23T can show strong induction of immune gene transcription and effectively clear a bacterial infection, so this lack of response is clearly not due to impaired immune responses (Pinto et al. 2012). Our Ae23 cell line showed an overall trend of innate immune activation following *Wolbachia* infection, but likewise did not display significant upregulation of defensin. This difference between the three *Aedes* mosquitoes in their responses to *Wolbachia* warrants further investigation, particularly into the potential interactions of native mosquito endosymbionts (*w*AlbA/*w*AlbB, *w*Flu) on uninfected *Ae. aegypti* mosquitoes.

Despite our cell lines being infected with the same *Wolbachia* strain, there was no single gene that was differentially expressed in the same direction in each insect line. This may have been due to the lack of biological replicates, and the authors suggest further studies should be undertaken for specific host-*Wolbachia* interactions of interest. Results for the moth cell line were comparable to *Wolbachia* infected ovaries of the parasitoid wasp *Asobara tabida,* both displaying higher levels of cactus and dorsal in infected vs uninfected (Kremer et al. 2012). In this same wasp, defensin is downregulated in ovaries infected with *Wolbachia* but our result was not significant. This difference is likely because *A. tabida* is dependent on the symbiont for oogenesis, where the reproductive system has had to respond to the parasitic nature of *Wolbachia* (Dedeine et al. 2005; Pannebakker et al. 2007). We therefore conclude that despite similarities in some areas of gene expression, there is no stereotypical response to *Wolbachia* infection across the cell lines. Tissue history of origin and species associated differences are probably contributing to this variation. This research cautions that findings from specific cell lines may not be broadly generalizable to whole insects.

CONCLUSION

The results presented here demonstrate that there are no fundamental avenues of immune responses by novel insect hosts to *Wolbachia* infection in cell lines and that the responses show very little evolutionary change in the first few passages. Our findings do suggest, however, that native hosts have suppressed TOLL and IMD activation compared to novel hosts. Future research may wish to focus on broader aspects of the host response beyond immunity or the response of the whole insect to novel infection. To implement a number of biocontrol strategies in vectors and agricultural pests, *Wolbachia* has and will need to be transinfected into uninfected hosts. Our work suggests that particular *Wolbachia* strains may be better suited to establishment given their native densities and that the nature of the host immune response to those particular strains is unpredictable and slow to evolve in initial culture.

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Chapter 4

Geographical distribution influences purifying selection on innate immunity genes in *Drosophila*

ABSTRACT

In general, species diversity, including that of pathogens, is negatively correlated with latitude (Bonds et al. 2012). We sought to determine whether flies restricted to the tropics versus those with more widespread distributions experiencing heightened selection in their antipathogen genes given their exposure to greater pathogen diversity. We tested for selection in 18 *Drosophila* species that varied by geographic restriction: tropical, temperate/tropical and cosmopolitan distributions. As saturation rapidly limits the ability to make genomic comparisons between distantly related taxa, we instead examined patterns of purifying selection on more conserved gene regions. We tested genes involved with generalised responses such as autophagy and melanisation as well as genes with more narrowly defined roles in antiviral and antibacterial pathways. Genes underpinning generalised responses did not experience differential purifying selection based on geography. We found that genes associated with the antibacterial TOLL pathway experienced greater purifying selection in flies with cosmopolitan distributions. This was contrary to our predictions, but as TOLL has dual roles in development and pathogen defense, the constraint may be driven instead by its primary functional role (development). In keeping with predictions, we found that antiviral response genes experienced greater constraint in species from the tropics. This pattern of selective constraint might be explained by greater viral diversity or by higher viral loads related to increased ambient temperatures in the tropics. It could also be a false signal derived from other factors that influence molecular evolution rates. Our work suggests that viruses may be playing an enhanced role in shaping the evolution of the immune system of insects restricted to the tropics compared to those with other distributions.

INTRODUCTION

Biodiversity is dramatically changing due to human population growth and associated land use around the world (Cardinale et al. 2012). Modelling has predicted that, as the biodiversity of free-living organisms decreases, pathogen burden becomes higher and thus human disease risk is increased (Bonds et al. 2012). This is particularly noticable in the tropical latitudes where the greatest of biodiversity in free-living organisms is found, including vectors of infectious and parasitic diseases (Bonds et al. 2012). Arthropod vectors are responsible for some of the greatest infectious disease burden in humans (Hill et al. 2005). These diseases include a range of parasitic infections, most notably malaria, but also viral diseases the disease burden of which is rapidly growing (World Health Organization 2015). Many viral families can infect insects (Huszar and Imler 2008; Possee and King 2001), including at least 12 viruses that also infect humans (Hughes et al. 2012; Weaver and Reisen 2010).

Disease vectors are not simply passive agents in the process of transmission; their immune response determines the outcome of the infection. To infect humans, viruses, just like parasites, must be able to infect a range of arthropod tissues and progress to the point where the pathogen loads are sufficiently high and in the right tissues (eg. salivary glands) to allow transmission (Hardy et al. 1983). While viral infections do not cause substantial outward signs of infection in vectors, there is evidence that vectors can suffer fitness costs in terms of longevity or reproduction. This has been documented for Dengue (Sylvestre et al. 2013), West Nile (Ciota et al. 2011) and Chikungunya (Reiskind et al. 2010) viruses. It is also clear that vectors mount active immune responses to these infections, most commonly detected via transcriptional response studies (Chotkowski et al. 2008; Paradkar et al. 2012).

Insects have an innate immune system that can recognise conserved pathogen motifs (Sackton et al. 2007). The two major immune responses to pathogens can be classified as humoral and cellular (Lemaitre and Hoffmann 2007). The humoral response has four major inducible pathways for dealing with pathogens, namely the TOLL and IMD pathways for bacteria (Buchon et al. 2014), and the JAK/STAT and RNAi pathways for viruses (Myllymaki and Ramet 2014; Xu and Cherry 2014). The designation of classes of pathogens targeted by these pathways is known to be overly simplistic, however, with evidence growing of cross-talk between anti bacterial and anti viral pathways (Agaisse and Perrimon 2004; Xi et al. 2008b) and variation in the immune response even within pathways in response to diverse pathogens (Dionne and Schneider 2008). Cellular immunity includes the pathways of autophagy, known to respond to bacteria, viruses and parasites (Moy and Cherry 2013) and melanisation, which responds to parasites (Tang 2009), fungi and bacteria (Binggeli et al. 2014).

Genes associated with the immune response commonly exhibit adaptive evolution (Schlenke and Begun 2003). We were

interested in testing whether there are distinct patterns of selection observable in the immune genes of insect species with varying levels of exposure to pathogens. Our prediction was that insect species that reside in the tropics would be exposed to a wider range of pathogens than those restricted to other geographic regions and hence experience differential patterns of selection. *Drosophila* has high rates of exposure to pathogens (Markow and O'Grady 2007) and has many complete genomes available (Drosophila 12 Genomes, 2007), making it ideal for studies of adaptive evolution in the immune system (Jiggins and Hurst 2003; Jiggins and Kim 2006; Obbard et al. 2006; Schlenke and Begun 2003). To date, a range of studies have focused on positive selection within narrow *Drosophila* groups (Jiggins and Kim 2007; Morales-Hojas et al. 2009; Sackton et al. 2007) as it is often difficult to detect such selection in sequences that have diverged substantially, due to too high a rate of synonymous substitutions.

We categorised *Drosophila* species into three groups based on their geographic ranges: (*i*) cosmopolitan species adapted for broad climatic conditions, (*ii*) species that reside in both temperate and tropical regions and (*iii*) species found only in the tropics. In selecting *Drosophila* sequence data, we focused on genes that were highly conserved across all species, thus to avoid saturation of the selection signal. Then, using likelihood-based models of molecular evolution, we aimed to identify patterns of purifying and positive selection by geographical range for genes representing different aspects of the immune response. We found an overabundance of purifying selection for genes that control viral infection in flies whose ranges are restricted to the tropics.

METHODS

Dataset and alignments

An initial set of genes was selected based on key immune genes outlined in recent reviews of *Drosophila* immunity (Buchon et al. 2014; Myllymaki and Ramet 2014; Xu and Cherry 2014), then the set was expanded by selecting genes from FlyBase annotations to supplement underrepresented immunity pathways (n < 8) where possible. The following pathways are included in the data set: Autophagy (13 genes), IMD (10 genes), JAK-STAT (13 genes), Melanisation (12 genes), RNAi (8 genes) and TOLL (19 genes). There is also an additional set of genes (9) that have known roles in antipathogen response but that are not clear members of the above pathways. Gene sequences were downloaded from FlyBase and e!Ensembl in November 2015.

Homologous genes were mined from the FlyBase database using BLASTn with the *Drosophila melanogaster* Coding Sequences as the search query to retrieve sequences from the *Drosophila* species (below). The best match in each *Drosophila* species was included in the manual multiple sequence alignment performed in Se_Al v2.0a11 (Rambaut 2002). Regions exhibiting any missing sequence data or major differences at the amino acid level were excluded on a case-by-case basis.

PhyML v3.0 was used to construct maximum likelihood gene trees, as described in the Supplementary Methods (Guindon et al. 2010). Trees were inspected using Figtree v1.4.0 (Rambaut 2009), and in some cases extremely long branches were removed to avoid oversaturation of d_N/d_S calculations (branch length > 0.5). This allowed the inclusion of very distant relatives for those genes that had been more strongly conserved. Alignments and phylogenetic trees are available in the supplementary materials.

Geographical Distribution

We selected 12 species with fully sequenced genomes (Drosophila 12 Genomes, 2007), plus six others with sequence data of multiple innate immunity genes. *Drosophila* species then were categorized based on their geographical distributions as described in Ashburner (1989), Markow and O'Grady (2007) and data available at the Molecular Evolution Group website, http://evolution.ibmc.up.pt/node/35. Species distributions of 'Cosmopolitan', 'Temperate/tropical' and 'Tropical' do not correlate with phylogenetic relatedness (Fig. 1). For example, *D. virilis, D. mojavensis* and *D. grimshawi* all share a recent common ancestor but each represents different geographical clusters, indicating recent differentiation with respect to distributions. Likewise, D. melanogaster and *D. erecta* have a common ancestor 5mya but have since differentiated with respect to distribution (Tamura et al. 2010). There were three species

that had cosmopolitan distributions (*D. ananassae*, *D. melanogaster*, *D. virilis*), five that were found in temperate/tropical regions (*D. albomicans*, *D. ficusphila*, *D. mojavensis*, *D. persimilis*, *D. takahashii*) and ten that were restricted to tropical regions (*D. biarmipes*, *D. bipectinata*, *D. elegans*, *D. erecta*, *D. eugracilis*, *D. grimshawi*, *D. kikkawai*, *D. rhopaloa*, *D. willistoni*, *D. yakuba*). The sequences of *D. pseudoobscura pseudoobscura* and *D. miranda* rarely differ significantly from *D. persimilis,* and as they are found in the same categorized area, these species were not included. Further, *D. simulans* and its sister species *D. sechellia* were not included as *D. simulans* developed a cosmopolitan distribution only very recently (Tamura et al. 2010) and the two species consistently clustered too closely to perform PAML analysis. A total of 103 immunity genes were assessed for study, representing six different innate immunity pathways[3].

PAML analysis

All PAML analyses were carried out with PAML version 4.8 (Yang 2007).

Identifying positive selection. Branch-site models (Zhang et al. 2005) were run on three randomly selected genes from each pathway tested. The null-hypothesis under both M0 (assumes a single ω for all sites) and M3, with the settings NSsites = 3; fix_kappa = 0, fix_omega = 0, ncatG = 3, was calculated. Several alternative hypotheses with one of the two models (0 or 3) and ncatG of 2 or 3 were then applied to identify positive selection in the geographical groups of interest. Each model was compared pairwise using a doubled log-likelihood difference in a chi-square test with 1 degree of freedom. These tests allow for purifying and neutral selection in sites along a branch, as well as two scenarios where the sites are under positive selection. Bonferroni corrections were performed to correct for multiple testing and to reduce the rate of false positives (Bland and Altman 1995).

Identifying purifying selection. For all alignments, we ran two branch models: model M0 that assumes a single ω across the whole tree, and M1 that estimates a different ω for each branch of the tree. These two models were compared using a doubled log-likelihood difference in a chi-square test where df was determined by the number of species included in the alignment. When ω was found to be significantly different between the two models, a further model of the *a priori* hypothesis was carried out using each species category as the foreground branch in turn. From this, ω values for foreground and background branches were compared to indicate the branches with the most purifying selection acting on them. In cases where ω of the background branches was smaller compared to the foreground branches, the individual ω for each species category was calculated. Differential purifying selection was deemed to be

[3] **Supplementary Table 1** A complete list of genes, known pathogen targets and FlyBase IDs used in this study.

occurring if two conditions were met: (1) a significant difference in ω between M0 and M1, and (2) a significant difference between the ω of one species category with the ω of the other two species categories.

An exact contingency test (r x c) was calculated followed by adjusted residual calculations to determine overrepresentation of certain purifying selection in each pathway.

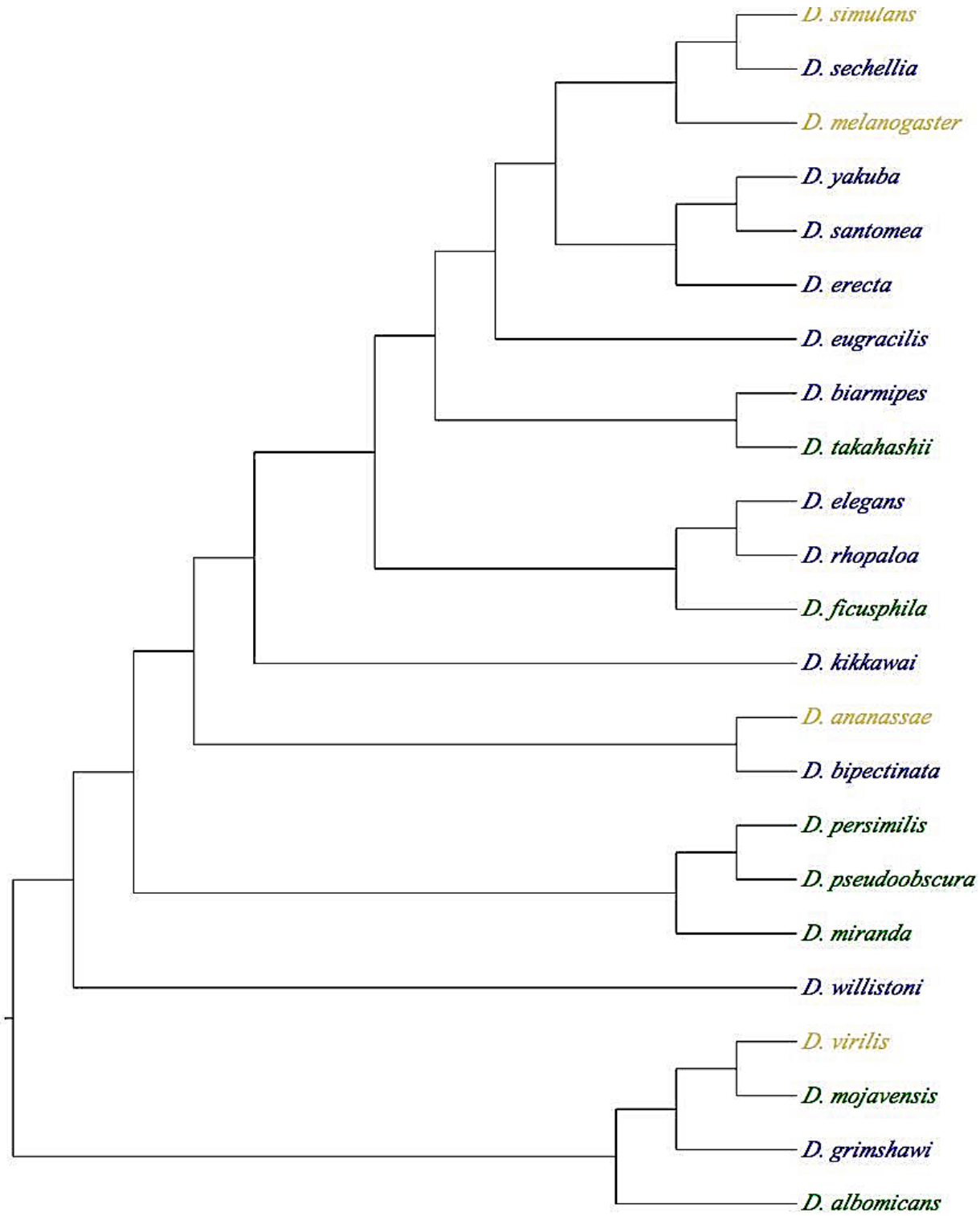

Figure 1 A phylogenetic tree of *Drosophila spp*. included in this analysis (adapted from Drosophila 12 Genomes et al. (2007)). Yellow indicates species classified as 'Cosmopolitan', blue indicates species classified as 'Tropical' and green indicates 'Tropical/temperate'.

