Developmental and Comparative Immunology 35 (2011) 360–365

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Infection with the *w*Mel and *w*MelPop strains of *Wolbachia* leads to higher levels of melanization in the hemolymph of *Drosophila melanogaster*, *Drosophila simulans* and *Aedes aegypti*

Pune Thomas [a], Nichola Kenny [a], Darryl Eyles [b], Luciano A. Moreira [a], Scott L. O'Neill [a], Sassan Asgari [a,*]

[a] School of Biological Sciences, The University of Queensland, St. Lucia, QLD 4072, Australia
[b] Queensland Brain Institute, The University of Queensland, Brisbane, QLD 4072, Australia

ARTICLE INFO

Article history:
Received 11 October 2010
Received in revised form 2 November 2010
Accepted 4 November 2010
Available online 21 November 2010

Keywords:
Wolbachia
Aedes aegypti
Drosophila melanogaster
Prophenoloxidase
Melanization
Immunity
Dopamine

ABSTRACT

Introduction of the life-shortening strain of *Wolbachia pipientis*, *w*MelPop, into the key dengue vector, *Aedes aegypti*, and the anti-pathogen effects in *Wolbachia*-infected hosts highlights the need for more research into its interactions with its original host, *Drosophila melanogaster*, and the novel mosquito host. The visual difference in darkness between the eggs of *w*MelPop *Wolbachia*-infected and uninfected mosquito hosts after egg deposition led to further investigation into melanization levels of the insects. Both *D. melanogaster* and *A. aegypti* infected with *w*MelPop showed increased levels of melanization, especially in females. This result was also seen in *D. melanogaster* and *Drosophila simulans* infected with the closely related *w*Mel strain. *D. simulans* infected with other strains of *Wolbachia* did not display this difference. HPLC analysis of hemolymph from mosquitoes showed that this difference was not due to dopamine levels in the host as they were no different in *w*MelPop-infected and control mosquitoes before or after blood feeding.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

The alphaproteobacterium *Wolbachia pipientis* is an obligate intracellular symbiont which infects a wide range of invertebrates. The majority of described infections are in Insecta where it is estimated that up to 70% of insect species may be infected (Jeyaprakash and Hoy, 2000). *Wolbachia* has been shown to have a range of effects on its hosts including reproductive manipulations such as feminization, parthenogenesis, male killing, and cytoplasmic incompatibility as well as other behavioral effects (Evans and Shapiro, 1997; Moreira et al., 2009; Peng et al., 2008; Turley et al., 2009; Werren et al., 2008). The *Wolbachia* strain *w*MelPop has been shown to have a life-shortening effect on its *Drosophila melanogaster* host (Min and Benzer, 1997). The effect was also seen when *w*MelPop was introduced into the dengue vector *Aedes aegypti* (McMeniman et al., 2009). Interestingly, Hedges et al. (2008) discovered that this *Wolbachia* strain delayed mortality of Oregon RC and w^{1118} *D. melanogaster* strains infected with Drosophila C virus (DCV) as well as Oregon RC flies infected with cricket paralysis virus and Flock House virus. The *w*MelPop strain has also been shown to limit the infection of *A. aegypti* by the mosquito-borne viruses, dengue and Chikungunya and the malaria parasite *Plasmodium gallinaceum* (Moreira et al., 2009).

The effects that symbionts or parasites may have on their arthropod hosts' physiology and immune response are of great interest due to implications in controlling mosquito-borne diseases. Melanization is a major component of the invertebrate immune system. Once hemocytes have encapsulated parasites or other foreign bodies with a multi-cell layer, the prophenoloxidase (proPO) activating system is used to enclose and kill them (Carton and Nappi, 1997). The proPO activating system has been shown to be especially important in the malaria vector *Anopheles gambiae*. Refractory strains have been shown to block the development of the malaria-causing *Plasmodium* by melanizing and encapsulating the parasite (Kumar et al., 2003). As melanin and its quinone intermediates are toxic to both host and parasite, activation of the system must be strictly regulated and localized. This is achieved by a cascade of inactive precursors which become enzymatically active after limited proteolysis by serine proteinases and several inhibitors (Cerenius and Soderhall, 2004). The activation of proPO to phenoloxidase (PO) is a terminal component of this cascade. PO catalyses many of the steps in this pathway, including the conversion of tyrosine to L-3,4-dihydroxyphenylalanine (DOPA), which can then either be used for melanization or sclerotization (Huang et al., 2005). Dopa decarboxylase (DDC) is the first enzyme used

* Corresponding author. +61 7 33652043.
E-mail address: s.asgari@uq.edu.au (S. Asgari).

