Assessing key safety concerns of a *Wolbachia*-based strategy to control dengue transmission by *Aedes* mosquitoes

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Mosquito-borne diseases such as dengue fever, chikungunya or malaria affect millions of people each year and control solutions are urgently needed. An international research program is currently being developed that relies on the introduction of the bacterial endosymbiont Wolbachia pipientis *into* Aedes aegypti *to control dengue transmission. In order to prepare for open-field testing releases of* Wolbachia*-infected mosquitoes, an intensive social research and community engagement program was undertaken in Cairns, Northern Australia. The most common concern expressed by the diverse range of community members and stakeholders surveyed was the necessity of assuring the safety of the proposed approach for humans, animals and the environment. To address these concerns a series of safety experiments were undertaken. We report in this paper on the experimental data obtained, discuss the limitations of experimental risk assessment and focus on the necessity of including community concerns in scientific research.*

Key words: *Aedes aegypti* - *Wolbachia* - dengue - risk assessment - safety - biological control

Biological control of agricultural, livestock and human pests has been undertaken successfully for centuries. For example, the biological control of citrus insect pests by ants has been described from as early as the 4th century AD in China (Hsi Han, cited in Wackers & van Rijn 2005). Biological control can be defined as the release into the environment of a biological agent to control a given pest through mechanisms such as predation, parasitism, herbivory or disease. In recent decades, more than 100 pest species have been targeted by the release of multiple biological control agents into the Australian environment. Iconic success include the release of myxoma virus to control rabbit populations (Saunders et al. 2010), the release of *Cactoblastis* moths to control prickley pear (*Opuntia* spp) (Dodd 1940), the introduction of dung beetles to manage cattle dung and the bush flies that breed in it (Edwards & Pavri 2007) and the control of floating *Salvinia* weed (Room et al. 1981) using the beetle *Cyrtobagous singularis*.

Dengue fever is a viral disease estimated to affect 50-100 million people annually and is primarily spread by the domestic mosquito *Aedes aegypti* (L.) (WHO 2009). The proposed approach aims to use the bacterium *Wolbachia* as a biological control agent to prevent the transmission of viruses such as dengue. *Wolbachia* are intracellular endosymbiotic bacteria naturally present in a large number of insects and other arthropod species (Werren et al. 2008). Some *Wolbachia* strains are also described as symbionts of filarial nematodes that infect humans and cause diseases such as river blindness and elephantiasis (Bandi et al. 1998). However, there is currently some controversy as to whether the *Wolbachia* that infect filarial nematodes should be classified as a separate species as their biology is quite distinct to the *Wolbachia* that infect insects (Pfarr et al. 2007). *Wolbachia* are able to actively spread into insect populations by manipulating insect reproduction (Werren et al. 2008). They are vertically transmitted across generations (via eggs) and confer reproductive advantages to infected females through a series of reproductive phenotypes that include cytoplasmic incompatibility, feminization, male killing or parthenogenesis (Werren et al. 2008). *Wolbachia* has long been considered as a potential biocontrol agent for insects and the pathogens they transmit, but has never been operationally deployed beyond pilot testing (Laven 1967, Beard et al. 1993, Sinkins & O'Neill 2000, Rasgon et al. 2003, Brownstein et al. 2003, Cook et al. 2007).

Wolbachia as a biological control agent against mosquito-borne diseases

Dengue viruses, like most pathogens transmitted by mosquitoes, need a significant period of development in the insect vector, termed the extrinsic incubation period (EIP), before they can be transmitted to a new host (Watts et al. 1987). Since the EIP is long relative to average mosquito lifespan, the majority of pathogen transmission is done by old mosquitoes. In 1997, a *Wolbachia* strain was discovered with virulent properties in its natural host, *Drosophila melanogaster*, halving adult lifespan (Min & Benzer 1997). It has been proposed that this strain, *w*MelPop, could be a biological agent to reduce lifespan of mosquitoes therefore reducing the

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proportion of infectious mosquitoes (Sinkins & O'Neill 2000, Brownstein et al. 2003, Cook et al. 2007). This strain has been successfully introduced into the dengue vector, *Ae. aegypti*, where it also reduces lifespan by up to 50%, while being maternally transmitted to 100% of the mosquito progeny (McMeniman et al. 2009).

A number of recent independent studies have shown that *w*MelPop and other *Wolbachia* strains can also confer resistance against a wide range of insect viruses as well as important human pathogens such as dengue and chickungunya viruses, *Plasmodium gallinaceum*, *Plasmodium berghei* or *Brugia pahangi* (Hedges et al. 2008, Teixeira et al. 2008, Kambris et al. 2009, 2010, Moreira et al. 2009a, Glaser & Meola 2010). This pathogen interference phenotype conferred by *Wolbachia* was observed in natural *Drosophila* hosts as well as in artificially introduced hosts such as *Ae. aegypti* (Moreira et al. 2009a, T Walker et al., unpublished observations). The discovery of pathogen interference effects opened a new approach where instead of reducing mosquito lifespan to reduce infectious mosquitoes in a population, the utilisation of *Wolbachia* as a biocontrol agent could rely on direct pathogen interference. Interestingly, even though *Wolbachia* is estimated to infect 65% of insect species, including multiple mosquito species (from the genus *Culex*, *Aedes*, *Coquillettidia*, *Mansonia* and *Uranotaenia)* the major vectors of dengue and malaria (*Ae. aegypti* and various anopheline mosquitoes, respectively) are not naturally infected by *Wolbachia* (Kittayapong et al. 2000, Ricci et al. 2002, Rasgon & Scott 2004, Hilgenboecker et al. 2008). The fact that many mosquito species are weak vectors could be due to the presence of *Wolbachia* symbionts, an interesting hypothesis that needs to be verified and could then provide evidence for a "natural" example of biological control by *Wolbachia*.