RESULTS & DISCUSSION

Positive selection

Numerous studies have been carried out to detect positive selection in *Drosophila* genes since 2000 (Begun and Whitley 2000; Jiggins and Kim 2007; Levine and Begun 2007; Obbard et al. 2009; Sackton et al. 2007; Schlenke and Begun 2003). Finding a signal of positive selection can be difficult, as it is biologically rare, and using divergent species groups tends to produce an overestimation of positive selection due to saturation of d_N/d_S estimations (Morales-Hojas et al. 2009) and variation in selective constraints (Obbard et al. 2009; Sackton et al. 2007).. However, It is possible to detect both lineage- (Jiggins and Kim 2005) and genus- (Jiggins and Kim 2006) specific positive selection in *Drosophila* genes. In studies like ours, based on diverse groups of Drosophila species, hypervariable regions tend to be excluded from alignments, with the aim of reducing the proportion of false positives (Heger and Ponting 2007). By limiting the length of sequence available for d_N/d_S calculations we also eliminated our ability to detect positive selection in our geographic comparisons, especially as positive selection would need to be the dominant feature of the remaining nucleotide sequence to be detected (Zhang et al. 2005). Our conclusions about patterns of selection therefore are restricted to evidence of purifying selection on sites experiencing greater constraint across the genus. To accurately assess the effects of positive selection, future approaches would need to look at narrower ranges of taxa with respect to phylogenetic diversity and/or focus on informed site-specific models of evolution (Nielsen and Yang 1998; Yang et al. 2000).

Purifying selection

We set out to test the hypothesis that immunity-associated genes are under differential purifying selection depending on the geographical range of the insect. Purifying selection is known to act strongly on antimicrobial peptides and pathogen recognition proteins (Jiggins and Hurst 2003; Jiggins and Kim 2005). Here we interpret greater purifying selection to mean that the function of the gene regions studied is more highly optimized or constrained than for the other five innate immunity pathways from flies of the same geographic distribution. We found evidence (Fig. 2) of greater purifying selection in the JAK/STAT pathway in insects from the tropics (4/13 genes, adjusted residual 1.744). The genes experiencing purifying selection are diverse in terms of function and include: Dome (receptor), Cdk2(kinase), Cdk4 (kinase) and upd2 (activator ligand). This pattern of heightened selection in response to viruses in the tropics (8/26 genes, $X^2 = 4.674$, $p = 0.0306$), but not in other regions, is recapitulated more broadly when we group genes by pathogen target (Fig. 3). Latitude is a known predictor of virus diversity, and so tropical areas have the greatest virus pathogen burden of the world (Guernier et al. 2004). The known viral diversity of wild insects spans at least 12 families (Huszar and Imler 2008; Possee and King 2001) and contains both native viruses and those that are vectored pathogens of plants

and animals (Hogenhout et al. 2003). In one study of *Drosophila melanogaster* populations from the wild, a minimum of 30% of insects were infected with one detectable virus (Webster et al. 2015). There is also evidence from a range of vector-virus associations (Davis 1932; Kay et al. 1989; Kilpatrick et al. 2008; Takahashi 1976) that viral replication rates increase with ambient temperature and so insects in the tropics will likely harbour higher viral loads than those from cooler climates (Patz et al. 2005). This consistent exposure would act as a strong selective agent. Tropical *Drosophila* species lack genetic variation, particularly in genes that enable adaptation to extreme temperature changes (Kellermann et al. 2009; Merila et al. 2001; Rodríguez-Trelles et al. 2013) and the specific environmental challenges of the tropics lead to narrower free-living species distribution (Addo-Bediako et al. 2000; Chown et al. 2002). This lack of genetic variation may be producing the purifying selection we observe in the JAK/STAT pathway.

While not significant (adjusted residual 1.058), 2/8 genes in the other antiviral pathway, RNAi, also exhibited purifying selection. It is not clear if this lack of significance relates to the much smaller number of genes studied in RNAi (7 vs. 13) or to differences between the two pathways in the level of functional constraint present in the genes or to the level of selection experienced. Both the RNAi and JAK/STAT pathways specifically target viruses, the former by recognition of viral dsRNA by Dcr-2 followed by RISC-mediated virus silencing, and the latter after activation by other cellular factors (Sabin et al. 2010). One key difference between the pathways is that RNAi is a general response, while JAK/STAT responds to specific viruses (Kemp et al. 2013). Additionally, RNAi can independently sense and enact the destruction of viruses, while the JAK/STAT pathway requires separate activation (Dostert et al. 2005). Lastly, suppressors of the RNAi pathway have evolved in some viruses that may instead lead to positive selection on these genes (Li et al. 2002). Three of the core genes in the RNAi pathway (Dcr-2, r2d2, and AGO2), in fact, are amongst the most rapidly evolving genes from the whole Drosophila genome (Obbard et al. 2006; Schlenke and Begun 2003).

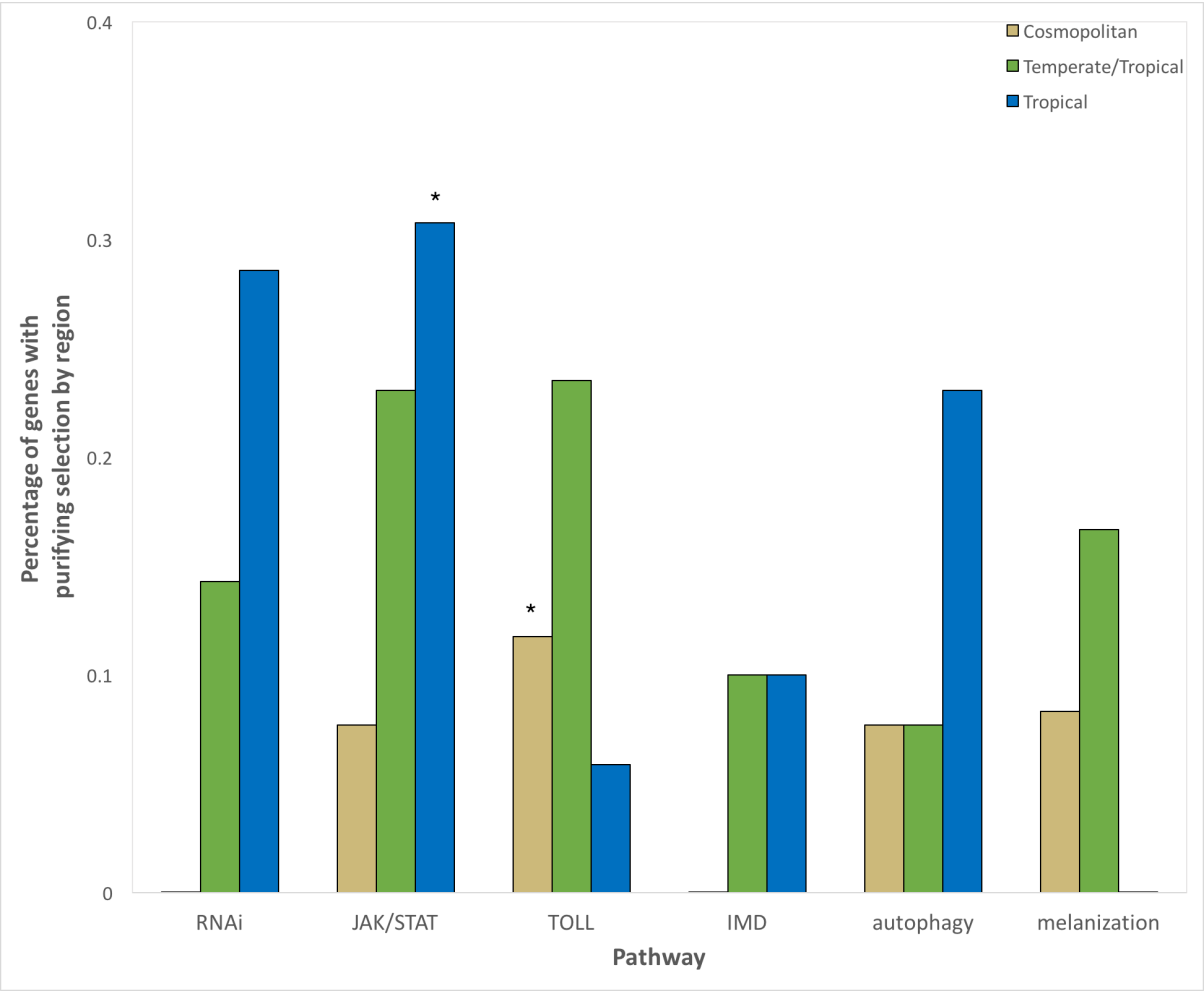

Figure 2 The proportion of genes experiencing purifying selection out of the total gene number tested within individual immune pathways by geographic region. Significance for any given pathway is relative to all other pathways within the same geographic region. *p < 0.05,**p < 0.01.

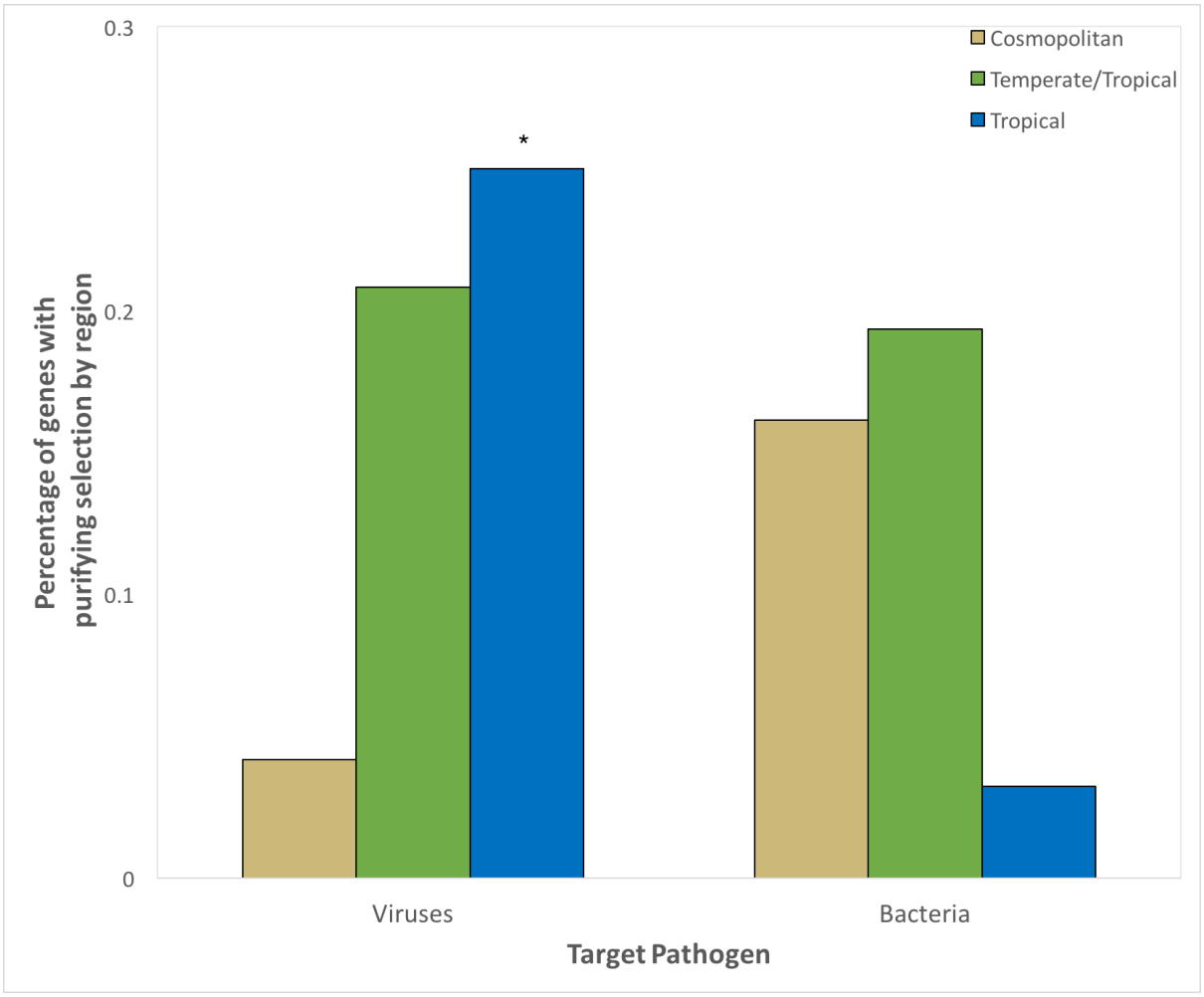

Figure 3 Proportion of genes experiencing purifying selection by category of pathogen targeted. Significance for virus or bacteria associated genes within a region is relative to the same genes from other geographic regions. *$p < 0.05$.

In contrast, there was no significant overrepresentation (Fig. 2) of generalist immune genes (autophagy, melanisation) for flies from any geographic range or of bacteria-targeting genes (TOLL, IMD) for flies with restricted geographic distributions (Tropics, Temperate/Tropics). Instead, there were fewer genes under selection in tropical regions than expected (0/11, adjusted residual -1.597). Unlike viruses, species richness of bacteria is not correlated with latitude (Guernier et al. 2004), so there is no prompt for antibacterial *Drosophila* genes to adapt differentially over geographical distributions. *Drosophila spp*. become systematically bacterially infected rarely in the wild compared to their rates of viral infections (Jaenike et al. 2007). Thus genes targeting bacterial pathogens are not under the same consistent pressure to evolve as are virus targeting genes. The elevated rate of purifying selection in TOLL genes (3/17, adjusted residual 1.502) in cosmopolitan species (Fig. 2) is therefore surprising. The genes experiencing constraint in the TOLL pathway in cosmopolitan species do not appear confined to particular functional roles: CG8595 Toll-7 (receptor), CG16705 SPE (activator) and CG4006 Akt1 (kinase). Given that these comparisons are relative however, the significance could be due to a lack of constraint seen in other pathways (RNAi, IMD) for these same flies. The pattern contrasts with Temperate/Tropical species that show similar levels of constraint for TOLL but also some constraint in all other pathways. It is also important to note that the TOLL pathway has known roles in development in addition to pathogen defense and so may experience greater pleiotropic constraints than other pathways (Lemaitre et al. 1996a). If this level of pleiotropic constraint varies with changing environmental conditions, such as temperature, it could play a role in the geographic patterns seen here. Lastly, as demonstrated in a mosquito species there appears to be cross talk, if not direct involvement of TOLL in the antiviral response (Xi et al. 2008b). If this is a common feature in other insects, pressure from viruses could also drive TOLL constraint as it does JAK/STAT.