0145-305X/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dci.2010.11.007

in the sclerotization part of this pathway to produce dopamine which continues on to form sclerotin (Li and Christensen, 1993). Dopamine can also be converted back into intermediates of the melanization pathway. When dopamine production was reduced by silencing DDC in the mosquito, *Armigeres subalbatus*, melanization was also significantly reduced (Huang et al., 2005).

The importance of dopamine and the PO cascade in other components of insect physiology has been shown; e.g. an increase in dopamine levels following blood-feeding in *A. aegypti* suggests the direct/indirect involvement of dopamine in ovarian and/or egg development (Andersen et al., 2006). PO also plays an important role in wound healing. Although it is not needed for the initial formation of hemolymph clotting, it is likely to act as a cross-linking agent to form hard clots (Bidla et al., 2005).

In this study, we investigated the effect of the *Wolbachia* strain *w*MelPop on tanning of *A. aegypti* eggs and PO activity. *Wolbachia*-infected *A. aegypti* hosts showed delayed tanning of the egg chorion. Subsequently, the effect of *w*MelPop on the proPO cascade was explored by measuring melanin formation in hemolymph samples and it was determined that the *Wolbachia* strain increased melanization levels in its *D. melanogaster*, *Drosophila simulans* and *A. aegypti* hosts. However, HPLC analysis of hemolymph from *Wolbachia*-infected and non-infected mosquitoes showed that the difference in melanization between infected and non-infected insects is not due to dopamine levels.

2. Materials and methods

2.1. Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Medical Research Ethics Committee at the University of Queensland (Project #2007001379). Volunteers were made aware of the risks of blood-feeding and the plans to analyze and publish all data prior to providing written consent to participate in the study.

2.2. Egg cuticle tanning

The *A. aegypti* line infected with the *w*MelPop strain *Wolbachia* was established as described (McMeniman et al., 2009). Persistence of *Wolbachia* infection in both mosquitoes and flies was confirmed regularly using PCR. Control line insects were previously treated with tetracycline and given at least two generations (2–4) to allow repopulation by gut microflora. Five- to seven-day-old PGYP1 (*Wolbachia*-infected) and PGYP1.tet (tetracycline-cured) *A. aegypti* mosquitoes were blood fed until repletion on a human arm. Two days after the blood meal, the mosquitoes were placed into individual oviposition tubes containing a filter paper and distilled water. The oviposition tubes were placed in a darkened environment for 1 h to allow the females to lay eggs. Females were then removed from the oviposition tubes, and each oviposition paper was placed on a sponge moistened with distilled water.

Using a dissecting microscope, observations of each paper were made every 10 min recording the number of eggs that had completely melanized. Eggs that had been hatched the previous week were used as a reference point to determine when complete melanization of eggs occurred. Observations continued for up to 3 h until all eggs had melanized, or until no additional eggs had melanized within a period of 30 min. Total number of eggs laid on each paper was scored and the percentage of eggs melanized per observation was determined. Data were analyzed by Mann–Whitney test.

2.3. Phenoloxidase assay

Flies were collected daily to control ages of flies at the time of assays. Mosquito pupae were separated out to narrow the age differences to 0–2 days. All insects were 4–8 days old when used. Hemolymph was extracted from mosquitoes and flies as described in Sidjanski et al. (1997). Briefly, 0.75 mL microcentrifuge tubes were first punctured several times at the bottoms with a 19-gauge needle, placed in 1.5 mL microcentrifuge tubes and chilled on ice. For each insect treatment and control, 30 males or females were anesthetized by chilling and incisions were made in the thorax and abdomen with a fine needle and collected in the chilled 0.75 mL microcentrifuge tubes. These were then centrifuged at 400 × *g* for 10 min to collect hemolymph in the 1.5 mL tubes. Equal volumes of hemolymph (0.5–1 μL) were then removed and added to 35 μL of ice-cold phosphate buffered saline (PBS) for each treatment and control. These were then centrifuged at 13,000 × *g* for 1 min to separate hemolymph from hemocytes. During centrifugation steps, L-3,4-dihydroxyphenylalanine (DOPA) (Sigma) was added to PBS to 20 mM and vortexed to dissolve. In each well of a 96-well Plate 10 μL of hemolymph was mixed with 90 μL of 20 mM DOPA by pipetting. Absorbance at 485 nm was then measured every 10 min for 300 min. Wells with 10 μL of PBS and 90 μL of DOPA were also made at the same time to measure auto-oxidation of DOPA which was then subtracted from the hemolymph values to indicate true melanization levels. For mosquito assays, 10–20 males or females were used and a larger amount of hemolymph was removed and added to 50 μL of PBS. The *D. melanogaster* lines used in this assay were w^{1118} and BNE infected with *w*MelPop and w^{1118} and da-Gal (P{Gal4-da.G32}UH1; Bloomington Stock Centre) infected with *w*Mel. The *D. simulans* lines were Me29 infected with *w*Mel (Poinsot et al., 1998), CO infected with *w*Au (Hoffmann et al., 1996) and HI infected with *w*Ha (O'Neill and Karr, 1990). Control, non-infected lines were previously created by treating fly lines with tetracycline and are denoted with a "T" after the fly-line name. The mosquito lines used were *A. aegypti* infected with *w*MelPop *Wolbachia* (PGYP1) and its control line (PGYP1.tet) which was originally generated using a tetracycline treatment (McMeniman et al., 2009).

