International shipments of *Wolbachia*-Infected Mosquito Eggs - Towards Scale-up of World Mosquito Program Operations

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Summary

The *Wolbachia* insect control method, employed by the World Mosquito Program (WMP), relies on introgressing *Wolbachia* through target *Aedes aegypti* populations to reduce the incidence of dengue. Since 2010 WMP has been producing *Wolbachia*-infected mosquitoes at numerous sites across the globe for release in 11 countries. As the technology has matured, greater focus has been placed on mosquito production at larger central facilities for transport to remote release sites, both domestically and internationally.

Of particular note is production of *Wolbachia*-infected mosquitoes at our Australian production facility for successful international deployments in Fiji, Vanuatu, Kiribati and Sri Lanka. This requires careful management of both production and supply-chain processes to ensure the quality, specifically hatch rate and *Wolbachia* infection rates, of mosquito eggs is maintained.

To ensure the cost-effectiveness and scalability of the *Wolbachia* method, these processes will be further refined to facilitate deployments from large central production facilities.

Keywords

Aedes aegypti – Egg shipment – Mosquito production – *w*Mel – *Wolbachia*.

Introduction

The *Wolbachia* method involves the introgression of *Wolbachia pipientis* through target *Aedes aegypti* populations to reduce incidence of vector borne diseases caused by flavi- and alphaviruses such as Dengue, Zika, Chikungunya and Yellow fever (1, 2). This method relies on the production, transport, and release of *Wolbachia*-infected *Ae. Aegypti,* which facilitates introgression through a target *Ae. aegypti* population.

Wolbachia pipientis, an obligate endosymbiotic alpha-proteobacterium, has been shown to be near ubiquitously dispersed globally and is estimated to infect up to 66% of insect species (3, 4, 5). The *Wolbachia* strain *w*Mel, derived from *Drosophila melanogaster*, was stably introduced into the Yellow fever mosquito, *Aedes aegypti*, in 2011 (2). *w*Mel confers three signature traits on *Ae. aegypti* that enables *Wolbachia*-infected *Ae. aegypti* to be deployed as a public health intervention (2). The first two, *Wolbachia*-mediated cytoplasmic incompatibility (CI) and maternal transmission, facilitate population introgression of *w*Mel into a target *Ae. aegypti* population. The third signature trait of *w*Mel, when present in *Ae. aegypti,* is that they interfere with viral replication. *Wolbachia*-infected *Ae. aegypti* have significantly lower vector competence (ability to transmit a pathogen) for all four serotypes of dengue virus, Chikungunya, Zika, Yellow fever and Mayaro virus when compared to mosquitoes not infected with *Wolbachia* (6, 7, 8, 9, 10). Moreover, *w*Mel is genomically and phenotypically stable in field deployments (11, 12, 13).

The World Mosquito Program (WMP) (formally Eliminate Dengue) first demonstrated the feasibility of the *Wolbachia* method as a public health intervention in 2011 through the introgression of the *w*Mel *Wolbachia* strain into naive *Ae. aegypti* populations using large semi-field cages located in James Cook University, Australia (2). Subsequently, *Wolbachia* (*w*Mel) has been fixed in *Ae. aegypti* populations throughout Northern Queensland, Australia via releases of *Wolbachia*-infected mosquitoes (14, 15). WMP's *Wolbachia* method is being deployed in 11 countries with release sites in Mexico and South

America (Colombia and Brazil), Southeast Asia (Sri Lanka, Indonesia, and Vietnam), as well as the Oceania region (Australia, Kiribati, Fiji, Vanuatu, and New Caledonia) (Fig. 1). Moreover, a non-WMP consortium has established *Wolbachia* in *Ae. aegypti* populations within Malaysia (16).

FIGURE 1 HERE

The *Wolbachia* method has been shown to be highly successful in reducing the incidence of dengue. In all areas where *Wolbachia* has been established and public health data is available, it has been demonstrated that the *w*Mel *Wolbachia* strain reduces the incidence of dengue (14, 15, 17, 18, 19). This has included a randomised controlled trial in Yogyakarta, Indonesia that demonstrated a 77% reduction in dengue incidence and an 86% reduction in dengue hospitalisations in clusters randomly allocated to receive *w*Mel deployments compared to untreated clusters (19).