However, these findings should be interpreted with extreme caution. The comparisons made in this study made relatively arbitrary assumptions not only the relative distribution and rate of advantageous, deleterious, neutral, and nearly neutral mutations (Razeto-Barry et al. 2012), but a range of other factors that can influence molecular evolution (Obbard et al. 2012). Phylogenetic relatedness may have had the most significant impact on skewing our results. In general, substitution rates are estimated by associating nodes of a phylogenetic tree with dated fossils or phylogeographic events or by estimating mutation rates from laboratory studies (Pybus 2006). A problem is that purifying selection or saturation of sites by high mutation rates produce divergent estimates of substitution rates that prevent accurate timescales of evolution (Obbard et al. 2012). Most timelines for *Drosophila* evolution are based on Hawaiian phylogeography (Tamura et al. 2004), which has now been proved to have multiple caveats (Heads 2011), yet we used the corresponding times to justify the exclusion of *D. simulans* and *D. sechellia* from our analysis (Tamura et al. 2010). Generation time, which also impacts *Drosophila* evolution timelines, was also not considered. Tropical species

are found to have longer generation times (eg. D*. silvestris* have 4 generations/year (Boake et al. 1998)), while most other species reproduce more rapidly (eg. *D. melanogaster* have 20 generations/year). This could have accounted for the so-called 'purifying selection' we saw in tropical flies, simply due to the slower rate of mutations in tropical species. Another factor that could have interfered with our results was effective population size, however the research of Petit and Barbadilla (2009) states that the populations we studied did not have different population sizes. Finally, differential pleiotropy among genes can stabilize selection (Griswold and Whitlock 2003; McGuigan et al. 2014).

CONCLUSION

Here we demonstrate that flies restricted to the tropics experience heightened selective constraints on the more conserved regions of their antiviral genes compared to flies with more cosmopolitan distributions. This pattern could result from differential exposure to viruses in the tropics that may include greater viral prevalence, diversity or load. These alternate hypotheses cannot be differentiated without a better understanding of the native viruses of these fly species relative to those documented for *D. melanogaster* and *D. simulans* (Webster et al. 2015). As tropical regions expand due to shifts in climate, so too will the distributions of insects, including a number of vectors (Régnière et al. 2012). Understanding how geographic region shapes the immune response to pathogens may support short and long-term predictions about insect susceptibility to pathogens. In addition, this study has identified specific genes that underpin the coevolutionary response of flies to viral pathogens. However, our data analysis did not consider the different rates of molecular evolution in *Drosophila* lineages, and so the results should be carefully examined before being used to support future studies. Future comparative work may help to reveal whether these patterns are generalizable for insects in the tropics and if there are consistent gene types of immune genes experiencing heightened selective constraints. Lastly, as we identify likely candidate anti-pathogen genes for genetic modification in insect vectors, we will need to understand the capacity for such genes to harbor and maintain genetic variation through time and over broad geographic landscapes.

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Chapter 5

General Discussion

In this thesis, I have examined the gene responses experienced by diverse hosts with novel and native infections of the endosymbiont *Wolbachia.* Novel *Wolbachia* infections in the mosquito vector *Aedes aegypti* affect the transmission of Dengue virus (DENV) (Iturbe-Ormaetxe et al. 2011) and other viruses (Ciota et al. 2011; Reiskind et al. 2010), and this forms the basis of the Eliminate Dengue (ED) program (www.eliminatedengue.com). Current field releases are of *Ae. aegypti* infected with the *Wolbachia* strain *w*Mel (Ritchie 2014) that can spread to fixation in populations via the method of cytoplasmic incompatibility (CI) (Hoffmann et al. 2011) and the antiviral effect remains stable (Frentiu et al. 2014). Several other novel infections have been created including *w*Mel and *w*MelPop from *Drosophila melanogaster* (McMeniman et al. 2009; Walker et al. 2011a), *w*AlbB from *Aedes albopictus* (Xi et al. 2005b) and a superinfection of *w*Mel/*w*AlbB (Joubert et al. 2016). However, there is always an inevitable threat of resistance in either mosquito or virus – potentially this can be predicted through examination of current endosymbiont-host-pathogen interactions.

Host-pathogen interactions are influenced by three major types of factors. The first of these is host factors such as genetic background and endosymbiont presence/absence. In **Chapter 2**, I explored this factor by use of a bioinformatics approach to characterize the host responses to *Wolbachia* across diverse insect species. I continued to look at Factor 1 in **Chapter 3,** where I used experimental infection of cell lines to examine the effects of *Wolbachia* on host immune gene expression. In **Chapter 4**, I researched the other two factors: pathogen diversity and environmental influences. I compared sequence evolution in disparate *Drosophila spp.*, by use of PAML, with the aim to assess the selective effects of different pathogen exposures, and environmental effects such as temperature and humidity.

SHARED RESPONSES IN ENDOSYMBIONT-HOST INTERACTIONS

Each freshly published transcriptional study of a *Wolbachia*-host relationship is couched in the relevant literature of closely related organisms, yet it is rare that the authors compare results across disparate species. This appears to be due to the assumption that the genomes of arthropods and nematodes are too different and difficult to compare. However, I used currently available transcriptome data from diverse hosts to ascertain whether there are biological processes altered by *Wolbachia* that are fundamental regardless of host.

My findings in **Chapter 2** confirm previous identifications of core biological functions involved in *Wolbachia*-host interactions and also highlighted a number of new areas of interest. A sample obtained in the absence of *Wolbachia* infection (compared to an infected sample) results in a spectrum of changes in the host physiology, and also the underlying transcriptomic profile. I have identified some of the underlying components in order to better understand fitness effects and pathogen blocking as well as potential drug targets. Of the areas I identified, a number of these have already been well investigated including metabolism (Darby et al. 2014; Evans et al. 2009; Ross et al. 2016), oxidative stress (Darby et al. 2014; Gill et al. 2014), ER/Golgi/membrane association (Chagas-Moutinho et al. 2015; Cho et al. 2011; Voronin et al. 2004), cytoskeleton (Ferree et al. 2005; Frydman et al. 2006; Newton et al. 2015), heme synthesis and iron provision (Darby et al. 2014; Kremer et al. 2009; Pfarr and Hoerauf 2005), and lipids (Caragata et al. 2013; Molloy et al. 2016). There emerged two new areas of interest: chitin and chaperones.

Chaperones may act upon the cytoplasmic incompatibility (CI) phenotype of an arthropod host. CI is important for biological control of vector diseases because it allows releases of *Wolbachia*-infected mosquitoes to spread and wipe out wild populations (Walker et al. 2011b; Werren et al. 2008). The CI phenotype has now been linked to the two prophage WO genes in *Drosophila* species (Beckmann and Fallon 2013; LePage et al. 2017) and a *Wolbachia* deubiquitylating enzyme (Beckmann et al. 2017). In *Drosophila,* chaperones may underlie the CI phenotype by impacting on spermatogenesis (Zheng et al. 2011a). My findings suggest that chaperone responses to *Wolbachia* are arthropod specific; *Wolbachia* infection state alters chaperone expression in *A. tabida*, *D. melanogaster* (*w*Ri), *D. melanogaster* (testis), *Ae. aegypti* and *An. gambiae.* This function could be exploited by genetically modifying *Wolbachia*-infected individuals to affect host reproductive fitness via changes in the CI phenotype. I could have also identified chaperones from my analysis because they may influence stress responses to the disturbed cellular homeostasis caused by *Wolbachia's* removal.

Altering cellular homeostasis is one way to combat filarial infections. Indeed, one of the current targets is the heme synthesis ability of *Wolbachia* (Slatko et al. 2010). Many host nematodes lack heme biosynthesis genes and rely in some

manner on *Wolbachia* to provide this resource (Pfarr and Hoerauf 2005). Thus inhibiting heme synthesis in *Wolbachia* affects nematode viability (Wu et al. 2009). As the nematode dies swiftly after antibiotic treatment, it is difficult to detect whether *Wolbachia* provides heme to the nematode directly or contributes to heme extraction from endogenous sources such as the vertebrate host (Gill et al. 2014). It is also difficult to differentiate between the effects of antibiotic toxicity and the effects of *Wolbachia* on the transcriptome. However, I showed that heme synthesis was affected regardless of whether a host was naturally *Wolbachia* free or treated with antibiotics. I found evidence that *Wolbachia* alters heme related gene expression in a range of non-nematode species (*Ae. aegypti, D. melanogaster* (*w*Ri), and *An. gambiae)*: this key endosymbiont-host interaction could be explored in those organisms to inform drug development.

Another target in the treatment of filarisis is chitin, primarily because of its function in formation of the cuticle (Kumar et al. 2007). I found that chitin biosynthesis is altered in the mosquitoes *Ae. aegypti* and *Anopheles gambiae* in the presence of *Wolbachia.* Before parasites and viruses can bodily infect a vector they must escape from the alimentary canal through the peritrophic matrix (Merzendorfer and Zimoch 2003; Zhu et al. 2016). This matrix is formed by chitin, and thus *Wolbachia's* alteration of chitin biosynthesis might create a barrier to the pathogens by alteration of homeostasis (Merzendorfer and Zimoch 2003). This is a key area that should be focussed on for further research in both nematode and virus control.

However, my results may reflect limitations in the form of data chosen for analysis. In several cases the transcriptomic data were obtained at the same time as assembly of the initial genome scaffold, and this may have led to incorrect assumptions about gene function or genome content (Chevalier et al. 2012; Kremer et al. 2012; Vigneron et al. 2012). It is therefore likely that I have missed areas that are either specific to poorly sampled host niches or didn't have enough evidence to be classified as of further interest. This would particularly affect the number of nematode genes identified, as there were only three datasets available and two came from the same organism (Ghedin et al. 2009; Rao et al. 2012; Strubing et al. 2010), whereas there were nine arthropod datasets. More datasets will mean wider host samples and thus more data to be exploited for medical and agricultural applications.

Additionally, my analysis examined only transcriptomic data, and did not take the discrepancy between gene transcription and protein expression into account (Haider and Pal 2013). This can be due to several factors, including mRNA half-lives, post-translation machinery, and the purification/quantification methods (Haider and Pal 2013). This discrepancy may affect our discussion of differential regulation of biological areas of interest, but it is likely that the areas I identified will still be of interest. For example, the correspondence of reads and abundance in an RML12 cell line (*Ae. albopictus*) and the *Wolbachia* strain *w*Mel-Pop-CLA is low, but when examined at a functional level, the two show

a convincing upregulation of cell wall, membrane and envelope biogenesis (Darby et al. 2014). Thus, a joint analysis of the transcriptomic and proteomic data can provide useful insights that may not be deciphered from individual analysis of mRNA or protein expressions.

There are still questions to be answered and more details that can be taken from these transcriptomic studies. An extensive comparison of homologues between the samples (within Diptera and Nematoda) could make further use of this dataset when combined with single gene studies such as my work in **Chapter 3**. There is a wealth of organisms yet to be sampled. I have compared data from only eight arthropods and two nematodes (Figure 1) but in fact *Wolbachia* is estimated to infect 40% of arthropods (Weinert et al. 2015; Zug and Hammerstein 2012; Zug et al. 2012) and within this prevalence it is found in 28% of vector mosquitoes such as *Ae. albopictus, Aedes fluviatilis, Culex pipiens* and *Culex quinquefaciatus* (Kittayapong et al. 2000). There are limited transcriptomic studies of *Wolbachia's* interaction with the variety of other organisms it infects (for a summary see Werren et al. (2008) and Weinert et al. (2015)). Since completion of my experimental work, the transcriptomic interaction of *Ae. fluviatilis* with its native *Wolbachia* strain has been published (Caragata et al. 2017).

While experimental comparisons become more difficult by the virtue of genome size and the wealth of genes available for study, studies that are bioinformatically based on others' data have advantages in that once the pipeline is generated, data can be rapidly processed by a variety of methods. Although full genome sequencing and transcriptomic studies are becoming more affordable, funding is still limited, particularly for species that appear to have no direct impact on human health. The genes I have identified in **Chapter 2** and **Chapter 3** could be used as a starting point of investigation into these species' interactions with *Wolbachia,* with no need to sequence the entire genome. A potential problem with such an approach is the inevitable significant sequence divergence of disparate species that could be addressed by the methods I developed in **Chapter 4**. Nevertheless, perhaps this technique could be used to compare nematodes that do not affect humans, or insects that do not vector prevalent human pathogens, to those that are implicated in human pathology, as shown in current available datasets.

Ultimately my work in **Chapter 2** and **Chapter 3** could inform instances to predict how Eliminate Dengue's *Wolbachia* pathogen blocking could fail or identify potential targets to assist the anti-Wolbachia Consortium (A-WOL). Experimental studies in this area will impact research into biological control, but bioinformatic studies will continue to provide a cost effective method for comparison of disparate data.

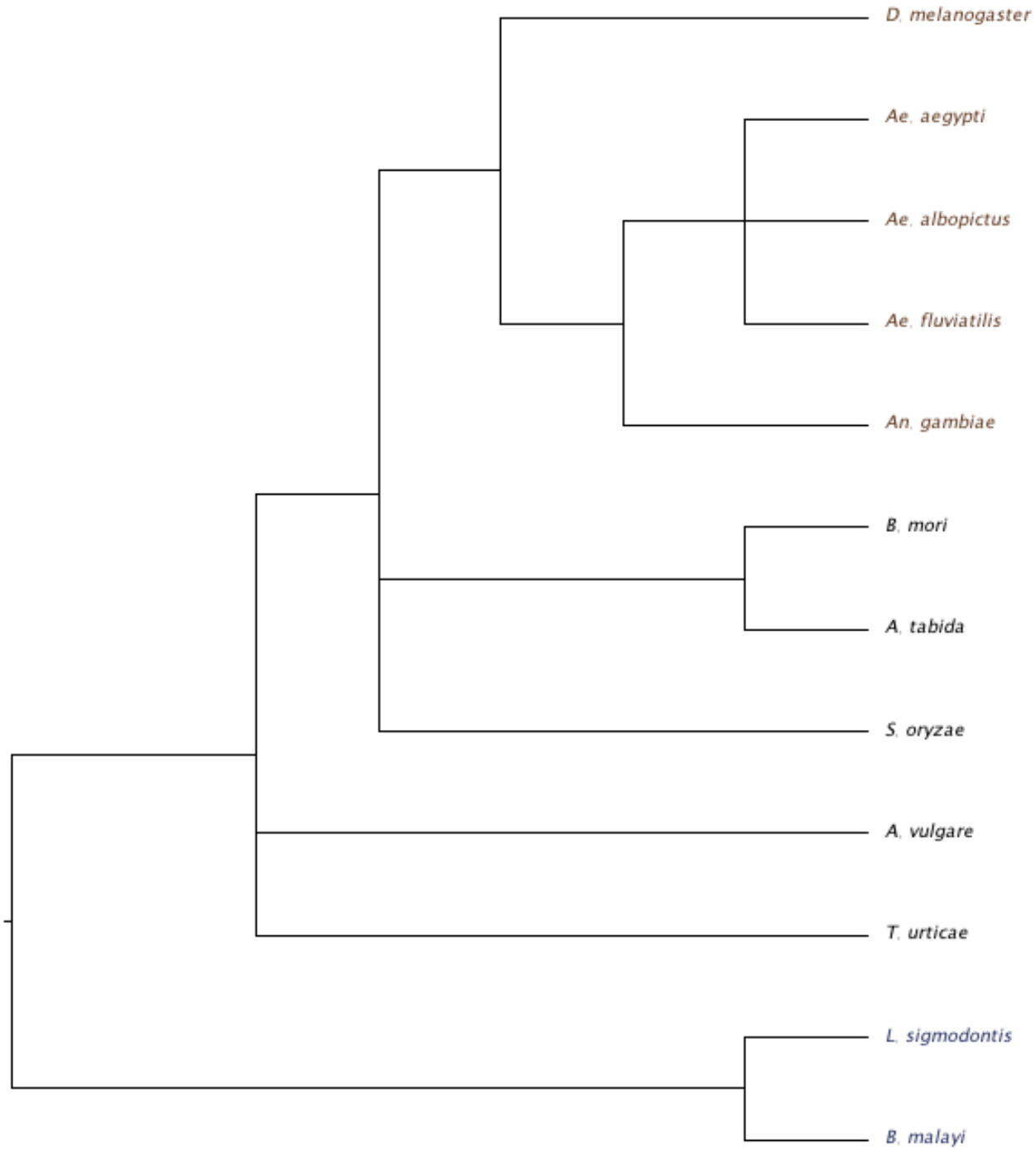

Figure 1 A cladogram of organisms with transcriptomic data comparing *Wolbachia*-infected and *Wolbachia*-uninfected sample*s* as of October 2017. The tree is rooted at the divergence of Nematoda (blue) and Arthropoda, the Diptera Order is shown in red.