2.4. Dopamine levels

For each replicate, hemolymph was isolated from 10 seven-day-old female mosquitoes of each type as described above with hemolymph added to 30 μL of PBS saturated with phenylthiorea (PTU) to avoid any further PO activity. Ten replicates of PGYP1 and PGYP1.tet hemolymph were extracted for analysis of dopamine levels using HPLC. Ten microliters of hemolymph solution was injected into a HPLC system that consisted of a degasser, autosampler and an isocratic HPLC pump (Model 1100, Agilent Technologies Inc., CA), a Sunfire C18 column, 4.6 mm × 150 mm, 5 μm (Waters Corporation, MA) and a Coulochem III (ESA Laboratories Inc., MA) electrochemical detector. The mobile phase was a 12% acetonitrile/75 mM potassium dihydrogen phosphate buffer containing 1 mM EDTA and 1.4 mM octane sulfonic acid adjusted to pH 4.13 with phosphoric acid with a flow rate of 1.2 mL/min. The conditioning cell (Model 5020, ESA Laboratories Inc., MA) operated at +350 mV with the first and second electrode of the analytical cell (Model 5014B, ESA Laboratories Inc., MA) set to −150 mV and +250 mV, respectively. Data were quantified by calculating peak-height ratios for dopamine relative to internal standard, deoxyepinephrine, and calibrated using standard curves. Data were stored and processed with Chemstation software (Rev B.01.03, Agilent Technologies Inc., CA). Samples were corrected for dilution and expressed as pg/μL sample.