Our approach in all regions is heavily dependent on community support and participation. Typically, community engagement will precede any planned release and releases will not commence until a community acceptance threshold is reached (17). We also engage local communities through educational programs in local schools, large information events and community facilitated releases.

As with all aspects of WMP's *Wolbachia*-infected *Ae. aegypti* release, the methods for generating community acceptance and support have evolved over time as the scale of the releases has increased. Initially releases involved a much higher individual household-based approach. As release sites grow in size and number, and with the approval of local regulatory agencies, the Public Acceptance Model (PAM) will be employed. PAM involves acceptance at the community level, rather than individual level (20). Currently PAM, and community level acceptance, has only been employed in limited fashion, but as deployments become larger it will become the dominant strategy. Regardless of the method employed, the rights of participating communities to be informed, raise concerns, and get involved in WMP projects are always respected. Releases of *Wolbachia*-infected *Ae.*

aegypti only ever occur with sufficient local awareness, understanding and support.

WMP *Wolbachia*-infected mosquito deployment relies on two methodologies for mosquito releases, 1) adult mosquitoes housed in release tubes or cups or 2) mosquito eggs deployed in mosquito release containers (MRCs). The choice of method depends heavily on the local climate and geography as well as the socioeconomic environment with some release sites employing mixed strategies. However, both methodologies require the availability of high quality, *Wolbachia*-infected *Ae. aegypti* eggs. WMP *w*Mel-infected *Ae. aegypti* are being mass produced in rearing facilities in Medellin, Colombia; Rio de Janeiro, Brazil; and Melbourne, Australia. Production is also occurring in several smaller, localised production facilities in New Caledonia, Mexico, Vietnam, and Indonesia. As the number and remoteness of *Wolbachia* release locations increase, deployments increasingly rely on the packaging of eggs at centralised production facilities and subsequent transport to target sites. Transportation of *Wolbachia*-infected *Ae. aegypti* eggs require rapid temperature-controlled shipping, while also complying with biosecurity containment requirements. WMP has successfully employed a strategy that has relied on production from the Monash production facility in Melbourne, Australia to release sites across the globe. This review will focus on methodology used for transport from this production facility to Fiji, Vanuatu, Kiribati, and Sri Lanka.

Production and storage

The manufacturing process for *Wolbachia*-infected *Ae. aegypti* requires strict quality assurance to ensure that mosquitoes for release are fit for purpose, comply with all local guidelines, and pose no risk due to inadequate safety or quality. Throughout the manufacturing process, regardless of location, WMP employs a continually refined quality management system. Reflecting this commitment, the WMP Monash facility has recently received ISO 9001:2015 certification. These physical and procedural processes are documented to ensure strict

compliance. Moreover, all staff members are trained and regularly assessed to ensure adherence to documented practices.

WMP mosquito production is conceptually divided into aquatic rearing, adult rearing, egg processing and material supply (Fig. 2). In addition, these processes are replicated over three levels of rearing numbers: a brood level with low density rearing conditions for stock maintenance; an amplification level with an intermediate mosquito density to high levels of egg production to feed the next level; and a mass production level capable of producing egg quantities required for release. There are quality assurance checks throughout this process to ensure material consistency and ongoing quality control to ensure effective facility operations. These checks include *Wolbachia* frequency during larval rearing, blood pathogen screening prior to feeding, and a colony fertility test at the end of production.

FIGURE 2 HERE

Although *Ae. aegypti* eggs can survive for extended periods of time, *Wolbachia*-infected *Ae. aegypti* eggs have a comparatively reduced lifespan (21, 22, 23, 24). This poses a major hurdle for the centralised production and transport of eggs for mosquito releases. Furthermore, storage conditions can have a major impact on the fitness of offspring derived from *Wolbachia* infected eggs (25). For these reasons, we have determined a set of storage and transport conditions that allows for *Wolbachia* infected eggs to be stored for up to eight weeks post-production, as well as developing a logistic chain to transport eggs to release sites in Kiribati, Fiji, Vanuatu as well as Sri Lanka.

When *w*Mel-infected eggs were stored between 18–22°C with a relative humidity of 70–80%, viability losses (hatch rate decline) of only 20% were observed over an eight-week period. Furthermore, *Wolbachia* density in offspring was not affected for the duration of storage (26). To facilitate transport, WMP contracted a specialist courier company that could provide stable shipping conditions (temperature and humidity), as well as provide logistical support to manage import and custom requirements. With this service, eggs could be delivered weekly to release sites. Fitness tests (egg hatch rate, *Wolbachia* density in

offspring and wing length of offspring) conducted concurrently at Monash and destinations demonstrated no adverse fitness effects because of shipping or storage.

