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Wolbachia infection in *Aedes aegypti* mosquitoes alters blood meal excretion and delays oviposition without affecting trypsin activity

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ABSTRACT

Blood feeding in *Aedes aegypti* is essential for reproduction, but also permits the mosquito to act as a vector for key human pathogens such as the Zika and dengue viruses. *Wolbachia pipientis* is an endosymbiotic bacterium that can manipulate the biology of *Aedes aegypti* mosquitoes, making them less competent hosts for many pathogens. Yet while *Wolbachia* affects other aspects of host physiology, it is unclear whether it influences physiological processes associated with blood meal digestion. To that end, we examined the effects of *w*Mel *Wolbachia* infection in *Ae. aegypti*, on survival post-blood feeding, blood meal excretion, rate of oviposition, expression levels of key genes involved in oogenesis, and activity levels of trypsin blood digestion enzymes. We observed that *w*Mel infection altered the rate and duration of blood meal excretion, delayed the onset of oviposition and was associated with a greater number of eggs being laid later. *w*Mel-infected *Ae. aegypti* also had lower levels of key yolk protein precursor genes necessary for oogenesis. However, all of these effects occurred without a change in trypsin activity. These results suggest that *Wolbachia* infection may disrupt normal metabolic processes associated with blood feeding and reproduction in *Ae. aegypti*.

1. Introduction

The act of blood feeding serves to make female *Aedes aegypti* a critical threat to human health, by facilitating the transmission of medically-important pathogens including the dengue (DENV), chikungunya (CHIKV), Zika (ZIKV) and yellow fever (YFV) viruses. The last 20–30 years have seen a great increase in the size and geographic range of *Ae. aegypti* populations (Kraemer et al., 2015), a resurgence in cases of dengue, and the emergence of novel arboviruses, most notably ZIKV (Duffy et al., 2009; Musso et al., 2015). This has been driven by increasing urbanization and changing climate bringing more people into contact with mosquitoes, and by the declining efficacy of common mosquito control strategies, due in part to the increasing prevalence of genetic resistance to commonly used mosquito insecticides (Smith et al., 2016). To that end, there is a need to develop novel strategies that reduce the transmission of arboviral infections.

One such technique involves the use of the endosymbiotic bacterium *Wolbachia pipientis* as a bio-control agent. *Wolbachia* naturally infects around 40% of all terrestrial insect species (Zug and Hammerstein, 2012). The bacterium is maternally transmitted and greatly alters host reproductive biology to promote that transmission (Werren et al., 2008). *Ae. aegypti* is not thought to be naturally infected, but there is recent evidence of *Wolbachia* in some field-collected larvae (Coon et al., 2016). Multiple transinfections of *Ae. aegypti* have been generated through embryonic injection (Joubert et al., 2016; McMeniman et al., 2009; Walker et al., 2011; Xi et al., 2005). Under this strategy, *Wolbachia*-infected mosquitoes are released into the field, where the reproductive manipulation cytoplasmic incompatibility promotes the spread of the bacterium to high levels in wild mosquito populations (Hoffmann et al., 2011). The bacterium also strongly inhibits the

Abbreviations: AA, amino acids; CHIKV, chikungunya virus; DENV, dengue virus; ILP, insulin-like peptide; TOR, target of rapamycin; YFV, yellow fever virus; YPP, yolk protein precursors; ZIKV, Zika virus.

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replication and transmission of DENV, CHIKV and ZIKV, and thus makes these mosquitoes less effective vectors (Aliota et al., 2016a, 2016b; Dutra et al., 2016b; Ferguson et al., 2015; Moreira et al., 2009; Walker et al., 2011) for key pathogens. This strategy is currently being deployed in several countries as part of the Eliminate Dengue Program (www.eliminatedengue.com).

Female *Ae. aegypti* mosquitoes are anthropophillic and must take a blood meal in order to obtain the nutrients required to produce eggs. Animal blood is protein-rich, and its digestion is primarily facilitated by proteinases - protein digesting enzymes, particularly trypsins (Barillas-Mury et al., 1995; Noriega et al., 1994, 2001). Blood meal-derived nutrients are stored as nutritional reserves (Naksathit et al., 1999), used in other metabolic processes (Zhou et al., 2004), or used for egg production (Ziegler and Ibrahim, 2001). A high percentage of blood meal-derived amino acids (AA) are sent to the fat body where they are used to produce the yolk protein precursor (YPP) proteins that play a critical role in vitellogenesis (egg yolk formation) (Attardo et al., 2005; Raikhel et al., 2002). Undigested blood components, and potentially harmful ammonia- or iron-containing compounds are metabolised, detoxified, and then excreted over the next 2–4 days (Graca-Souza et al., 2006).