IMMUNE GENE RESPONSES TO *WOLBACHIA*

Wolbachia-host interactions are often unique to the particular endosymbiont strain / host species relationship under examination. In *Ae. albopictus* the native *Wolbachia* infection (*w*AlbA, *w*AlbB) does not affect immune gene expression (Bourtzis et al. 2000) but can influence DENV transmission dynamics (Mousson et al. 2012). When *w*AlbB is artificially transfected into *Ae. aegypti*, DENV blocking effects are seen (Xi et al. 2005b). The same occurs in *Ae. aegypti* with artificially introduced *w*Mel from *D. melanogaster* (McMeniman et al. 2009; Walker et al. 2011a). The current biocontrol of DENV relies on field-release of the latter mosquitoes, currently under extensive tests in Vietnam, Brazil and Indonesia (McGraw and O'Neill 2013; Ritchie 2014) (see www.eliminatedengue.com). However, although the mosquitoes spread to fixation in the population (Hoffmann et al. 2011; Walker et al. 2011b; Xi et al. 2005b) and DENV blocking has remained stable to date (Frentiu et al. 2014), the pathogen blocking effects of the novel *Ae. aegypti* + *w*Mel pair are potentially fallible. An alternative strain of *Wolbachia, w*MelPop, exhibits exceptionally strong blocking abilities (Moreira et al. 2011), however, due to a significant impact on host physiology (McMeniman et al. 2009; Yeap et al. 2011), it cannot be maintained in field releases (Nguyen et al. 2015). There are therefore ongoing investigations into further strains that could facilitate: (1) stronger virus blocking against DENV; (2) possible blocking against other viruses; or (3) provide options to overcome resistance. Recently a *w*Mel/*w*AlbB double infection was created in *Ae. aegypti* that can block DENV more strongly than the single *w*Mel infection (Joubert et al. 2016).

Due to the current interest in these two *Wolbachia* strains, in **Chapter 3** I focussed on them to investigate the effects of novel *Wolbachia* infections on host immune gene expression. As previously discussed, *Wolbachia* strains show different DENV blocking abilities. At present, it is not fully understood why this is the case. Understanding the ways that hosts react might make a difference to how the ED team can tailor novel *Wolbachia*-host relationships to the evolution of resistance in either the mosquito or virus. Unfortunately, I found that novel infections established with *w*Mel were transient, somewhat as observed in other studies for *w*Ri in a *D. melanogaster* S2 cell line (Xi et al. 2008a) and *w*Mel in an *Ae. albopictus* Ae23.tet cell line (Voronin et al. 2010). It is stable in C6/36 (Voronin et al. 2010). Thus I was unable to mirror the new *Ae. aegypti* double strain – ideally, I could have introduced a *w*Mel infection into *w*AlbB infected cells to parallel the mosquito infection protocol order of Joubert et al. (2016). I was further hampered by the limited number of cells available for extraction and sequencing in the early stages of *Wolbachia* infection. This prevented me from adopting a transcriptomic approach, which would have required more material. The dropout of the *w*Mel infection also prevented me from looking at responses to early verses late challenges to dengue. To date, no early and late term infections with *Wolbachia* followed by virus infection have been performed. Nevertheless, compared to *in vivo* transfections, cell lines still provide a simpler method of looking at *Wolbachia's* effects on its hosts. Future studies may

be able to perform this comparison by adopting passaging techniques that better maintain *w*Mel infection in the population of cells, or by focussing on infecting a single cell line rather than multiple cell lines.

To an extent, it is possible to predict the success of *Wolbachia* transfer based on the *Wolbachia* density of the donor cell line. Suboptimal passaging procedures for the donor *Ae. albopictus* + *w*AlbB cell line (Ae23 + *w*AlbB) caused the *Wolbachia* infection of this cell line to drop out completely despite an initial high infection level. Despite attempts to revive the *Wolbachia* density using optimum passaging conditions (**Chapter 3**), the *Wolbachia* density could not be increased. As *Wolbachia* density dropped in the donor line, it became increasingly difficult to infect novel cell lines. However, I did not encounter the same problem for the *Ae. aegypti* + *w*Mel cell line (Aag2 + *w*Mel) because the *Wolbachia* infection remained stable over time. This could have been due to three factors: (1) the passaging proceedures established at the expense of the *w*AlbB infection could effecively maintain stable *Wolbachia* density; (2) *w*Mel is found at higher densities than *w*AlbB in host cells (Voronin et al. 2010; Walker et al. 2011b; Xi et al. 2005b) and this is mirrored in cell lines; (3) *w*Mel is better cell line adapted than *w*AlbB and thus is maintained more easily than *w*AlbB. The flow through problem of (3) is that cell line adaptation affects the genome of *Wolbachia* and potentially fixes rare gene mutations into the cell population that convey a growth advantage in cells, but may have a deleterious effect on the original whole insect. I found this manifested in an inability to infect the uninfected host cell line (for wAlbB, Ae23.tet and for wMel, Aag2). There is implicit acceptance of the cell line adaptation problem when *Wolbachia* is transferred from cell cultures to infect whole insects (McMeniman et al. 2008).

Although my study focusses on cell lines through transfer of *Wolbachia* from one cell line to another, there are limitations to this approach. The transfection of novel hosts is achieved in mosquitoes by egg injection (McMeniman et al. 2009; Walker et al. 2011a). A current oversight is that in nature there are additional means for transmission of symbionts between species that include: wounding by parasitoid wasps (Ahmed et al. 2015; Gehrer and Vorburger 2012) and mites (Jaenike et al. 2007), cross-infection by infected and uninfected hosts during copulation (Moran and Dunbar 2006) and cofeeding (Caspi-Fluger et al. 2012), and directly from the environment (Kikuchi et al. 2007). These methods may hide some secrets about how to improve notoriously difficult infection rates (Bian et al. 2013a; McMeniman et al. 2009; Xi et al. 2005a). A single paper that developed a short-term cell line of *Brugia malayi* was published over 10 years ago (Higazi et al. 2004), but since then no cell lines have been established. Similarly, establishing nematode cell lines is difficult, possibly due to loss of *Wolbachia* during the procedure.

In addition, underlying differences in *Wolbachia* species or differences in the host cell lines may affect transfection. Uninfected cell lines are maintained in the laboratory for many years and this potentially causes the genome content

to shift away from the original insect. I took *w*Mel maintained in a mosquito cell line that had been established from *Wolbachia*-infected *D. melanogaster* ovaries: a source known to impact the ability of *Wolbachia* to effectively infect its original host (McMeniman et al. 2008). This may have impacted my experiment at several stages: the insect cell lines that could be infected with *Wolbachia,* the persistence of the *Wolbachia* infection and the associated changes in innate immune gene expression. Thus although my conclusion was that that there are few commonalities in the nature of the early host immune response across fly, mosquito and moth cell lines, this might not be true of all *Wolbachia* strains or all cell lines. Attempts to infect different *Ae. albopictus* cell lines (eg. Ae23.tet, C6/36 and Rml12) may therefore evoke different immune responses. Previous work supports this hypothesis in both cell lines and whole insects (Bian et al. 2010; Bourtzis et al. 2000; Kambris et al. 2009; Moreira et al. 2009a; Pan et al. 2012; Rances et al. 2012; Xi et al. 2008a) (See also: Table 2 of **Chapter 1**, General Introduction). However my results from **Chapter** 2 suggest that I may have been able to detect a pattern of gene expression if more strains of *Wolbachia* had been investigated.

In hindsight, it would have been of interest to test the genes identified in **Chapter 2,** in the cell lines of **Chapter 3**. The *Drosophila* and *Aedes* genomes show some similarities; it is possible to identify homologous genes between the two genera. A problem with testing 'homologues' is that where genomes have not been well characterized and without further experimental validation, it is not possible to detect whether matches are legitimate. For example, I was unable to design a set of primers for dFADD and cecropin in *Spodoptera frugiperda* that amplified the genes. This may reflect technical issues or perhaps the genes were not expressed. Without use of RNAi or specific sequence analysis of the gene, I cannot confirm the finding. A related limitation of transcriptomic studies is that the overwhelming amount of data available for analysis makes it difficult to isolate areas of interest. The majority of transcriptomic studies focus on immune responses (arthropods) and heme/iron exploitation (nematodes) because these have been well established, but as shown in **Chapter 2**, it is a severely limited scope. This problem can be linked to funding and the focus on medically relevant research outcomes.

The highest priority follow up research should seek to develop *Wolbachia* infections in nematode cell lines of interest, for the A-WOL consortium to test. My results suggest that the approach taken by the A-WOL consortium using *Ae. albopictus* cells infected with *Wolbachia* to test potential drug types (Taylor et al. 2014) is filled with pitfalls, as we have found that despite similarities in endosymbiotic relationships (**Chapter 2**) the specific interactions, particularly within the immune system (**Chapter 3**), are probably host background and *Wolbachia* strain dependent.

HOST-PATHOGEN INTERACTIONS IN THE LIGHT OF A CHANGED BIODIVERSITY LANDSCAPE

Population growth and land use has dramatically changed biodiversity to date, but climate change is also increasingly a driving factor (Cardinale et al. 2012). Arthropod species make up a large portion of animal diversity and much of this diversity is found in the tropics (Bonds et al. 2012). The specific environmental challenges of the tropics lead to narrower species distribution (Addo-Bediako et al. 2000; Chown et al. 2002). This is linked to a lack of genetic variation, particularly in genes that enable adaptation to extreme temperature changes (Kellermann et al. 2009; Merila et al. 2001; Rodríguez-Trelles et al. 2013). Temperature changes will therefore ultimately reduce insect and plant species biodiversity in tropical areas (Bonds et al. 2012)(Guernier et al. 2004). To fill the ecological gap, invasive widely distributed species may become primary pathogen vectors due to rapid virus adaptation in the absence of its native tropical vectors (Addo-Bediako et al. 2000; Bonds et al. 2012; Deutsch et al. 2008). The interaction of naïve hosts with pathogens may skew towards pathogen survival with a flow through effect creating an even greater pathogen burden in tropical areas (Guernier et al. 2004). These effects are already apparent for parasitic diseases such as malaria, and viral diseases such as Dengue fever (World Health Organization 2015).

In **Chapter 4**, I discovered a link between tropical temperatures and evolutionarily heightened constraint in antiviral response genes in *Drosophila* species. Studies into sequence evolution in Drosophila are usually limited by the determination to examine only positive selection. However, with an *a priori* hypothesis such as mine, that the pathogen burden and temperature exposure alter sequence evolution, it is possible to draw on parts of the sequence that have not evolved adaptively, to infer useful information. I identified a set of genes with sequences that are highly conserved between *Drosophila* species, based on where the species is found.

Although there are 12 *Drosophila* genomes available I did not source data from them all, in **Chapter 4**, due to extensive sequence similarities that create short phylogenetic branch lengths which destroy the ability to detect selection. The most distant species included in this analysis, *D. virilis*, split 62.9 MYA from *D. melanogaster* (Tamura et al. 2010). *D. melanogaster, D. simulans* and *D. sechillia* exhibit sequence similarities that have been previously exploited to detect positive selection. Interestingly, molecular estimates of *Drosophila* divergence times generally depend on speculative inferences from the phylogeography of these three species (Obbard et al. 2012). In my analysis, I removed *D. simulans* and *D. sechillia* because the former became cosmopolitan only very recently (Tamura et al. 2010), and the two are sister species. *D. melanogaster* also became cosmopolitan only 10'000 years ago but can be considered to have considerable alterations to its genome after its exit from tropical Africa, because it then underwent a series of selective sweeps that reduced genetic diversity (Kauer et al. 2003).

While *Drosophila* is not a vector of human pathogens, its genome shows some similarity to *Anopheles* and *Aedes* mosquitoes, rendering investigations pertinent. *An. gambiae* mosquitoes are highly efficient vectors of *P. falciparum* malaria and their global spread in response to climate change (Mangili and Gendreau 2005) could be devastating (Killeen et al. 2002). The distribution of malaria is expected to be sensitive to climate change, but predictions of disease spread are inconsistent (Gething et al. 2010; Pascual et al. 2006; Reitera et al. 2004; Rogers and Randolph 2000). Models show that daily temperature fluctuations have a significant effect on malarial transmission dynamics: cooler temperatures increase malarial transmission effects while higher temperatures decrease it (Paaijmans et al. 2010). Additionally, *Wolbachia* mediated *Plasmodium* blocking is altered by temperature (Murdock et al. 2014). This suggests that the increases in mean temperature by global warming could lessen malarial transmission (Paaijmans et al. 2009). Similarly to *Drosophila, Anopheles* species live in a range of climates that includes hot and wet (*An. gambiae sensu stricto* and *An. melas*), dry (*An. arabiensis*), and cool (*An. quadriannulatus*) zones. I suggest that *Anopheles'* innate immune genes could be examined as per my method to help inform climate change models of disease transmission.

Ae. aegypti's transmission of DENV is likewise altered by temperature (Lambrechts et al. 2011). Only *Ae. albopictus* (Chen et al. 2015) and *Ae. aegypti* (Nene 2007) genomes have been published to date, and the focus has turned to transcriptomics (Caragata et al. 2017) from which I mined the primary data for **Chapter 2**. Nevertheless, using the methods and results from **Chapter 4** in combination with work on *Anopheles* to identify further genes of interest could be valuable.

In **Chapter 3**, when examining novel gene expression in response to a new *Wolbachia* infection, I found few commonalities between the three different cell lines established with *w*Mel. The gene list generated, in **Chapter 2,** did not contain any of the genes I tested, as previously discussed. However, there were six innate immune genes that were examined in both **Chapter 3** and **Chapter 4**. *Drosophila* cell line, Cactus, TAB2 and myd88 have significantly altered expression profiles following *Wolbachia* infection. These genes are not differentially selected upon based on pathogen exposure or geography. The three other genes, Relish, dFADD and Imd, did not significantly change in expression and showed no differences in selection.

I suggest that future research to stem from **Chapter 4** could be in two distinct areas. Firstly, examination of *Anopheles* species data to identify genes that could be linked to malaria spread, potentially to improve predictions of temperature-influenced vector and disease spread. Secondly, utilising the candidate gene list I have produced to select genes from *Ae. aegypti* to experimentally test for roles in environmental adaptation.

CONCLUSION AND IMPLICATIONS

This thesis has contributed to the body of knowledge about *Wolbachia's* interactions with its hosts, both novel and native. Such knowledge assists with two disease control strategies that depend on *Wolbachia*: combatting Dengue virus (DENV) by using *Wolbachia's* anti-virus effects in novel hosts, and removing native *Wolbachia* infections to kill the nematodes responsible for filarisis. My findings from comparison of transcriptome changes in a range of *Wolbachia*-infected hosts identifies key *Wolbachia*-host interactions that could be exploited by A-WOL. However, the specific interactions of *Wolbachia*-host relationships, particularly innate immunity gene expression in response to novel *Wolbachia* infections, can be vastly different so caution is advised. For both ED and A-WOL, the essential next step is to develop cell lines that directly model *Wolbachia*-host relationships of interest. My finding of a link between tropical temperatures and sequence evolution of antiviral response genes in *Drosophila* species could usefully form a starting point to predict insect responses to changed environmental conditions, but extensive replications and work using the new methods I developed must be performed before relying on this data.

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Appendix 1

Breeding status affects the ability to measure the heritable susceptibility of *Aedes aegypti* to oral infection with Dengue virus

This study has been placed in the Appendix as its subject matter differs substantially from the 3 other data chapters. This study was performed early during PhD candidature, and as such, has now been written as a paper for PLOS Pathogens, where the candidate will be the second author. **This work is titled: The Biological determinants of dengue virus extrinsic incubation period in *Aedes aegypti*.**

ABSTRACT

The mosquito *Aedes aegypti* is the primary vector of dengue virus (DENV) that poses a growing threat of human disease globally. Populations of *Ae. aegypti* exhibit differential abilities to spread DENV, depending on their geographical distribution and family history. This is measured in terms of the extrinsic incubation period (EIP): the time from when the mosquito takes an infected blood meal to when the virus can be secreted in the saliva. In order to uncover the genes underlying a mosquito's ability to spread DENV we compared the EIP of inbred parent/offspring pairs collected from one geographical region. Due to experimental variables, repeatable estimations of EIP heritability were not achieved. We conclude that there may be a trade-off between egg laying ability and susceptibility to DENV. Additionally, other factors, including the mosquito's previous blood meals and age, confound the ability to isolate this trait. Here we offer suggestions for future work, as these results have the potential to change common laboratory practices in mosquito line maintenance.