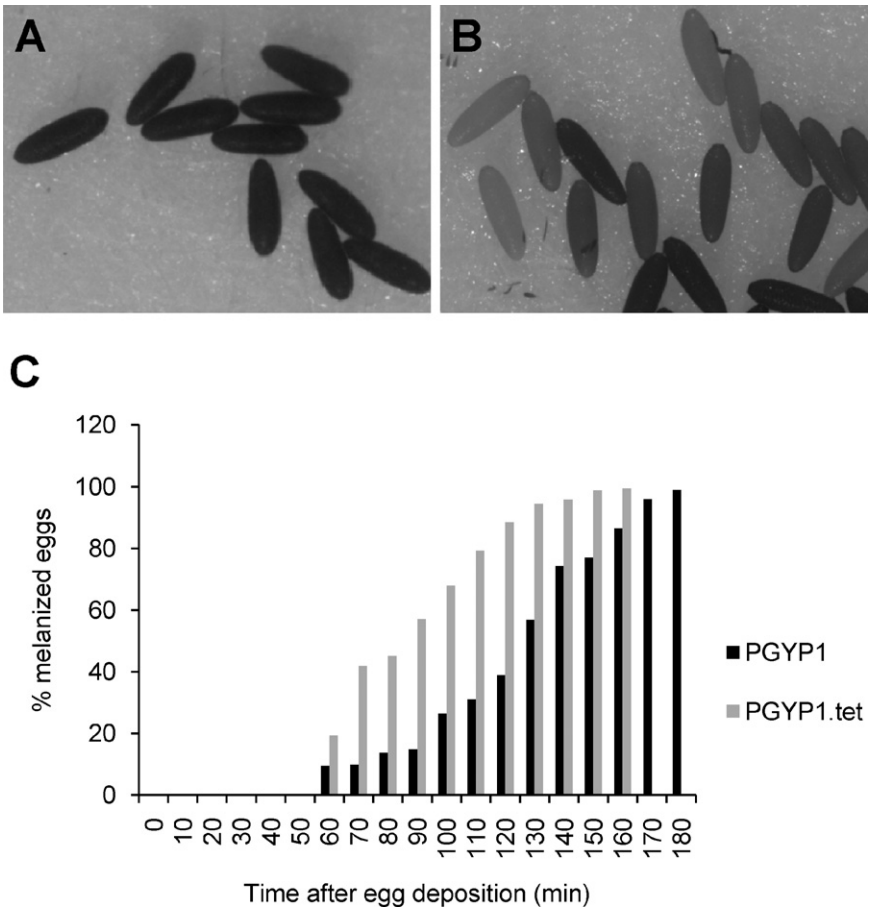

Fig. 1. Effect of *Wolbachia* infection on egg melanization in *A. aegypti*. Images of *A. aegypti* mosquito eggs from (A) control (tetracycline-treated) and (B) *w*MelPop-infected mosquitoes at 1.5 h after oviposition. (C) Number of melanized eggs was scored over 180 min after deposition by *Wolbachia*-infected (PGYP1) and control tetracycline-treated (PGYP1.tet) mosquitoes ($n = 100$ eggs for each).

3. Results

3.1. Egg melanization

In most insects, including *A. aegypti*, egg melanization is observed after oviposition. Initially, we observed a difference in the degree of egg melanization between *Wolbachia*-infected and tetracycline-treated mosquitoes. Eggs deposited by *Wolbachia*-infected mosquitoes were significantly lighter than those laid by control mosquitoes (Fig. 1A and B). To compare and quantify the speed of egg melanization in *Wolbachia*-infected and control mosquitoes, melanization was monitored over a 3 h period following oviposition. The results clearly showed that the rate of melanization is significantly delayed in *Wolbachia*-infected mosquitoes (Z value = 7.107, $P < 0.0001$; Fig. 1C); however, they all melanized eventually.

3.2. Phenoloxidase assays

To determine the effect of *Wolbachia* infection on melanin formation, melanization assays were carried out in the presence of a PO substrate, DOPA. The hemolymph of female *D. melanogaster* w^{1118} and da-GAL flies infected with *w*Mel showed increases in absorption at 485 nm with statistically significant values in *t*-tests ($P = 0.0004$ and 0.0005, respectively). w^{1118} and BNE females infected with *w*MelPop also showed a significant rise in PO activity ($P < 0.001$ and 0.0320). Male flies of the lines infected with *w*Mel or *w*MelPop showed no change in PO activity compared to control males. The w^{1118} data is shown in Fig. 2 as a representative result.

In mosquitoes, when *w*MelPop-infected and control lines were used in similar assays, the increase in melanization caused by infection in both males and females was also observed (Fig 3A and B; $P = 0.0040$ and 0.0180). This difference was also observed in mosquitoes 24 h after blood feeding (Fig. 3C; $P < 0.0001$). Interestingly, PO activity was about 10 times higher in female mosquitoes than that of male mosquitoes or that of female *Drosophila*. This difference is most likely due to increases in dopamine levels in the hemolymph in females after blood feeding (see Section 3.3).

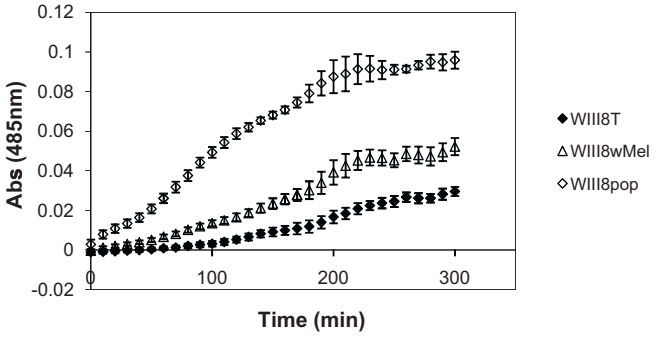

Fig. 2. *w*Mel and *w*MelPop increase melanization in host female *D. melanogaster*. Absorbance readings over 300 min for hemolymph PO assays using DOPA as substrate. w^{1118}T represents control line flies and w^{1118}*w*Mel and w^{1118}MelPop represent *Wolbachia*-infected lines. Error bars indicate standard deviations of averages from three replicates.