The midgut during blood digestion is likely an important site of interaction between mosquito, virus and *Wolbachia*, as the tissue is the first point of contact between the virus and the *Wolbachia*-infected host. While the midgut also serves as an important barrier to viral infection during the extrinsic incubation period, and *Wolbachia* has been shown to extend the extrinsic incubation period, at least for DENV (Ye et al., 2015). Blood feeding has also been demonstrated to increase *Wolbachia* levels (Frentiu et al., 2014). Critically, the effects of infection with the *w*Mel *Wolbachia* strain on blood meal digestion, excretion and oviposition have not been well characterised. In non-blood fed *Ae. aegypti*, *w*Mel alters the expression of serine proteases and trypsins, which may be indicative of changes in digestive processes (Rancès et al., 2012). *w*Mel also affects the expression of many genes involved in the biosynthesis, metabolism, and nutrient transport, and decreases levels of free cholesterol, all of which could impact on oogenesis (Attardo et al., 2005; Caragata et al., 2014; Rancès et al., 2012). *Wolbachia* infections in mosquitoes also alter the host stress response, which could feasibly influence the biochemical processes associated with blood meal detoxification, and could influence host fitness (Bian et al., 2013; Caragata et al., 2017; Pan et al., 2012; Rancès et al., 2012).

Analysis of the *w*Mel genome suggests that the symbiont is likely to be metabolically reliant on its host, and energetically dependent on AA (Wu et al., 2004). While in *Ae. aegypti* infected with the more virulent *Wolbachia* strain, *w*MelPop, competition for blood meal AA decreases fecundity and egg viability (Caragata et al., 2014; McMeniman et al., 2011). As such, processes associated with blood meal digestion, and oogenesis potentially represent important areas of metabolic interaction between host and symbiont that could affect host competitiveness in the field. Furthermore, *Wolbachia*-induced changes in blood digestion could also play an important role in host-symbiont-pathogen tripartite interactions, given the link between metabolism and the decreased fitness of pathogens in *Wolbachia*-infected mosquitoes (Caragata et al., 2013, 2016).

To that end we conducted experiments in order to gain insight into the effects of *w*Mel infection on physiological processes associated with blood meal digestion in *Ae. aegypti*. We compared blood meal intake, post-blood meal survival under starvation, and the excretion and oviposition rates for *w*Mel-infected (Mel) and -uninfected (Tet) mosquitoes. We then looked at molecular processes associated with digestion and oogenesis, examining the effect of *w*Mel infection on trypsin activity, and the expression of key genes involved in vitellogenesis.

2. Material and methods

2.1. Mosquitoes

Two *Ae. aegypti* lines were utilised across all experiments. The first was infected with the *w*Mel *Wolbachia* strain (Mel). This infection was originally established in Australian mosquitoes (Walker et al., 2011), and subsequently backcrossed to obtain a Brazilian genetic background as previously described (Dutra et al., 2015). The second was a *Wolbachia*-uninfected line established by tetracycline treatment (Tet) more than 4 years prior to the start of experiments, as previously described (Dutra et al., 2015). Genetic similarity between lines was maintained by regular introduction of wildtype male mosquitoes into colony cages at a rate of 10 per 100 mosquitoes every 3 generations. These wildtype males were the F_1 or F_2 generation of *Ae. aegypti* that were captured in Rio de Janeiro, Brazil between 2014 and 2016.

Mosquitoes were maintained in a climate-controlled insectary with a mean temperature of 27 ± 1 °C, mean relative humidity of 70± 10%, and a photoperiod of 12 h light: dark. For experiments, eggs were hatched in 3L of RO water containing one half tetramin tropical tablet (Tetramin®), ground with a mortar and pestle. Larvae were reared at a density of 250 in 3L of RO water, and were fed an average of one half tetramin tablet per day. Pupae were collected in cups with clean water and moved to BugDorm-1 cages (bugdorm) ($h = l = d = 27.5$ cm). Adult mosquitoes were maintained on 10% sucrose solution, supplied *ad libitum* and changed three times per week unless specified below. In all experiments described below where blood feeding was performed, mosquitoes were fed on fresh human blood for approximately 15 min per cage. Blood feeding was conducted in accordance with The Committee for Ethics in Research (CEP)/FIOCRUZ (License - CEP 732.621), and with Brazilian laws 196/1996 and 01/1988, which govern human ethics issues in scientific research in compliance with the National Council of Ethics in Research (CONEP). Afterwards, mosquitoes were rendered unconscious using carbon dioxide, maintained on ice, and screened for the presence of blood. Mosquitoes that were not fully engorged were removed from experiments. This represented a very small number of mosquitoes, and we observed no difference in the rate of engorgement between the Mel and Tet lines. These mosquitoes were excluded because their digestion, excretion, and egg development would likely have been greatly altered by to the low volume of blood that they ingested. In all experiments, mosquitoes were blood fed at 5 days post-eclosion.

2.2. Blood feeding & blood meal weights

Pre- and post-blood meal weights were compared between Mel and Tet mosquitoes to determine whether *Wolbachia* influenced the total amount of blood imbibed. 1.5 mL eppendorf tubes were weighed three times using a fine balance (Mettler Toledo XS205 Dual Range). The first time the tubes were empty, the second they contained an individual non-blood fed female mosquito, and then finally an individual blood fed mosquito that was collected less than 10 min after feeding. Both blood-fed and non-blood fed mosquitoes were starved for approximately 16 h prior to weighing in order to encourage blood feeding and to eliminate between individual variation in weight due to sugar feeding. To calculate blood meal weights, the weight of each empty tube was subtracted from the weight of each blood fed and non-blood fed mosquito. The average weight of non-blood fed mosquitoes was calculated independently for the Tet and Mel cohorts, and this value was subtracted from the