Egg shipment to multiple locations

Regulatory Pathway & export process mechanism

The manufacturing process requires moving *Wolbachia*-infected mosquitoes across the globe. To ensure containment of *Wolbachia*-infected *Ae. aegypti*, eggs are shipped in either capsules or on paper to target countries. Rapid transit from a production facility to the release site is achieved by working with local and international biosecurity agencies and transport companies. *Wolbachia*-infected *Ae. aegypti* present regulatory advantages over some other technologies. They have been repeatedly defined not to be genetically modified (GM) and as such not subject to international or domestic GM regulatory frameworks (27, 28, 29).

However, *Wolbachia*-infected mosquitoes are still subject to biosecurity regulatory requirements and must be handled correctly. The shipment process is highly specific, as every country operates their own biosecurity regulatory frameworks. Typically, each country requires both an export and import permit for *Wolbachia*-infected *Ae. aegypti* transport. These are guided by a pre-established authorisation from relevant biosecurity authorities. Depending on the specifics of individual shipments, transit/transhipment permits can also be required. Although pre-existing agreements may be in place, each shipment typically requires its own permits and certification. To facilitate rapid transport and minimise risk, partnerships with logistics and support companies are pursued.

Within Australia, WMP partners with LabCabs (Murarrie, Queensland, Australia) to facilitate shipments from the Melbourne-based production facility to field sites. WMP typically employs CoolPac shippers (Doveton, Victoria, Australia) for all egg shipments, which, coupled with WMP packaging, ensure *Wolbachia*-infected *Ae. aegypti* eggs are contained via six layers (Fig. 3). Each batch of eggs is given an internal

identification number that is linked to their *Wolbachia* screening, human blood pathogen report and their equine blood pathogen report. This comprehensive supply-chain management ensures that the cause of any adverse events, from early development to release, can be rapidly identified and corrected. This product traceability and careful monitoring ensures product integrity throughout all WMP operations.

FIGURE 3 HERE

As there is no generic regulatory pathway providing oversight to transport, WMP handles this process on a release site by release site basis. Typically, this involves engagement with regulatory agencies in both the country of manufacture, in our case Australia, and the country importing the material. The process relies on three separate types of approval - import, export, and transit permits. Each of these permits ensures maintenance of biosecurity.

Import permits: are required for shipping local strains to a production facility for strain development, backcrossing, and for transfer of *Wolbachia*-infected release material to country sites.

Export permits: are required to ensure appropriate containment procedures are followed. However, they are also critical in many countries, such as Brazil, where strong protections to prevent the illegal export of biological samples exist.

Transit permits: are required to ensure appropriate procedures are followed when transporting mosquitoes. For example, as there are no practical direct flights from Australia to Kiribati, *Wolbachia*-infected *Ae. aegypti* for release need a transit permit from the Fijian authorities for their transport.

Almost exclusively, WMP transports eggs, as opposed to adults, of both *Wolbachia*-infected and uninfected *Ae. aegypti*. Transport of eggs provides a strong barrier to escape, for this species they are more durable than adult stages in terms of exposure to conditions during shipment and have reduced bulk and weight and therefore lower shipment costs.

Projects - Monash to Sri Lanka, Vanuatu, Fiji and Kiribati

WMPs Melbourne-based production facility has shipped locally adapted strains of eggs to field sites across the Western Pacific Region (Fiji, Kiribati and Vanuatu) and South-East Asia (Sri Lanka). From 2018 until this year, WMP regularly shipped eggs to each of these locations. The LabCab (Australia) shipments ensured rapid transportation to each of these release sites. Typically, within 24–48 hours to Sri Lanka but up to five days to Kiribati due to the remote location. The LabCab service includes door to door shipment. Moreover, they also provide considerable assistance with navigating customs procedures.

Costs associated with the international transport of mosquito eggs can vary, with the common factors impacting logistics (fuel prices, package size, etc) fluctuating regularly. The specific requirements mandated by the port of entry and/or export may also influence the cost of shipment. Regulatory requirements for export permits and potential additional certifications incur a fee for provision of said documents for each instance of shipment. For example, transportation to Kiribati was, on average, twice the cost of shipping to Fiji. This was primarily due to the lack of direct shipment. This necessitated transportation through an intermediate country and therefore additional regulatory/compliance hurdles.