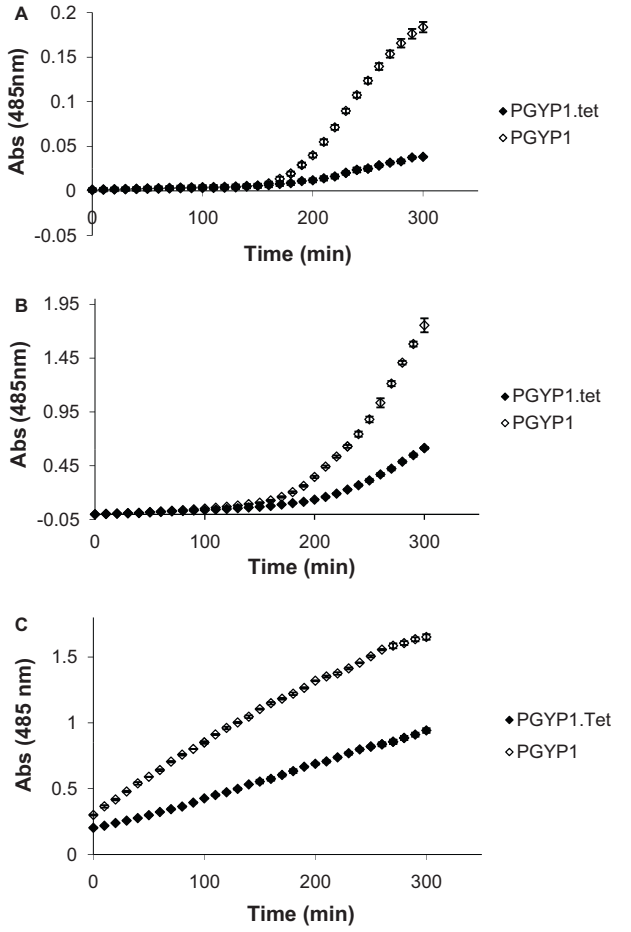

Fig. 3. *w*MelPop infection increases melanization in *A. aegypti* mosquitoes. Hemolymph PO assay results in (A) male, (B) female (pre-blood feeding) and (C) 24 h after blood-feeding mosquitoes. Absorbance readings over 300 min were recorded using DOPA as substrate. PGYP1.tet represents control line mosquitoes and PGYP1 represents the *Wolbachia*-infected line. Error bars indicate standard deviations of averages from three replicates.

To find out if the effect of *Wolbachia* infection is host or strain dependant, *D. simulans* lines Me29*w*Mel, HI*w*Ha and CO*w*Au were analyzed. The results showed a large increase in PO activity in Me29 flies infected with *w*Mel when compared to the Me29T control line (Fig. 4; $P < 0.0001$). No reduction in melanization levels were seen in the other *D. simulans* lines when compared to their control lines HIT and COT (data not shown). This indicated that the effect that *w*Mel has on *D. melanogaster* is also seen in *D. simulans* and that the other *Wolbachia* strains used do not have this effect in *D. simulans* either.

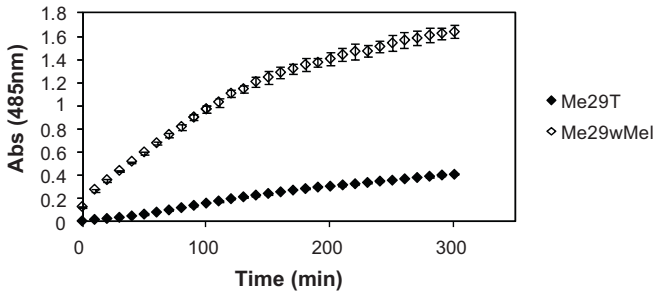

Fig. 4. *w*Mel increases melanization in host female *D. simulans*. Absorbance readings over 300 min for fly PO assays using DOPA as substrate. Me29T represents control line flies and Me29*w*Mel represents *Wolbachia*-infected lines. Error bars indicate standard deviations of averages from three replicates.

3.3. Dopamine levels

Dopamine levels in the hemolymph were measured in uninfected and *w*MelPop-infected female mosquitoes to determine if this was related to increases in PO activity observed in *Wolbachia*-infected insects. HPLC analysis of hemolymph showed concentrations of 45–60 pg/μL of dopamine in both *w*MelPop-infected and uninfected female mosquitoes (Fig. 5A). There was no significant difference between the dopamine levels in PGYP1-infected mosquitoes and PGYP1.tet, the control line ($P = 0.24$). In addition, we measured the dopamine levels in the hemolymph of PGYP1-infected mosquitoes and PGYP1.tet mosquitoes 24 h after blood feeding and did not find any significant difference between the two (Fig. 5B; $P = 0.85$). However, dopamine levels were about 5 times higher (ca. 250 pg/μL) in the hemolymph of mosquitoes after blood feeding compared to non-blood fed mosquitoes. This is consistent with a previous study which showed increased levels of dopamine in *A. aegypti* mosquitoes following blood feeding (Andersen et al., 2006).