INTRODUCTION

Mosquito-borne diseases are a major threat and cause of mortality in humans worldwide, with dengue fever estimated to affect more than 390 million people annually in over 100 countries (Bhatt et al. 2013). Dengue viruses (DENVs) cause a spectrum of symptoms ranging from a mild fever to the life-threatening dengue haemorrhagic fever (Guzman et al. 2010; Halstead 2008; Kyle and Harris 2008; Ross 2010). Transmitted by the vector *Aedes aegypti,* and to a lesser extent its sister species *Aedes albopictus,* it has become the most prevalent arthropod-borne virus affecting humans (Gubler 1998; Gubler 2002; Wilder-Smith et al. 2012). The risk of contracting DENV is spreading throughout tropical and subtropical regions due to human population growth and urbanization, which allows an increase on the distribution of the vector (Colon-Gonzalez et al. 2013; Kyle and Harris 2008; Wilder-Smith et al. 2012).

Vectorial capacity is a measurement of the efficiency of vector-borne disease transmission (Kramer and Ebel 2003; Macdonald 1951). Several factors determine vectorial capacity, including vector density, probability of a vector biting a human, vector competence, extrinsic incubation period (EIP), and vector lifespan (Kramer and Ebel 2003; Macdonald 1951). The extrinsic incubation period (EIP) is an epidemiological determinant of how likely the mosquito is to cause disease (Smith 1987). EIP is the time from when the mosquito takes an infected blood meal to when the virus can be secreted in the saliva upon feeding, having undergone replication in the gut and disseminated to infect the salivary glands (Smith 1987). The earlier the pathogen arrives in the saliva, the more cases of disease the mosquito has the potential to cause (Macdonald 1951). It is known that complex immune interactions between vector and pathogen are also important for the ability of the vector to transmit disease (Kramer and Ebel 2003). Across the geographical range of *Ae. aegypti,* the ability of a pathogen to establish an infection of the virus depends on both the local mosquito strain and the serotype of DENV present (Gubler et al. 1979). Other work has clearly illustrated that susceptibility, or vector competence in general, is determined by mosquito genotype, virus genotype and their interactions (Fansiri et al. 2013; Lambrechts et al. 2009; Miller and Mitchell 1991; Ye et al. 2014).

EIP ideally should be estimated detecting the amount of virus in mosquito saliva (Zompi and Harris 2012). Most assays for mosquito saliva are destructive, killing mosquitoes at specific time points in order to collect discontinuous data, and requiring large numbers of individuals in order to obtain results across a timespan. A recent study examining the genetic linkage involved in EIP phenotype used the infectious titre of disseminated virus as a measure of infectivity (Fansiri et al. 2013). However, previous studies illustrated that the titre of virus in each mosquito body part can vary dramatically, also depending on the virus isolate (Lambrechts et al. 2009).

Our lab has developed a non-invasive technique that can be used to repeatedly sample DENV-infected mosquitoes (Ye et al. 2015). Here we attempted to estimate heritability of the EIP trait by comparing the EIP phenotype of parents and their offspring using this innovative new method for measuring EIP. The goal was to identify the genes responsible for this trait. To do so, we carried out a half-sib breeding design as per previous approaches (Falconer and Mackay 1996), with the additional goal of obtaining offspring from mosquitoes with either long or short EIP phenotypes. What we found instead indicated a strong influence of age and breeding status on EIP length and repeatability making the study system not appropriate for genomic analysis.

METHODS

Family design and mosquito rearing

Ae. aegypti eggs collected from Queensland, Australia in 2013 were hatched. *Ae. aegypti* species identification was based on specific morphological characteristics. After hatching the larvae were reared at a standard density of 150 individuals per 3L RO water. Larvae were maintained on fish food (Tetramin Tropical Tablets; Tetra, Melle, Germany) until pupation, at which time pupae were sexed and allowed to emerge in 30 cm^3 square cages at a density of approximately 400 individuals (Gerberg et al. 1994). The mosquitos were raised at 25^oC, and 65-70% humidity, under a 12 hour light/dark cycle; adult mosquitoes were maintained on a 10% (w/v) sucrose solution (Moreira et al. 2009b).

A half-sibling breeding design was followed to obtain G2 (Falconer and Mackay 1996). G1 individuals were mass fed and individually mated at 5-6 days post emergence followed by egg collection from multiple gonotrophic cycles (G2). At 5-9 days post emergence, G2 and F2 mosquitoes were mass mated, then given access to a human blood meal. Egg collections were carried out for the period of 1 week, followed by an infectious blood meal at age 16-21 days. These F2 eggs were stored under standard humidity conditions for 3 months while EIP testing of the parental mosquitoes (G2) took place. Following this, mosquito lines (F2) with either short or long EIPs were mass mated as before for egg collection followed by the EIP assay.

Virus

Dengue virus serotype 3 (DENV-3) was grown and collected from *Aedes albopictus* C6/36 cell lines using standard methods (Frentiu et al. 2010). In brief, C6/36 cells were passaged in supplemented RPMI 1640 media (Invitrogen, Carlsbad, CA) at 26ºC until 80% confluency was obtained, then inoculated with virus.

Live virus was harvested by centrifugation at 3200g for 15 minutes from the infected cells supernatant 7 days after inoculation.

DENV infection

For infection, 20-80 mosquitoes aged 16-21 days from each line were given 2 hours of access to a DENV-laden defibrinated sheep's blood meal (mixed at a ratio of 1:1) through a desalted porcine membrane feeder (Moreira et al. 2009a; Rutledge et al. 1964). At 4 hours post feeding, blood engorged mosquitoes were sorted and placed into solitary urine cups.

Saliva Collection

EIP was non-invasively assayed by collecting mosquito saliva in sucrose, using a unique assay developed by Ye et al. (2015). In brief, DENV-fed females were isolated in 250ml polypropylene cups (Sarstedt, Germany) and provided with a source of sucrose in the form of 200µl of 10% sucrose solution pipetted into the cap of a 2ml polypropylene screw-cap tube (Sarstedt, Germany). After 2 days, the cap was changed in order to collect the expectorated saliva to be assayed for DENV.

Mosquito saliva samples were purified using the PureLink™ Pro 96 Viral RNA/DNA Kit (Invitrogen). Viral cDNA was reverse transcribed from RNA using Superscript-III (Invitrogen) and copy numbers were determined using RT-PCR methods described by Moreira et al. (2009a) and Richardson et al. (2006). A Lightcycler480® was used to identify the presence or absence of DENV mRNA in the mosquito saliva samples against a standard curve.

Data Analysis

The average EIP of each mosquito line was calculated using Prism GraphPad software. The data were then plotted, and examined for trends in EIP length using a general linear model. An unpaired t-test was performed to examine significant differences in repeated EIP measures for those family lines assayed over two generations.

RESULTS

EIP was non-invasively assayed across 38 inbred mosquito lines at G2. The first time point at which DENV became detectable in the sucrose feeding solution defined the EIP value. Overall there was a continuum between a mean EIP of 5.33 days and a mean EIP of 12 days for 35 lines (Figure 1), with 3 families not exhibiting a detectable EIP (lines 261, 265, 266). Although there was a linear trend across the dataset, 65.7% of lines had at least one mosquito spitting at DPI 5, with 27.4% of mosquitos spitting at DPI 5. This suggests that regardless of differences in the mean EIP between families there was substantial variation among whereas previous studies had demonstrated clear and consistent family based means (Ye et al. 2015).

Mosquito lines observed to have mean EIPs between 5.33-6.33 DPI (lines 3, 248, 257, 274) or 9-12 DPI (lines 233, 251, 256, 310), in addition to two lines (261, 266) that did not have detectable DENV RNA in the first round of EPI assays were assayed. A continuum between a mean EIP of 7.44 days and mean EIP of 10.17 days was observed (Figure 2). For three out of four fast lines the mean significantly changed between generations ($p < 0.05$), universally becoming slower by 1.5 or more days (Table 1). For the final fast line (line 3) significance was approached but not met ($p = 0.058$); this is likely due to the small original dataset. It can be seen from Figure 2 that individual EIP measures did not tightly cluster around the mean, regardless of the traits of the previous generation.

As the fast phenotype appeared to have been lost within one generation the experiment was halted.

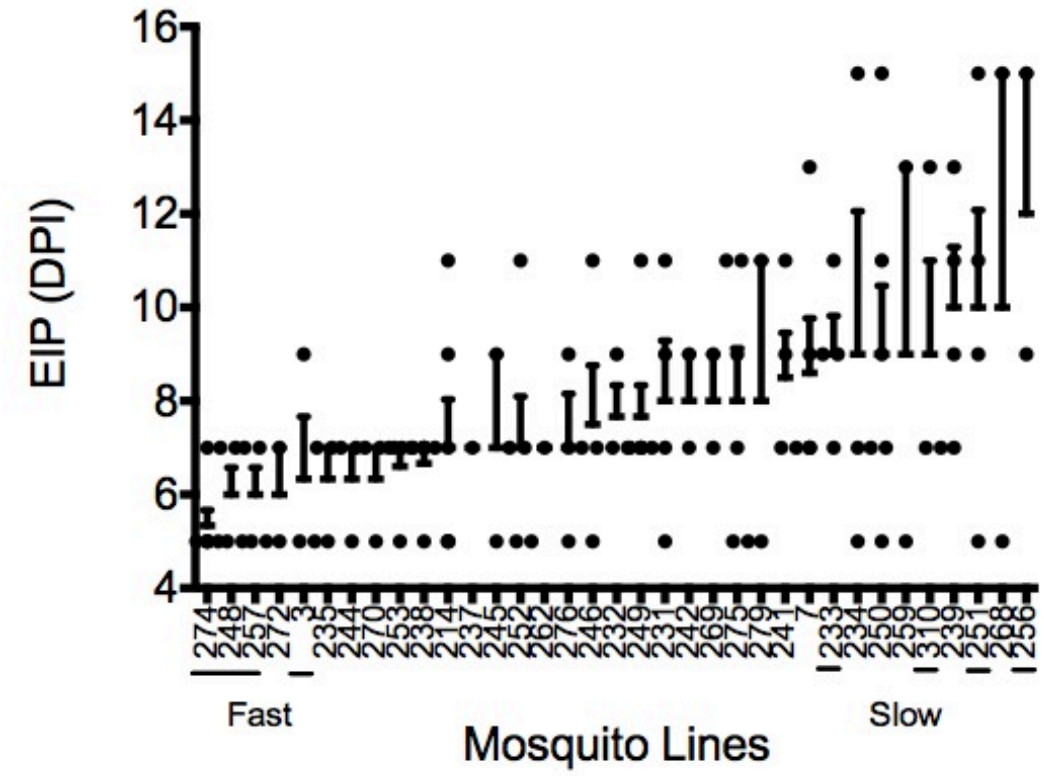

Figure 1 EIP of 35 inbred lines of second generation *Ae. aegypti*. A total of 135 mosquitoes provided a positive DENV sample. Bars depict means ± S.E.M. Fast and slow lines selected for analysis at generation 3 are highlighted.

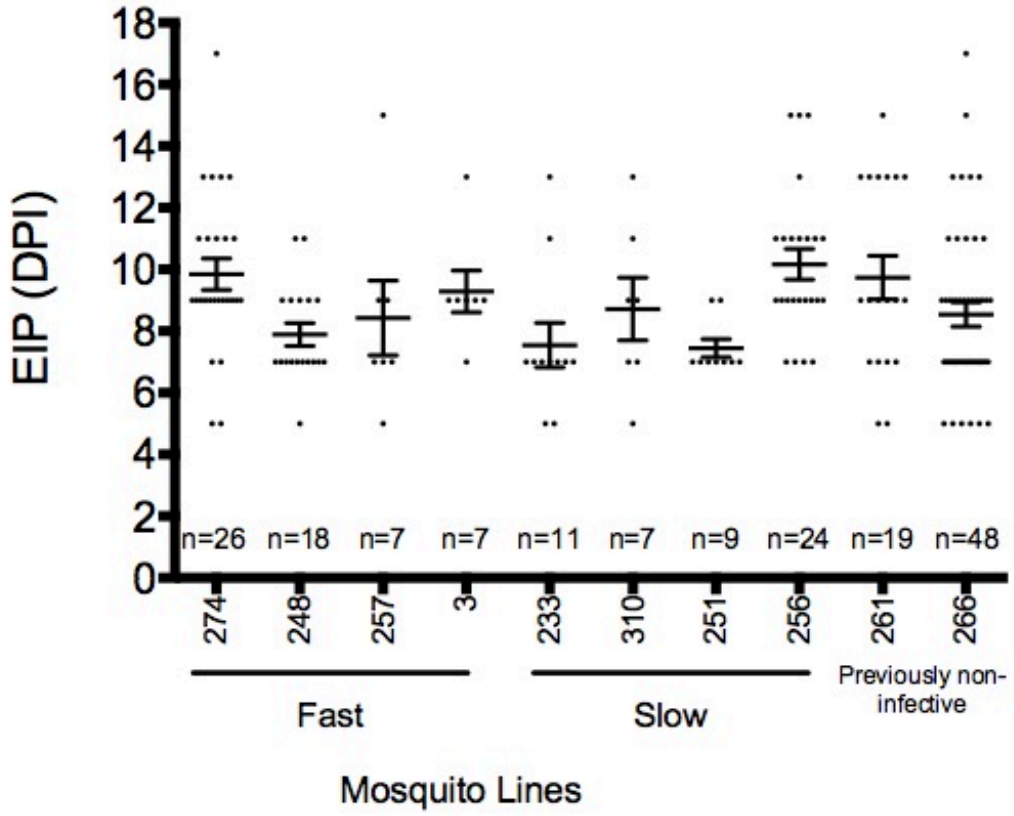

Figure 2 EIP of 10 inbred lines of *Ae. aegypti* assayed at generation 3. Bars depict means ± S.E.M.

Table 1 Mean EIPs of 8 inbred *Ae. aegypti* lines selected as fast or slow from generation 2, and assayed again at generation 3. * indicates p-values that show significant difference between means across generations.

Fast	G2 mean EIP	G3 mean EIP	p-value for mean comparison between G2 and G3
3	6.33	9.29	0.0585
248	6.00	7.89	0.0352 *
257	6.00	8.43	0.0429 *
274	5.33	9.85	0.0003 *
Slow			
233	9.00	7.55	0.2844
251	10.00	7.44	0.0916
256	12.00	10.17	0.3342
310	9.00	8.71	0.8901

DISCUSSION

Other work has clearly illustrated that the variation in mosquito population susceptibility to DENV is due to underlying genetic factors (Miller and Mitchell 1991). Within Australia, DENV resistance differs between geographically isolated *Aedes aegypti* populations (Ye et al. 2014) and also depends on both the local mosquito strain and the serotype of DENV present (Gubler et al. 1979). Thus the hunt for universal infectivity genes is complicated. It is known that the heritability (personal communication) of EIP varies across genetically distinct families at G2 (H^2 = 0.346). To tease out the factors involved in this heritability we attempted to select for lines at each extreme of the EIP variability curve (Figure 1) to isolate the genomic basis of traits. However there were a number of confounding factors that contributed to failure to observe this heritability in our own dataset.