Finally, ongoing factors associated with the COVID-19 pandemic have greatly hampered almost all transportation and release. This included disruption of the Sri Lankan releases due to the inability to safely conduct and monitor field operations, thus forcing their stoppage. Once releases continued, the transportation costs had risen by approximately 25%. It is currently unknown when, or even if, the supply chain and transportation will return to normal. However, it seems almost certain there will be ongoing increases in the costs associated with the global transportation of materials in the foreseeable future.

Typically, cost reduction can be achieved by decreasing the frequency of shipments, however the decision to ship more eggs less frequently is

dictated by the facilities weekly rearing capacity, robustness of the material being shipped and the on-site storage capacity.

Conclusion

WMP has successfully deployed *Wolbachia*-infected *Ae. aegypti* in 11 countries. These deployments have resulted in widespread reductions in dengue incidence. Initially, releases were of locally produced mosquitoes that involved limited transport to deployment sites. However, as the technology has matured, greater emphasis has been placed on production from central facilities to more remote international sites. Process refinement permitted the successful transport of *Wolbachia*-infected mosquito eggs from the production facility in Monash University (Melbourne, Australia) to release sites in Fiji, Vanuatu, Kiribati, and Sri Lanka.

Establishment of production facilities can be met with considerable financial and regulatory hurdles. Moreover, small facilities lack the efficiencies needed for cost-effective deployment of the *Wolbachia* method. This means it remains highly impractical to establish facilities in every release area, especially if the life of a particular project is unknown. Thus, WMP developed the necessary frameworks and processes, using transport to Pacific release sites as a pilot, for global distribution from centralised facilities. Critical to this was stability, both in production output and product quality.

Inconsistency in the number of *Wolbachia*-infected eggs supplied could have serious negative impacts, both in cost and effectiveness, of *Wolbachia* introgression at a target site. Therefore, throughout the aforementioned deployments, consistent egg production was maintained through constant refinement and monitoring of involved processes. This culminated in ISO 9001 certification of the Monash production facility. Although not a requirement, we would encourage any parties looking to undertake centralised production to investigate the feasibility of certification. ISO 9001 provides a management framework that greatly facilities harmonious high-quality output of the target product.

Likewise, maintaining egg quality throughout the transport chain is critical for an effective deployment. Sharp declines in egg viability (hatch rate) or *Wolbachia* infection rates could also adversely affect deployment. Thus, these remained critical quality assurance/control checks. To facilitate consistency, transportation partners and highly specific packing were employed. Moreover, a considerable body of work has been carried out to define the optimal conditions for storage and transport. In transport to Fiji, Vanuatu, Kiribati and Sri Lanka, typically no significant declines in either hatch rates or *Wolbachia* infection rates of transported material was observed relative to material from the same batch stored at the production facility. Given production and logistical costs, a deployment may continue, on a case-by-case basis, for egg batches with low hatch rates. However, diminished *Wolbachia* infection rates would likely result in the destruction of the failed batch. Moreover, any failures will also trigger process reviews.

WMP will constantly review these processes to ensure consistency is maintained as to move towards large scale centralised production. A key focus of this ongoing review will be cost effectiveness of material transport. Some of this will likely come from scaling up, however gains must also be made in cost per shipment if WMP's goals are to be achieved. There are several proposed material packaging options aimed at reducing costs in either transportation or deployment.

Acknowledgements

We would like to thank all members of the WMP Product Development & Supply teams. In addition, we would like to thank all partner organisations and their staff.

This research was funded in whole, or in part, by Wellcome Trust (Grant 212914/Z18/B) for the purpose of open access, the author has applied a CC BY public copyright licence to any Author Accepted Manuscript version arising from this submission.

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Fig. 1 - Worldwide WMP release countries for *Wolbachia*-infected *Aedes aegypti*. At present WMP, and local partners, have undertaken deployments in a total of 11 countries.