4. Discussion

The symbiotic bacterium, *Wolbachia*, which is widespread among insects, has a variety of effects on its hosts including reproductive manipulations, behavioral effects and protection against viral infection. However, there are only few examples known about the effects of the symbiont on the hosts' immune systems. Moreira et al. (2009) showed that *Wolbachia* infection in *A. aegypti* reduced infection with three mosquito-borne pathogens, Dengue and Chikungunya viruses and *P. gallinaceum.* In another report, overall induction of the immune system in *Wolbachia*-infected *A. aegypti* was documented and that the presence of the endosymbiont limited the development of filarial nematodes in the mosquitoes (Kambris et al., 2009).

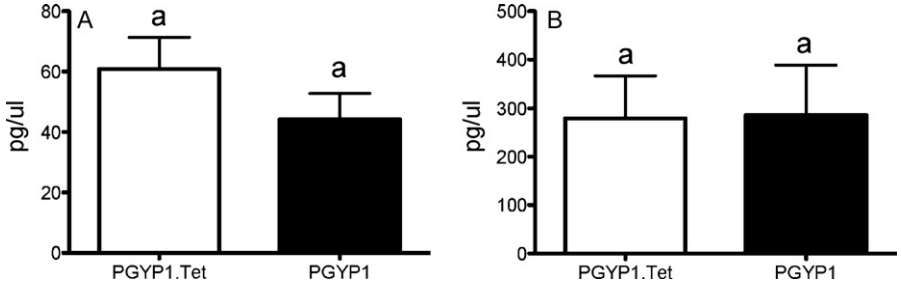

Fig. 5. High pressure liquid chromatography (HPLC) analysis of dopamine levels in the hemolymph from 7 days old (A) non-blood fed and (B) blood fed *A. aegypti* mosquitoes. PGYP1.tet represents control line mosquitoes and PGYP1 represents the *Wolbachia*-infected line. Same letters on top of the bars indicate that they are not statistically significant ($P > 0.05$).

Melanization of foreign invaders is a key component of the insect innate immune system (Cerenius et al., 2008; Theopold et al., 2004), which is involved in wound healing, cellular and humoral encapsulation and antiviral responses. In many host-parasite interactions suppression/evasion of melanization has been reported as a mechanism of survival from host immune responses (Asgari, 2007; Lambrechts et al., 2007; Osta et al., 2004). In this study, we explored the impact of *w*Mel and its closely related life-shortening strain, *w*MelPop, of *Wolbachia* on melanization in *D. melanogaster*, *D. simulans* and *A. aegypti*. In all the hosts, an increase in melanization levels was demonstrated in the hemolymph. This is consistent with the expression analysis data generated by microarray analysis of *A. aegypti* infected with *w*MelPop (Kambris et al., 2009). The data indicated that expression of components of proPO activation cascade, such as proPOs and CLIP-serine proteinases homologs (SPH) were upregulated in *Wolbachia*-infected mosquitoes in comparison to control mosquitoes.

Interestingly, Hedges et al. (2008) and Teixeira et al. (2008) demonstrated that *w*MelPop and *w*MelCS (a closely related strain) have an antiviral effect. Two strains of *D. melanogaster*, infected with these *Wolbachia* strains, were challenged with three RNA viruses, Drosophila C virus (DCV), cricket paralysis virus (CrPV) and Flock House virus (FHV). *Wolbachia*-infected flies showed delayed mortality, as compared to control counterparts, when challenged with DCV or CrPV, and reduced mortality from FHV infection. However, *w*Mel did not confer any resistance to Insect Iridescent Virus 6, a DNA virus (Teixeira et al., 2008). Further, *Wolbachia* has been shown to induce resistance in *A. aegypti* to Dengue virus (Moreira et al., 2009; Bian et al., 2010). Although the mechanism of resistance/tolerance and possible involvement of melanization in protecting insects against RNA viruses has not been investigated, elevated levels of melanization in *Wolbachia*-infected mosquitoes may directly or indirectly be responsible for the protection and lower mortality rates in flies harboring the symbiont. Certainly, the role of melanization in protection/resistance of insects against baculoviruses (DNA viruses) has been shown (Washburn et al., 1996; Saejeng et al., 2010).