Our fastest lines became significantly slower in in G3, rather than remaining at the extreme of < 6 days as assayed at G2 (Figure 2, Table 1). This does not discount Ye *et al* (2015)'s findings. While our test mosquitoes were generated using the same breeding design and assayed in the same manner as Ye *et al.* mosquitoes, the G2 mosquitoes used in Ye's assay were younger at the DENV feed, ranging between 5-9 days post emergence compared to 16-21 day old mosquitoes in our assay. Mosquitoes become more susceptible to DENV as they age, due to decreased immunity function and decreased detoxification capacity associated with senescence (Styer et al. 2007). Delaying the DENV+ blood meal by 7 days to produce eggs effectively ages the mosquitoes past the usual window of experimental EIP testing. This age dependant effect means that all mosquitoes assayed here would have displayed greater susceptibility to DENV compared to those of Ye et al. (2015) simply due to aging.

Aging and immune function are intricately linked both with themselves and blood feeding. The most informative gene for age-grading mosquitoes, AAEL008844 (Caragata et al. 2011; Hugo et al. 2014), had previously been thought to not have expression altered by a blood meal (Hugo et al. 2010). More recent research (Bonizzoni et al. 2011) demonstrates that gene expression is significantly decreased after blood feeding, and significantly increased after a sucrose meal. If these genes are in any way associated with the aging mosquito phenotype, the initial blood meal given to our mosquitoes may have primed these genes to decrease, and then the age phenomenon kept them at decreased levels, allowing the DENV to more effectively infect the host mosquitoes. Blood feeding has been shown to activate immunity, leading to increased mortality in DENV2 infected mosquitoes (Maciel-de-Freitas et al. 2011). Our mosquito population included those who took two blood meals (human, and DENV laden) and those that took a single blood meal (DENV laden meal only). The repeated blood feeding necessary here to extract eggs from each generation likely primed the

immune response of these mosquitoes to DENV3 and encouraged a higher death rate. This means that some of the mosquitoes that contributed eggs may have exhibited falsely fast EIP rates.

In studies where field and laboratory colonies are exposed to DENV and then assayed for fecundity, regardless of the experimental mosquitoes' life history, mosquitoes that are refractory for DENV suffer from poor egg laying abilities, while susceptible mosquitoes are likely to lay more eggs (Maciel-de-Freitas et al. 2011; Sylvestre et al. 2013). In this light, our observation of the move towards the mean group EIP by refractory mosquito lines in just one generation does not seem farfetched. Although the majority of the members of the line may have been refractory, the most susceptible members may have contributed to a greater extent to the following generation.

This work has the potential to influence the 'freshening' practices performed in most mosquito lines maintained under laboratory conditions to prevent the effects of inbreeding on line characteristics. Likewise, there should be more investigation into the routine act of producing inbred lines and the optimum number of generations to maintain before seeing fixation of the EIP phenotype. The difficulty of this will be directly reflected in what causes the loss of the highly susceptible mosquito phenotypes – if the issue is fitness costs on the mosquito, rather than age influences on susceptibility, this may cause a tandem shift in what inbred lines can be selected for without losing the entire population.

CONCLUSION

Although this experimental approach has not been able to answer the question of the basis of genetic variability that underlies the heritability of EIP, it has elucidated other valuable information. Work that may come out of this area includes a further investigation into the effect of intervening human blood meals potentially priming the mosquito for DENV dissemination, or the effect of mosquito susceptibility on producing inbred lines.

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Appendix 2

The nature of the immune response in novel *Wolbachia*-host associations

Chapter 3 of this thesis was published in the peer-reviewed journal Symbiosis in August 2017. The following pages consist of the final published PDF.

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SHORT COMMUNICATION

The nature of the immune response in novel *Wolbachia*-host associations

Rosemarie I. Herbert[1] · **Elizabeth A. McGraw**[1]

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Abstract *Wolbachia* is an obligate, intracellular symbiont that is commonly found in insects and causes a diverse array of reproductive manipulations. Normally transmitted vertically, the occasional horizontal host species jump can be seen in the lack of concordance between *Wolbachia* and host phylogenies. In the laboratory, the symbiont can be artificially introduced into novel hosts and selected to produce persistent infections. In the case of the vector of dengue virus, *Aedes aegypti*, the symbiont was successfully introduced with the aim of developing the bacterium for biocontrol. In this insect and others, *Wolbachia* limits co-infection with pathogens including viruses, bacteria and parasites. Here we have novelly infected cell lines derived from diverse insect species with *Wolbachia* in an attempt to determine if there are commonalities in the early host response to the symbiont. We then monitored the expression of genes in the antibacterial Toll and Imd pathways in the first several passages. We focused on immunity gene expression as it underpins the bulk of the transcriptional response to *Wolbachia* and because it may play a role in the pathogen blocking effect. We found that successful cell infections of *Wolbachia* were difficult to achieve and often required repeated rounds of reinfection. We saw significant variation in the nature of the transcriptional changes across cell lines and no attenuation of gene expression changes in the first several passages. These results suggest that insect species are likely to exhibit distinct responses to *Wolbachia* infection. They also reveal that any evolution of an attenuated transcriptional response, as predicted by long-standing *Wolbachia* x host associations, is not likely to occur rapidly. The findings will have implications for biocontrol programs that rely on the novel infection of naïve hosts.

Keywords Evolution · Innate immunity · *Wolbachia* · Dengue · Endosymbiont · Insects

1 Introduction

Wolbachia is a gram-negative, maternally inherited endosymbiotic bacterium that is present in 40–60% of known insect species (Zug and Hammerstein 2012). *Wolbachia* is also known to infect non-insect arthropods including mites (Breeuwer and Jacobs 1996), spiders (Oh et al. 2000), crustaceans (Cordaux et al. 2001; Cordaux et al. 2012) and nematodes (Sironi et al. 1995). Numerous *Wolbachia* strains have undergone extensive host jumps between these invertebrates, primarily identified by the lack of strong concordance between host and *Wolbachia* phylogeny (Werren et al. 1995; Zug et al. 2012). The range of possible means for transmission of symbionts between species include, but are not limited to; wounding by parasitoid wasps (Ahmed et al. 2015; Gehrer and Vorburger 2012) and mites (Jaenike et al. 2007), cross-infection by infected and uninfected hosts during copulation (Moran and Dunbar 2006) and cofeeding (Caspi-Fluger et al. 2012) and directly from the environment (Kikuchi et al. 2007). While these transfer events appear common on phylogenetic timescales, they are rare in real time.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13199-017-0503-6) contains supplementary material, which is available to authorized users.

✉ Elizabeth A. McGraw
beth.mcgraw@monash.edu

Rosemarie I. Herbert
rosemarie.herbert@monash.edu

[1] School of Biological Sciences, Monash University, Clayton Vic 3800, Australia

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While the history of successful host jumps can be seen in the phylogeny, many more cross species infection events must occur that are not successful. Researchers that attempt to create artificially transinfected host species in the laboratory, are fully aware of the difficulty of transitioning *Wolbachia* between species (Bian et al. 2013a; McMeniman et al. 2009; Xi et al. 2005a). Often many thousands of embryos must be infected to obtain a single female that harbors *Wolbachia* in the germline and then transmits to the symbiont to her offspring. Frequently these newly infected insects fail to produce offspring. These transitions work best when the donor and recipient host are closely related. The assumption is that long associated *Wolbachia* and host species have coadapted with time. This notion is supported by improved transinfection rates in insects following periods of co-culture of *Wolbachia* in cells of the target species (McMeniman et al. 2008).

Wolbachia infection in insects has been shown to interfere with the replication of a broad range of co-infecting pathogens including; viruses, filarial nematodes, bacteria and the malaria parasite (Hedges et al. 2008; Kambris et al. 2010; Kambris et al. 2009; Moreira et al. 2009; Teixeira et al. 2008; Ye et al. 2013). *Wolbachia* was artificially transinfected into the mosquito vector of dengue virus (DENV), *Aedes aegypti* in a bid to develop the symbiont as a form of biocontrol. A range of *Wolbachia* strains have been introduced to date: *w*Mel and *w*MelPop from *Drosophila melanogaster* (McMeniman et al. 2009; Walker et al. 2011a), *w*AlbB from *Aedes albopictus* (Xi et al. 2005b) and a superinfection of *w*Mel/*w*AlbB (Joubert et al. 2016). In the novelly infected *Ae. aegypti, Wolbachia* appears to evoke a more substantial immune response than in the donor hosts as measured by the number of genes with changed expression and the magnitude of that expression change (Pan et al. 2012; Rancès et al. 2012; Ye et al. 2013) (Table 1). While this induced immune response may play a part in *Wolbachia*-mediated pathogen blocking (Bian et al. 2010; Pan et al. 2012; Rancès et al. 2012) it is not sufficient to explain the effect (Caragata et al. 2013; Moreira et al. 2009). Given the limited immune response of native hosts to *Wolbachia*, the hyperactivated response in novel hosts is expected to decline with time via coevolution (McGraw et al. 2002). As native hosts tend to harbor lower *Wolbachia* densities and contracted tissue distributions as a result of coadaptation (Bian et al. 2013b; Osborne et al. 2012; Pan et al. 2012) this may offer a potential path by which reduced activation of the immune response emerges.

The main insect defense mechanism against pathogens is the innate immune system. There are four major inducible pathways, namely the Toll and Imd pathways for bacteria (Buchon et al. 2014), and the JAK/STAT and RNAi pathways for viruses (Myllymaki and Ramet 2014; Xu and Cherry 2014). Some of these pathways may participate in the production of antipathogen effects. In addition, some of these pathways may directly affect *Wolbachia* loads in the host, particularly the pathways that target bacteria – Toll and Imd. The activity of these pathways is commonly measured by expression change in genes encoding the antimicrobial peptides diptericin, cecropin and defensin. Native *Wolbachia* infections in *Drosophila* have no effect on the transcription of these antimicrobial peptide genes (Bourtzis et al. 2000; Rancès et al. 2012; Wong et al. 2011), but a strain of *Wolbachia* introduced from *Drosophila simulans* into *Drosophila melanogaster* lead to increased expression of diptericin expression (Xi et al. 2008) (Table 1). *Ae. albopictus* and *Ae. aegypti* mosquitoes upregulate expression of these key peptide

Table 1 Summary of previous studies' results on innate immune genes in *Wolbachia* infections of *Aedes* and *Drosophila* species. Blank cells indicate that gene expression for that cell line was not tested

	Host organism	*Wolbachia* strain	Immune Genes				Reference
			Relish	Defensin	Cecropin	Diptericin	
Aedes species	*Ae. albopictus*	*w*AlbB		Unaffected			Bourtzis et al. (2000)
	Ae. aegypti	*w*AlbB	Upregulated	Upregulated	Upregulated	Upregulated	Bian et al. (2010)
	Ae. aegypti	*w*AlbB	Upregulated	Upregulated	Upregulated	Upregulated	Pan et al. (2012)
	Ae. aegypti	*w*Mel		Upregulated	Upregulated	Upregulated	Rancès et al. (2012)
	Ae. aegypti	*w*MelPop	Upregulated	Upregulated	Upregulated		Kambris et al. (2009)
	Ae. aegypti	*w*MelPop		Upregulated	Upregulated	Upregulated	Rancès et al. (2012)
	Ae. fluviatilis	*w*Flu	Unaffected	Unaffected	Unaffected	Unaffected	Caragata et al. (2017)
Drosophila species	*D. melanogaster*	*w*MelCS		Unaffected		Unaffected	Wong et al. (2011)
	D. melanogaster	*w*Mel		Unaffected	Unaffected	Unaffected	Rancès et al. (2012)
	D. melanogaster	*w*MelPop		Unaffected	Upregulated	Unaffected	Rancès et al. (2012)
	D. melanogaster (S2 cell line)	*w*Ri	Upregulated	Unaffected	Unaffected	Upregulated	Xi et al. (2008)
	D. simulans	*w*Ri		Unaffected	Unaffected	Unaffected	Bourtzis et al. (2000)

genes, particularly in response to novel *Wolbachia* infections (Bian et al. 2010; Kambris et al. 2009; Pan et al. 2012; Rancès et al. 2012). However, in another *Aedes* species that is naturally *Wolbachia* infected, *Ae. fluviatilis,* Toll and Imd immune gene transcription is unaffected (Caragata et al. 2017). Evidence thus far therefore suggests that *Wolbachia* infections affect the expression of immune pathways in their native hosts less than in their novel hosts.

While in vivo experiments provide valuable information about adaptation, very low transinfection rates of insects render them virtually intractable for study of repeated introductions of *Wolbachia*. Here we have examined the nature of the early host response to *Wolbachia*, by infecting a range of cell lines from diverse species with two *Wolbachia* strains, *w*Mel from supergroup A and *w*AlbB from supergroup B. In the newly infected lines represented by two mosquito species (*Ae. albopictus*, *Anopheles gambiae*), a moth (*Spodoptera frugiperda*) and *D. melanogaster*, we have examined the nature of the immune response immediately following infection and across several passages. We have sought to determine whether there are commonalities in the nature of the early host immune response across these species and if they exhibit rapid evolution.

2 Materials & methods

2.1 Cell culture

The following recipient cell lines are naturally *Wolbachia* free: Aag2 (Peleg 1968), RML12 (Kuno 1983), Mos55 (Pudney et al. 1972), S2 (Schneider 1972) and SF9 (Vaughn et al. 1977). We also used the Ae23T cell line (O'Neill et al. 1997) and the Aeg2*w*Mel.tet cell line (Terradas et al. 2017), both of which had been previously tetracycline treated to remove the *Wolbachia* infections. Aag2 cells with *w*Mel (Terradas et al. 2017) or Ae23 cells naturally infected with *w*AlbB (O'Neill et al. 1997) served as donors of *Wolbachia*. The latter cell line was established from eggs of *Ae. albopictus* that were naturally infected with both *w*AlbA and *w*AlbB, but only one of these co-infecting *Wolbachia* strains were present in the resulting cell line (O'Neill et al. 1997). Further details for the cell lines used can be found in Table 2.

While maintaining cell lines, different methods were piloted to best encourage *Wolbachia* infection levels, including altering the percentage of FBS used to supplement cells (5%–25%), the length of passaging times (3–14 days), degree of cell detachment, flask size when expanding cell numbers (12 well plate - 75-cm^2 flask) and amount of introduced media at the point of passage. All uninfected cell lines were cultured in 25-cm^2 cell culture flasks at 26 °C in 5 ml of growth medium, supplemented with 10% (*v*/v) of heat-inactivated foetal bovine serum and penicillin/streptomycin (50 U/50 μg ml^{-1}). Aag2, Ae23 and RML12 cell lines were maintained in medium consisting of equal volumes of Mitsuhashi&Maramorosh and Schneider's insect medium (Sigma, France) (O'Neill et al. 1997). Aa23T cells were maintained in the same medium, with an additional 10% of FBS added. S2 and Mos55 cells were maintained in Schneider's insect medium, and SF9 cells were cultured in SF9 medium. Cells newly infected with *w*Mel were maintained for only five passages with the *Wolbachia* infection dropping out by passage six. In contrast, cells newly infected with *w*AlbB exhibited greater stability and we maintained for 10 passages without any loss of *Wolbachia*. *Wolbachia* was assessed at each passage by FISH.

Table 2 Characteristics of the cell lines used in this experiment, including *Wolbachia* donor sources

Cell line abbreviation	Source organism of cell line	Source material	Reference
Aag2 + *w*Mel	*Ae. aegypti* *Wolbachia* strain from *D. melanogaster* (*w*Mel)	Cell line: embryos *Wolbachia* source: ovaries	Terradas et al. (2017)
Ae23 + *w*AlbB	*Ae. albopictus* *Wolbachia* strain from *Ae. albopictus* (*w*AlbB)	Cell line: Embryos *Wolbachia* source: naturally infected	O'Neill et al. (1997)
Aag2	*Ae. aegypti*	Cell line: Embryos Naturally *Wolbachia* free	Peleg (1968)
RML12	*Ae. albopictus*	Cell line: Larvae Naturally *Wolbachia* free	Kuno (1983)
Mos55	*An. gambiae*	Cell line: Larvae Naturally *Wolbachia* free	Pudney et al. (1972)
S2	*D. melanogaster*	Cell line: Embryos Naturally *Wolbachia* free	Schneider (1972)
SF9	*S. frugiperda*	Cell line: Ovaries Naturally *Wolbachia* free	Vaughn et al. (1977)
Ae23T	*Ae. albopictus*	Cell line: embryos Native *Wolbachia* removed by tetracycline	O'Neill et al. (1997)
Aag2*w*Mel.tet	*Ae. aegypti* *Wolbachia* strain from *D. melanogaster*	Cell line: embryos *Wolbachia* source: ovaries *Wolbachia* removed by tetracycline	Terradas et al. (2017)

2.2 Infection experiments

Infection of cell lines with *D. melanogaster*-derived *Wolbachia* was carried out using the shell vial technique, as previously described (McMeniman et al. 2008; Terradas et al. 2017; Voronin et al. 2010).