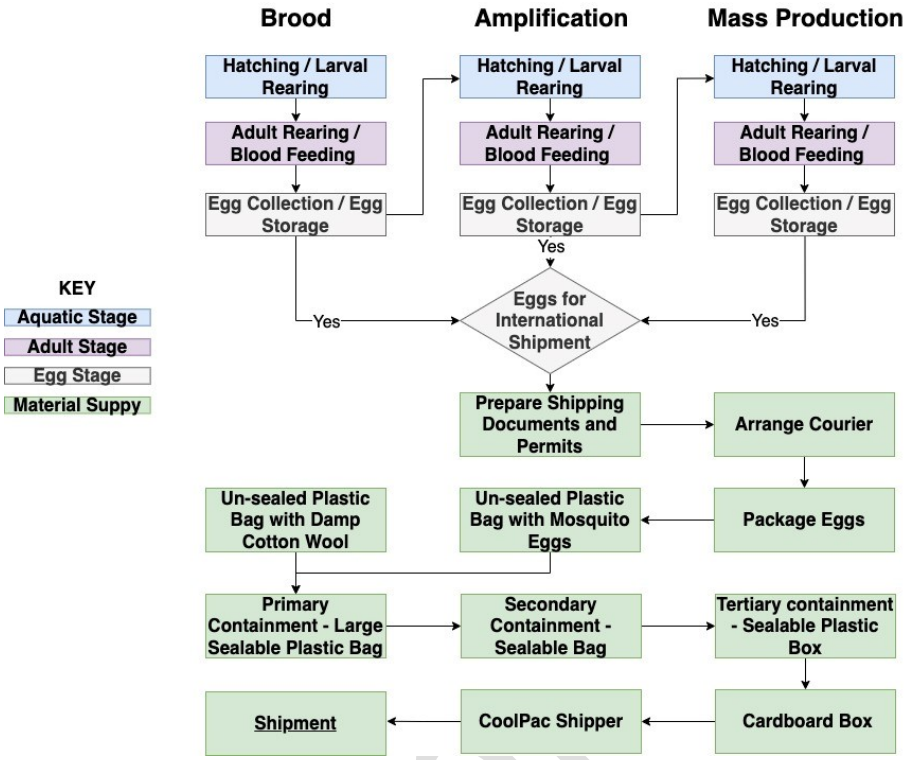

Fig. 2 - Representation of production and shipment of WMP *w*Mel *Wolbachia*-infected *Aedes aegypti*. Production is divided into three levels with increasing mosquito density - Brood, Amplification and Mass Production. Although eggs from each stage can be used for deployments, typically only those from Mass Production are packaged for transport. Shipment requires that Wolbachia-infected *Aedes aegypti* eggs are contained in several layers to ensure biosecurity compliance and to maintain quality.

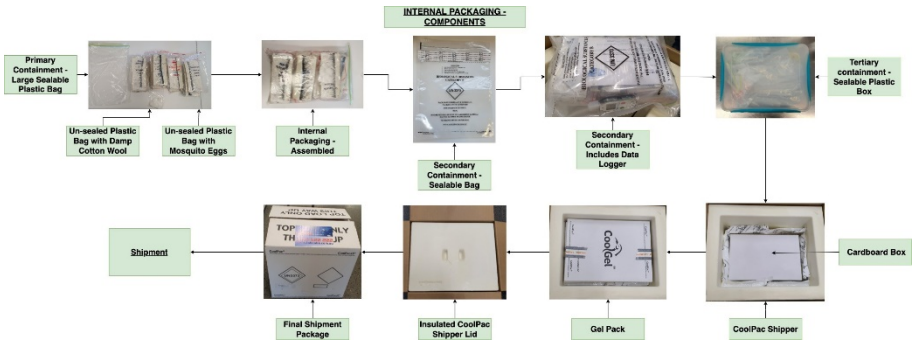

Fig. 3 - Shipment packaging and containment. Mosquito eggs shipped from World Mosquito Program Melbourne production facility are packaged to maintain optimal viability through transit. The internal packaging contains a packet of damp cotton wool that provides the eggs with a humid environment to minimise desiccation. The CoolPac Polyurethane Insulated Shipper box is a multi-use cold chain packaging system that uses an insulated container and refrigerant (gel packs) to ensure the contents are maintained at 15-25°C. The Australian biosecurity regulatory framework requires that all shipped material is appropriately contained to prevent the risk of quarantine material escaping. The World Mosquito Program ships its mosquito eggs within six levels of containment, including a solid plastic box to prevent accidental release of material.