Since dopamine may feed into the PO cascade, melanization can be affected by dopamine levels (Nappi and Christensen, 2005). This suggests that the higher PO activity measured in infected hosts could be caused directly or indirectly by *Wolbachia* affecting dopamine availability. Dopamine levels in the hemolymph were measured to determine whether *w*MelPop was increasing PO activation in hosts by directly increasing dopamine levels. The similar levels of dopamine in both *w*MelPop-infected and control mosquitoes suggested that there is no increase of dopamine levels in *Wolbachia*-infected mosquito hemolymph. Therefore, an increase in melanization in mosquitoes prior to blood feeding further confirms that the increased levels are most likely related to elevated expression levels of genes involved in the proPO activation cascade (Kambris et al., 2009).

An increase in dopamine levels following blood feeding in *A. aegypti* has been shown (Andersen et al., 2006). In our experiments, we also had a similar observation. However, in terms of dopamine levels, there was no difference between *Wolbachia*-infected and non-infected mosquitoes. In addition, melanization levels were still higher in *Wolbachia*-infected mosquitoes compared to non-infected mosquitoes. The difference in the colouration observed in *Wolbachia*-infected *A. aegypti* eggs versus a control line suggested a variation in melanization levels. Eggs deposited by control mosquitoes were darker than *Wolbachia*-infected *A. aegypti* eggs. Further, the process of darkening was significantly faster in eggs from control mosquitoes compared to that of *Wolbachia*-infected mosquitoes. This is not consistent with the observation that melanization in the hemolymph of *Wolbachia*-infected mosquitoes after blood feeding is still higher than that of uninfected mosquitoes. This may relate to differences in the allocation of resources in *Wolbachia*-infected versus non-infected mosquitoes, especially that ovaries are the main tissue in which *Wolbachia* replicates.

The increase in hemolymph melanization observed in *Wolbachia*-infected mosquitoes may be a side effect of the endosymbiont's other modifications to its host or may be due to direct alterations on the host's immune system to provide a less hostile environment. Further investigation revealed that dopamine levels were no different in mosquito hosts before or after taking a blood meal. Further studies are required to determine how *w*MelPop affects the host's melanization pathway and to determine any other effects that this might have on the host's fitness and interactions with arboviruses, especially with respect to the use of *Wolbachia*-infected hosts to control vector-borne diseases.

Acknowledgements

This project was funded by a Ph.D. scholarship to PT from the School of Biological Sciences at UQ as well as a grant from the Foundation for the National Institutes of Health through the Grand Challenges in Global Health Initiative of the Bill and Melinda Gates Foundation.

References

Andersen, J.P., Schwartz, A., Gramsbergen, J.B., Loeschcke, V., 2006. Dopamine levels in the mosquito *Aedes aegypti* during adult development, following blood feeding and in response to heat stress. J. Insect Physiol. 52, 1163–1170.

Asgari, S., 2007. Endoparasitoid venom proteins as modulators of host immunity and development. In: Rivers, D.B., Yoder, J. (Eds.), Recent Advances in the Biochemistry, Toxicity, and Mode of Action of Parasitic Wasp Venoms. Research Signpost, India, pp. 57–73.

Bian, G., Xu, Y., Lu, P., Xie, Y., Xi, Z., 2010. The endosymbiotic bacterium *Wolbachia* induces resistance to Dengue virus in *Aedes aegypti*. PLoS Pathog. 6, e1000833.

Bidla, G., Lindgren, M., Theopold, U., Dushay, M.S., 2005. Hemolymph coagulation and phenoloxidase in *Drosophila* larvae. Dev. Comp. Immunol. 29, 667–679.

Carton, Y., Nappi, A.J., 1997. *Drosophila* cellular immunity against parasitoids. Parasitol. Today 13, 218–227.

Cerenius, L., Soderhall, K., 2004. The prophenoloxidase-activating system in invertebrates. Immunol. Rev. 198, 116–126.

Cerenius, L., Lee, B.L., Söderhäll, K., 2008. The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol. 29, 263–271.

Evans, H., Shapiro, M., 1997. Viruses. In: Lacey, L. (Ed.), Manual of Techniques in Insect Pathology. Academic Press, London, pp. 17–54.

Hedges, L.M., Brownlie, J.C., O'Neill, S.L., Johnson, K.N., 2008. *Wolbachia* and virus protection in insects. Science 322, 702.

Hoffmann, A.A., Clancy, D., Duncan, J., 1996. Naturally-occurring *Wolbachia* infection in *Drosophila simulans* that does not cause cytoplasmic incompatibility. Heredity 76, 1–8.