Infection of cell lines with *Wolbachia* sourced from established cell lines was carried out using a blend of the protocols found in Dobson et al. (2002) and Lu et al. (2012). *Wolbachia* was sourced was from Aag2 cells hosting *w*Mel or Ae23 cells hosting *w*AlbB then infected into *Wolbachia* uninfected cell lines (Fig. 1). In brief, source *Wolbachia* cells were cultured in 75-cm^2 flasks containing 12 ml of medium. Cells were collected by shaking flasks vigorously, then centrifuged at 1000 RPM at 4 °C before discarding the supernatant and resuspension of cells in 10 ml of SPG buffer (218 mM sucrose, 3.8 mM KH_2PO_4, 7.2 mM MK_2HPO_4, 4.9 mM L-glutamate, pH 7.5) per 12 ml original media (Lu et al. 2012). Two washes were performed by centrifugation at 1000 RPM at 4 °C followed by resuspension in 10 ml of SPG buffer per 50 ml of original cell culture. Cells were sonicated on ice for 2 bursts of 10 s (18–23% power), and then cell debris was pelleted at 1000 RPM at 4 °C. The resultant supernatant was filtered successively through a 5 μm filter and 2.7 μm filter and cells were resuspended in SPG buffer at a ratio of 100 μl per 50 ml original source media, then 50 μl of this was immediately overlaid on an 80% confluent monolayer (2 ml media) of each of the cell lines of interest in a single well of a 12-well plate (Corning Life Sciences). The cells were transferred into a 25-cm^2 flask with 3 ml of new medium 3 days later when cell approached 95% confluency. When cells started detaching from the flask, 2 ml was spun down at 400 RPM at 4 °C and resuspended in 200 μl of TRIzol® (Life Technologies, Carlsbad, California, USA). The remaining cell-containing medium (~2–3 ml) was transferred into 75-cm^2 flasks with 10 ml of fresh medium.

2.3 Fluorescence in situ hybridization (FISH)

FISH was carried out as per (Moreira et al. 2009) using the *Wolbachia* probes [50-ACCAGATAGACGCCTTCGGCC-30] and [50-CTTCTGTGAGTACCGTCATTATC-30] (Xi et al. 2005a). For negative controls of FISH, pre-infection cell lines were used.

2.4 Quantitative PCR

The TRIzol® method from Invitrogen (Life Technologies, Carlsbad, California, USA) was used to extract total RNA from cell samples. RNA quality and quantity was checked with a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Inc.). Synthesis of cDNA was performed with Superscript II Reverse Transcriptase (Invitrogen) as per the manufacturer's instructions. The genes assayed were: attacin, cactus, cecropin, defensin, dFADD, dorsal, IMD, MyD88, relish and TAB2 (primer sequences listed in Online Resource 1). Amplification was carried out using the LightCycler LC480 system (Roche, Meylan, France) with a

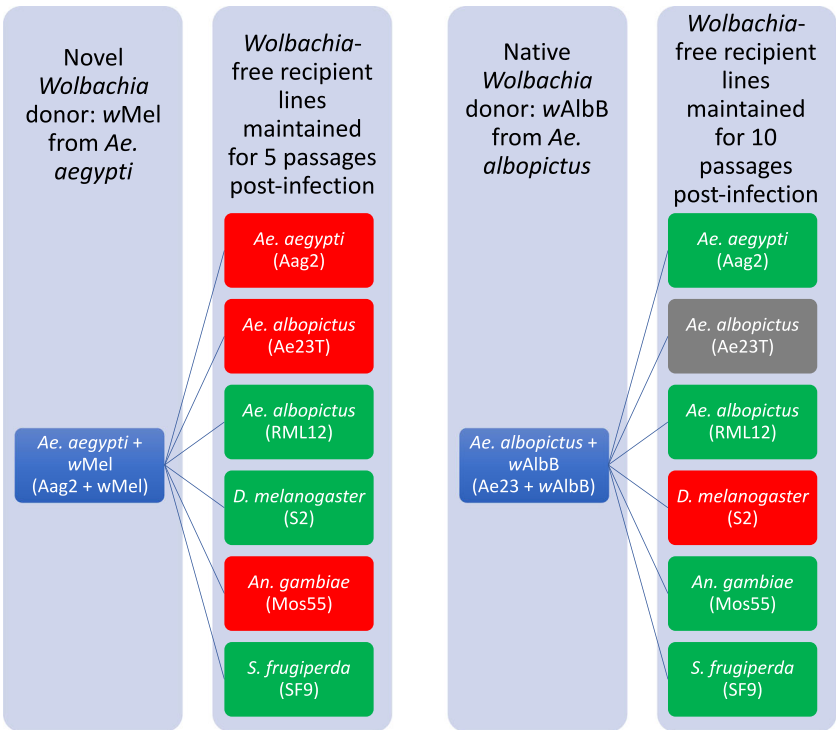

Fig. 1 Flow chart illustrating the two *Wolbachia* source cell lines (*blue*), the novel cell line infections established and the number of passages for which the novel cells lines were maintained. Colors of novel cells correlate with successful infection (*green*), unsuccessful infection (*red*) and not attempted (*grey*)

Platinum SYBR Green qPCR superMix (Invitrogen). qRT-PCR reactions were conducted using a 2 min step at 50 °C, 2 min step at 95 °C and 40 cycles of 15 s at 95 °C and 30 s at 56 °C. A fluorescence measurement was made at the end of each cycle. A melting curve analysis was performed at the end of the amplification program to examine for primer-dimers or nonspecific amplification. Duplicate qRT-PCR reactions were performed for each sample. Relative expression of genes was calibrated against the reference gene using the $\Delta\Delta C_T$ calculation method (Livak and Schmittgen 2001).

2.5 Statistical analysis

Multiple infection experiments were carried out (Table 3). In most cases only a subset of the recipients became infected. The final successful experiment for each donor line was defined by achieving successful infections in parallel in all of the recipients. Statistical analysis was carried out on the final successful study. To determine whether there was an association between gene expression and cell line in this experiment we performed a general linear model on log transformed expression data. Posthoc comparisons were then carried out and multiple corrections accounted for using a Bonferroni correction (Bonferroni 1935). Pearson chi-squared test was used to assess whether there was a significant difference in the direction (suppression or increase) of gene expression by insect compared to the original uninfected cell line, with a minimum of 1.5 fold change. We then tested for the effect of passage number on expression for all insects and genes, individually and as a group (ANOVA). All analyses were carried out in SPSS (IMB Statistics for Windows, Version 20.0 Armonk, NY).

3 Results

3.1 Cell culture

To optimize conditions for *Wolbachia*-infected cells, several cell passaging procedures were trialed. We found that using 20% FBS promoted *Wolbachia* growth, as did extending the length of passaging times to 14 days and allowing at least 50% of cells to detach prior to passaging. Upon each passage, 60% of the new flask's volume was composed of the previous flask's detached cell-containing media and the remainder was fresh media. This encouraged healthy cell growth in all cell lines, and prevented the early loss of cell lines in the first passage after *Wolbachia* infection.

3.2 Establishing persistent *Wolbachia* infections

We aimed to infect novel cell lines with *w*Mel (*D. melanogaster* origin) as it is the current strain of *Wolbachia* being released into the field as a biocontrol agent. Using *D. melanogaster* ovaries, we attempted to infect all the *Wolbachia*-negative cell lines at our disposal five times in parallel. However, each time we experienced bacterial contamination from the raw ovary preparation. Instead, by using a Aag2 cell line previously infected with *w*Mel (Terradas et al. 2017) it was possible to produce a single *w*Mel infected line (n = 1) at ~85% infection frequency (Fig. 2) for each of Ae23T, S2 and SF9 but not Aag2, RML12 or Moss55. This level of success required seven independent attempts (Table 3) and by passage 4 the *Wolbachia* had largely disappeared from the lines. In contrast, more of the recipient cell lines were successfully infected with the *w*AlbB strain. It took five independent infection attempts to achieve persistent high density *w*AlbB infections in Aag2, RML12, Mos55 and SF9 cell lines (Fig. 3) but not S2 or Ae23Tcells (Table 3). The successful *w*AlbB infections were maintained for >10 cell passages, and the cell infection frequency approached 90% in some cell lines. These findings confirm the role of the *Wolbachia* genome, not just the host genome in dictating the success of partnerships.

3.3 Innate immune gene expression

Eight different immunity genes were tested for changes in expression through time in *D. melanogaster*, *S. frugiperda* and *Ae. albopictus* cells following *Wolbachia* introduction (comparative to the uninfected cell line). First, we examined the magnitude of expression change across cell lines, grouping

Table 3 Summary of novel cell lines infected with *Wolbachia* in this experiment. Blank cells indicate that *Wolbachia* infections for that cell line could not be established

Cell line	Number of attempts at *w*Mel infection	*w*Mel infection established?	Number of attempts at *w*AlbB infection	*w*AlbB infection established?
Aag2	7		5	single successful infection
Ae23T	7	single successful infection		
RML12	7		5	single successful infection
S2	7	single successful infection	3	
Mos55	7		5	single successful infection
SF9	7	single successful infection	5	single successful infection

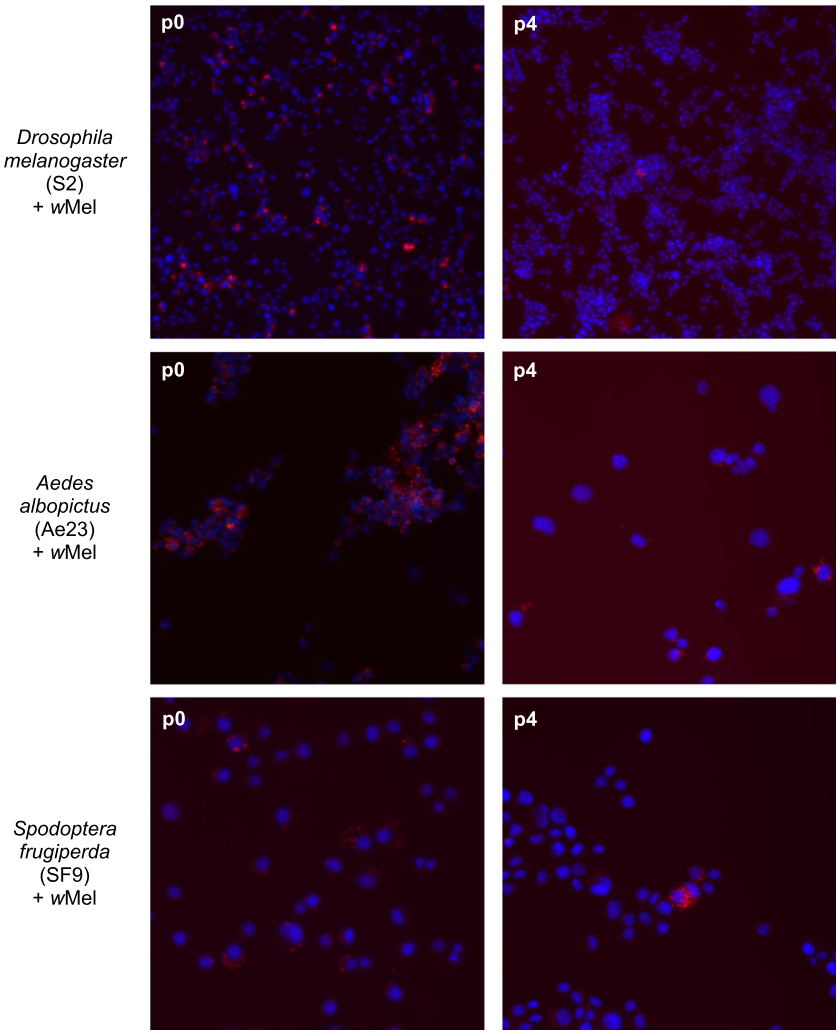

Fig. 2 FISH for wMel at ×100 magnification in three cell lines at two time points: passages 0 and 4 post-infection. Host nuclei stained in blue (DAPI) and *Wolbachia* 16 s rRNA stained in red (specific probe labeled with rhodamine). The number of cells imaged reflects the number of cells fixed onto the FISH slide. Infections wane by passage 4

all passage results together. We observed a significant effect ($F = 22.489$, $df = 2$, $p < 0.001$) of cell line, marked by suppression of immune gene expression in *D. melanogaster* cells and increased expression of genes in *S. frugiperda* and *Ae. albopictus* ($X^2 = 37.363$, $df = 2$, $p < 0.001$). We then compared each pair of insects. We detected significant differences between the fly and each of the other two insect samples, with the fly suppressing immune genes while mosquito and moth samples increased gene expression (fly:mosquito $X^2 = 23.587$, $df = 1$, $p < 0.001$, fly:moth $X^2 = 28.390$, $df = 1$, $p < 0.001$). No significant difference was observed between the moth and mosquito samples ($X^2 = 0.313$, $df = 1$, $p > 0.05$). This may be because both the moth and mosquito are naturally *Wolbachia*-uninfected, while the fly is the original source of the *Wolbachia* strain.

These results were recapitulated when examining the fold change in expression for each species individually. *D. melanogaster* cells infected with *w*Mel suppressed expression of Toll and Imd pathway genes in addition to 2 out of 3 other antimicrobial genes tested (Fig. 4). In contrast, the *Ae. albopictus* cell line showed an overall trend of innate immune activation following *w*Mel infection (4 promoted and 2 suppressed). Relish was not expressed in *Wolbachia*-free cells. Finally, in *S. frugiperda* cells, the majority of genes were unaffected, with only Cactus, Dorsal and Attacin showing greater than 1.5 fold changes in expression levels compared to the original *Wolbachia*-free line. This range of responses suggests that there is no one gene or pathway defines the novel host response.

We then compared the direction of regulatory change of genes by cell line. Overall there was a significant effect of cell line ($X^2 = 37.363$, $df = 2$, $p < 0.001$). Post hoc comparisons revealed that fly vs mosquito cell lines ($X^2 = 23.587$, $df = 1$, $p < 0.001$) and fly vs moth cell lines ($X^2 = 28.390$, $df = 1$, $p < 0.001$) differed from one another. The fly sample suppressed the majority of immune genes tested, while the mosquito and moth samples increased gene expression. There was no difference in expression between the mosquito and moth cell lines ($X^2 = 0.313$, $df = 1$, $p > 0.05$).