Huang, C.Y., Chou, S.Y., Bartholomay, L.C., Christensen, B.M., Chen, C.C., 2005. The use of gene silencing to study the role of dopa decarboxylase in mosquito melanization reactions. Insect Mol. Biol. 14, 237–244.

Jeyaprakash, A., Hoy, M.A., 2000. Long PCR improves *Wolbachia* DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. Insect Mol. Biol. 9, 393–405.

Kambris, Z., Cook, P.E., Phuc, H.K., Sinkins, S.P., 2009. Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. Science 326, 134–136.

Kumar, S., Christophides, G.K., Cantera, R., Charles, B., Han, Y.S., Meister, S., et al., 2003. The role of reactive oxygen species on *Plasmodium* melanotic encapsulation in *Anopheles gambiae*. Proc. Natl. Acad. Sci. U. S. A. 100, 14139–14144.

Lambrechts, L., Morlais, I., Awono-Ambene, P.H., Cohuet, A., Simard, F., Jacques, J.C., et al., 2007. Effect of infection by *Plasmodium falciparum* on the melanization immune response of *Anopheles gambiae*. Am. J. Trop. Med. Hyg. 76, 475–480.

Li, J.Y., Christensen, B.M., 1993. Involvement of L-tyrosine and phenol oxidase in the tanning of *Aedes aegypti* eggs. Insect Biochem. Mol. Biol. 23, 739–748.

McMeniman, C.J., Lane, R.V., Cass, B.N., Fong, A.W.C., Sidhu, M., Wang, Y.-F., et al., 2009. Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. Science 323, 141–144.

Min, K.-T., Benzer, S., 1997. *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. Proc. Natl. Acad. Sci. U. S. A. 94, 10792–10796.

Moreira, L.A., Iturbe-Ormaetxe, I., Jeffery, J.A., Lu, G.J., Pyke, A.T., Hedges, L.M., et al., 2009. A *Wolbachia* symbiont in *Aedes aegypti* limits infection with Dengue, Chikungunya, and Plasmodium. Cell 139, 1268–1278.

Nappi, A.J., Christensen, B.M., 2005. Melanogenesis and associated cytotoxic reactions: applications to insect innate immunity. Insect Biochem. Mol. Biol. 35, 443–459.
O'Neill, S.L., Karr, T.L., 1990. Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. Nature 348, 178–180.
Osta, M.A., Christophides, G.K., Kafatos, F.C., 2004. Effects of mosquito genes on *Plasmodium* development. Science 303, 2030–2031.
Peng, Y., Nielsen, J.E., Cunningham, J.P., McGraw, E.A., 2008. *Wolbachia* infection alters olfactory-cued locomotion in *Drosophila* spp. Appl. Environ. Microbiol. 74, 3943–3948.
Poinsot, D., Bourtzis, K., Markakis, G., Savakis, C., Mercot, H., 1998. *Wolbachia* transfer from *Drosophila melanogaster* into *D. simulans*: host effect and cytoplasmic incompatibility relationships. Genetics 150, 227–237.
Saejeng, A., Tidbury, H., Siva-Jothy, M.T., Boots, M., 2010. Examining the relationship between hemolymph phenoloxidase and resistance to a DNA virus, *Plodia interpunctella* granulosis virus (PiGV). J. Insect Physiol. 56, 1232–1236.
Sidjanski, S., Mathews, G.V., Vanderberg, J.P., 1997. Electrophoretic separation and identification of phenoloxidases in hemolymph and midgut of adult *Anopheles stephensi* mosquitoes. J. Parasitol. 83, 686–691.
Teixeira, L., Ferreira, A., Ashburner, M., 2008. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLoS Biol. 6, 2753–2763.
Theopold, U., Schmidt, O., Soderhall, K., Dushay, M.S., 2004. Coagulation in arthropods: defence, wound closure and healing. Trends Immunol. 25, 289–294.
Turley, A.P., Moreira, L.A., O'Neill, S.L., McGraw, E.A., 2009. Wolbachia infection reduces blood-feeding success in the dengue fever mosquito, *Aedes aegypti*. PLoS Neglect. Trop. D 3, 6.
Washburn, J.O., Kirkpatrick, B.A., Volkman, L.E., 1996. Insect protection against viruses. Nature 383, 767.
Werren, J.H., Baldo, L., Clark, M.E., 2008. *Wolbachia*: master manipulators of invertebrate biology. Nat. Rev. Microbiol. 6, 741–751.