We then tested for the effect of passage number on expression and found that there was no significant difference in magnitude of expression by cell line (fly $F = 0.613$, $df = 4$,

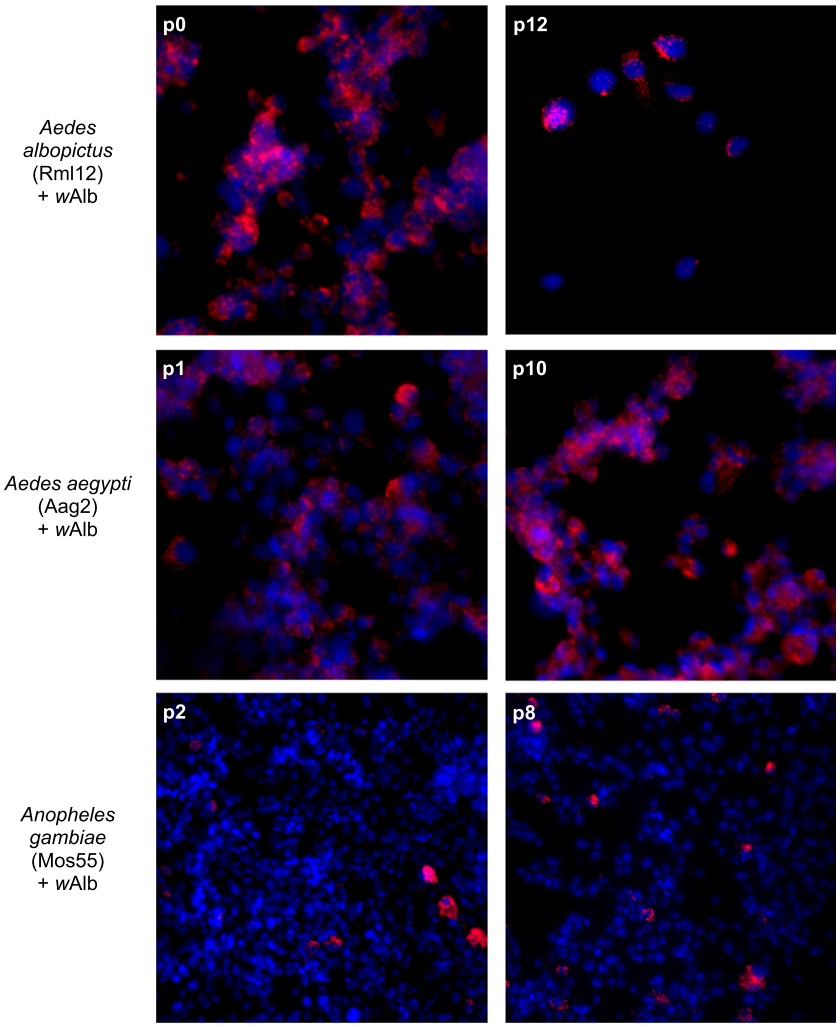

Fig. 3 FISH for *w*AlbB at ×200 magnification in three cell lines at early and late time points following infection. Host nuclei stained in blue (DAPI) and *Wolbachia* 16 s rRNA stained in red (specific probe labeled with rhodamine). Infections remain stable over time

$p > 0.05$, mosquito $F = 0.475$, $df = 3$, $p > 0.05$, moth $F = 2.005$, $df = 4$, $p > 0.05$) or individual genes (attacin $F = 0.057$, $df = 4$, $p > 0.05$, cactus $F = 0.273$, $df = 4$, $p > 0.05$, cecropin $F = 0.379$, $df = 4$, $p > 0.05$, defensin $F = 0.312$, $df = 4$, $p > 0.05$, dFADD $F = 0.151$, $df = 4$, $p > 0.05$, dorsal $F = 0.254$, $df = 4$, $p > 0.05$, IMD $F = 0.828$, $df = 4$, $p > 0.05$, MyD88 $F = 0.066$, $df = 4$, $p > 0.05$, relish $F = 0.384$, $df = 4$, $p > 0.05$, TAB2 $F = 0.160$, $df = 4$, $p > 0.05$). Given the drop in *Wolbachia* infection levels (Fig. 2) we had predicted we would see corresponding declines in immune activation (Fig. 4).

4 Discussion

A specific analysis of potential immune genes important in the *Wolbachia*-host interaction has been conducted in vitro using insect cell lines of mosquito, fly and moth origin. We assessed the transcription of 2 major immunity gene pathways (Toll, Imd), and found that in *Drosophila* these pathways were suppressed, while the opposite was true in the mosquito and moth samples. We also examined whether this response changed over several cell passages, but saw no significant differences.

4.1 Establishing persistent *Wolbachia* infections

To replicate the biological conditions when establishing *Wolbachia* infections for biocontrol (McMeniman et al. 2009; Walker et al. 2011a), we attempted to use *D. melanogaster* ovaries to infect the *Wolbachia*-negative cell lines. Although we could establish a *w*Mel infection in naïve Aag2 cells, this infection was at very low levels (less than 10% of cells). We were unable to establish additional cell lines for comparative studies. Instead, we used a previously developed cell line of Aag2 (Terradas et al. 2017) as a source of *w*Mel as the *Wolbachia* was cell line adapted. In all *w*Mel infected cell lines *Wolbachia* infection was lost by five passages post-infection. Previous studies have also found that *w*Mel does not persist in S2 and Ae23 cell lines (Voronin et al. 2010; Xi et al. 2008). The earlier loss of *w*Mel in our case may be explained by our method, as we infected all cells once in parallel instead of multiple sequential times. In the *Ae. albopictus* cell line,

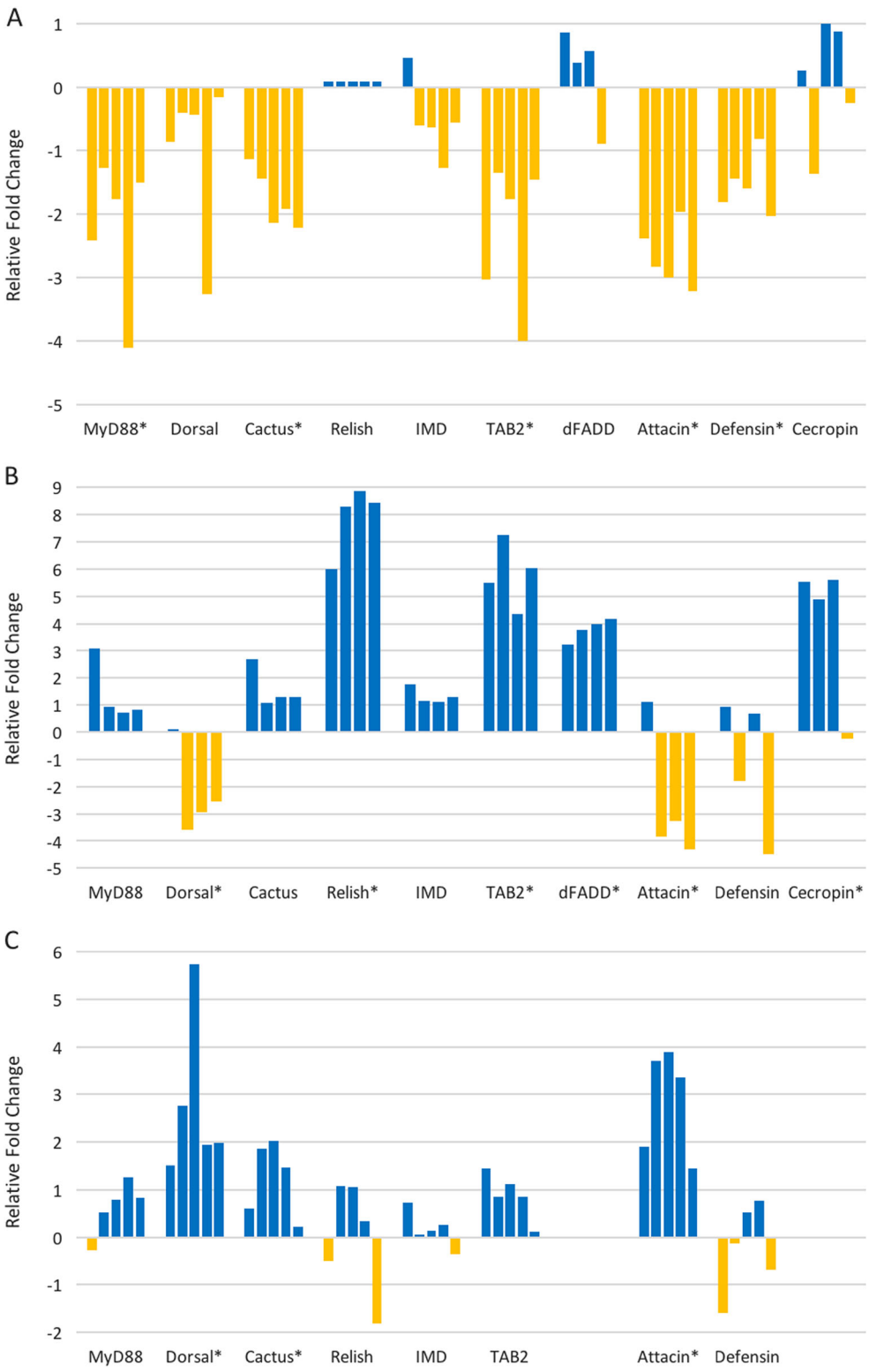

Fig. 4 Fold change of Toll, Imd and antimicrobial peptide genes in *Wolbachia* (*w*Mel) infected samples, relative to uninfected cell line. **a** *Drosophila melanogaster* (S2). **b** *Aedes albopictus* (Ae23). **c** *Spodoptera frugiperda* (SF9). For each gene, the bars represent passages 0–4. *denotes >1.5 fold change in the majority of passages. Colors correlate with upregulation (*blue*) and down (*yellow*)

loss may also be assisted by the upregulated innate immune response (Blagrove et al. 2012; Xi et al. 2008). In *Ae. aegypti* mosquitoes, *w*Mel exhibits higher symbiont loads, broader tissue distributions and a greater fitness cost than in its native fly host (Ross et al. 2014; Turley et al. 2013; Voronin et al. 2010; Walker et al. 2011b). These greater fitness costs of *w*Mel likely explain our inability to establish *w*Mel infections in other cell lines as well, particularly the naïve Aag2 line. Cell-line adaptation of *Wolbachia* in mosquito cells caused it to lose its ability to infect its original host *D. melanogaster*

(McMeniman et al. 2008). The Aag2 + *w*Mel line was separately established more than 5 years ago. We suggest that *w*Mel from Aag2 is cell-line adapted but that there has been a lack of selection to maintain diversity in the genome of *w*Mel *Wolbachia* which prevents it successfully reestablishing itself in a new host.

The high density *w*AlbB infections we created were stable over 10 subsequent passages. However, the loss of *w*AlbB infection in our donor cell line prevented us from assaying the changes in immune gene reactions in the earliest passages following infection. Interestingly, *w*AlbB exhibits low levels of infection in its native mosquito host (Walker et al. 2011b; Xi et al. 2005b) and following transinfection into the novel mosquito host, *Ae. aegypti* (Axford et al. 2016; Xi et al. 2005b). The tetracycline treated *Ae. albopictus* cell line (Ae23T) has previously been re-infected with a persistent *w*AlbB infection and with a wide range of other *Wolbachia* strains (Dobson et al. 2002), suggesting that native hosts are more readily receptive to new *Wolbachia* infections than *Wolbachia*-naïve hosts. Likewise, novel *Wolbachia* infections using *w*Ri from *Drosophila simulans* were previously established in Ae23T, S2, C6/36 and SF9 cell lines using the shell vial technique (Dobson et al. 2002). The infection levels varied based on cell type (10% of the S2 cells and >90% of the SF9 cells) but were always less than in the 100% infection rate found in the original host. In whole insects, *w*Ri transferred by microinjection from *D. simulans* to *D. melanogaster* also occurred at lower densities in the new host than in the native (Boyle et al. 1993). These studies suggest that the behavior of individual host:*Wolbachia* pairings may be affected by their history/lack of history of adaptation as well as *Wolbachia*-strain specific characteristics.

The differences seen in infection success in the cell lines for *w*AlbB and *w*Mel are mirrored in *Ae. aegypti*, where recently a double infection of the two strains was created (Joubert et al. 2016). The former strain when alone has a reduced tissue density compared to *w*Mel, causes fewer fitness consequences and induces weaker pathogen blocking (Axford et al. 2016; Lu et al. 2012; Pan et al. 2012; Walker et al. 2011b; Xi et al. 2005a; Xi et al. 2005b). In the double infection, the two *Wolbachia* strains co-localise. The *w*AlbB strain exhibits a similar density to when it is alone in *Ae. aegypti*, while *w*Mel exhibits a higher density. The behavior of *w*AlbB reflects that of its native host and a history of coadaptation in a closely related mosquito. The *w*Mel strain, in contrast, with a history of adaptation in both species and potentially greater selection for stronger growth during passaging in mosquito cells, responds differently to coinfection.

4.2 Innate immune gene expression

Relish is critical in Imd signaling in response to challenge with gram-negative bacteria in *Drosophila* (Dushay et al. 1996). Interestingly, we found that *w*Mel suppressed expression of Imd and Toll pathway effectors but did not express Relish basally or after *Wolbachia* infection. Thus we hypothesize that *Wolbachia* does not activate the immune response of *Drosophila* in a typical Imd signaling manner. Novel *w*Ri infected *Drosophila* cell lines have multiple genes in the Toll and Imd immune signaling pathways that respond with higher expression (Xi et al. 2008). In contrast, native host fly samples infected with *w*Mel, *w*MelCS or *w*MelPop-CLA in *D. melanogaster* and *w*Ri in *D. simulans* show no changes in antimicrobial expression (Bourtzis et al. 2000; Rancès et al. 2012; Wong et al. 2011). The difference between the results of Bourtzis et al. (2000); Rancès et al. (2012) and Xi et al. (2008) are likely due to native vs non-native strains of *Wolbachia* used for the respective infections. We suggest that our finding, that *w*Mel affects gene expression in its native *D. melanogaster,* could be due to the long history of *w*Mel adaptation in *Ae. aegypti* cells. Alternatively, our results may differ as we captured the time-period directly following infection, while the previous study on S2 cells (Xi et al. 2008) took snapshots of expression after multiple shell vial infection procedures.

Defensin is the main antibacterial peptide produced in *Ae. aegypti* in response to both Gram positive and Gram negative bacteria (Lowenberger et al. 1995). Novel infections of *w*AlbB, *w*Mel and *w*MelPop-CLA in *Aedes* mosquitoes induce transcription of defensin, cecropin and diptericin (Bian et al. 2010; Kambris et al. 2009; Pan et al. 2012; Rancès et al. 2012), possibly due to the upregulation of Relish and Cactus (Bian et al. 2010; Kambris et al. 2009). However the two mosquito native symbioses, *Ae. albopictus* and the closely related species *Ae. fluviatilis,* do not show changes in Toll and Imd pathways dependent on *Wolbachia* presence/absence (Bourtzis et al. 2000; Caragata et al. 2017). Ae23T can show strong induction of immune gene transcription and effectively clear a bacterial infection, so this lack of response is clearly not due to impaired immune responses (Pinto et al. 2012). Our Ae23 cell line showed an overall trend of innate immune activation following *Wolbachia* infection, but likewise did not display significant upregulation of defensin. This difference in the responses between the three *Aedes* mosquitoes to *Wolbachia* warrants further investigation, particularly into the potential interactions of native mosquito endosymbionts (*w*AlbA/*w*AlbB, *w*Flu) on uninfected *Ae. aegypti* mosquitoes.

Despite our cell lines being infected with the same *Wolbachia* strain, there was no single gene that was differentially expressed in the same direction in each insect line. Results for the moth cell line were comparable to *Wolbachia* infected ovaries of the parasitoid wasp *Asobara tabida,* both displaying higher levels of cactus and dorsal in infected vs uninfected (Kremer et al. 2012). In this same wasp, defensin is downregulated in ovaries infected with *Wolbachia* but our

result was not significant. This difference is likely because *A. tabida* is dependent on the symbiont for oogenesis, where the reproductive system has had to respond to the parasitic nature of *Wolbachia* (Dedeine et al. 2005; Pannebakker et al. 2007). We therefore conclude that despite similarities in some areas of gene expression, there is no stereotypical response to *Wolbachia* infection across the cell lines. Tissue history of origin and species associated differences are likely contributing to this variation. This research cautions that findings from specific cell lines may not be broadly generalizable.

The results presented here demonstrate that there are no fundamental avenues of immune responses of novel insect hosts to *Wolbachia* infection in cell lines and that the responses show very little evolutionary change in the first few passages. Our findings do suggest, however, that native hosts have suppressed Toll and Imd activation compared to novel hosts. Future research may wish to focus on broader aspects of the host response beyond immunity or the response of the whole insect to novel infection. For a number of biocontrol strategies in vectors and agricultural pests, *Wolbachia* has and will need to be transinfected into uninfected hosts. Our work suggests that particular *Wolbachia* strains may be better suited to establishment given their native densities and that the nature of the host immune response is unpredictable and slow to evolve in initial culture.

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Compliance with ethical standards

Conflict of interest RIH and EM declare that they have no conflict of interest.

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