The proposed approach of using *Wolbachia* infections of *Ae. aegypti* as a biological control approach for dengue transmission is nearing open field testing in the course of an international research program associating collaborators from Australia, Vietnam, Thailand, USA and Brazil (www.mosquitoage.org/en/HOME.aspx). If these strains can spread in natural populations as they do in the laboratory, they could be an effective control solution against dengue transmission. A trial release of *Wolbachia*-infected *Ae. aegypti* is proposed for the 2010-2011 "wet season" in Cairns, Australia, prior to subsequent releases in Vietnam and Thailand.

Integrating community concerns in the scientific approach

Concomitantly with the scientific work on this approach, a social research and community engagement program was developed (2008-2009) and undertaken (2009-2010) in the city of Cairns - including the proposed release areas. Its aim was to develop an ethical, effective, culturally sensitive, stakeholder-directed framework for community engagement and authorisation that was sensitive to different socio-political settings (D McNaughton, unpublished observations).

Using a mixed methods approach, extensive social research and engagement were used to examine (i) how communities at the potential release sites wished to be engaged, (ii) what would constitute authorisation and (iii) acceptability and non-acceptability issues (McNaughton 2009). The results of this research were then used to develop a community engagement strategy and to develop mechanisms for addressing and responding to community questions and concerns (McNaughton et al. 2010, D McNaughton, unpublished observations).

"Community acceptability is critical to the future use and success of this program and public engagement, collaborative partnerships and community and regulatory authorisation have been recognised as an 'ethical requirement' of this type of project." (Newman et al. 2006, Lavery et al. 2008, D McNaughton, unpublished observations). Engaging communities early and identifying any concerns is critical to ethical engagement. It also provides a window for the research team to respond more effectively to community concerns, through new scientific experiments such as those described here as well as the creation of new communication materials, capacity building, education and ongoing engagement (D McNaughton, unpublished observations).

Social research into lay knowledge of dengue fever and acceptability of the *Wolbachia* method was undertaken in 2008-2009. Qualitative techniques including focus groups, in-depth interviews, questionnaires and participant observation were used initially to gauge the full range of views and then confirmed by data from quantitative measures such as telephone surveys which used representative population samples and randomised sampling techniques (McNaughton 2009, D McNaughton, unpublished observations).

The most common community concerns related to the safety of the approach and its capacity for transfer, namely: is the *Wolbachia* approach safe for people? Is it safe for animals and other organisms? Is it safe for the environment (D McNaughton, unpublished observations)?

Initially, these questions were answered by drawing on the large number of well-documented arguments in the extant literature, incorporating this information into communication materials and engagement activities. However, as these arguments rely on baseline knowledge of the biology of *Wolbachia*, dengue virus and *Ae. aegypti* it was thought they may not in themselves be enough to reassure the community who, in some instances, expected to be provided experimental data that would directly assess those risks. It was determined that answering these questions relied on experimental evaluation as well as on previous data, available observations and knowledge of the approach.

Can *Wolbachia* affect/be transferred to humans? Baseline data

A major concern the community repeatedly expressed was whether *Wolbachia* could be transferred to humans through the bite of infected mosquitoes (D McNaughton, unpublished observations). *Wolbachia* are specialized endosymbionts that infect insects as well as spiders, mites, terrestrial crustaceans (Breeuwer & Jacobs 1996, Bouchon et al. 1998, Taylor & Hoerauf 1999, Oh et al. 2000, Bandi et al. 2001, Rowley et al. 2004). *Wolbachia* have never been found in humans or

other mammals, neither in birds, reptiles or fish. When *Wolbachia* were first discovered in the 1930's they were suspected of being potential human rickettsial pathogens and were tested accordingly (Hertig 1936). Preparations from *Wolbachia* infected ovaries were injected into chicken embryos but *Wolbachia* did not grow. They were also injected into baby mice both intraperitoneally and intracerebrally without symptoms or mortality that could be attributed to *Wolbachia*. The conclusion of these studies was that *Wolbachia* was a non-pathogenic symbiont of insects (Hertig 1936).

Humans have been exposed to *Wolbachia* for thousands of years. *Wolbachia* are extremely common in the environment naturally infecting a large range of insect species including pests of stored food products as well as insects that bite humans such as nuisance mosquitoes like *Aedes albopictus* and *Culex quinquefasciatus*. There is no evidence showing neither that residues of *Wolbachia*-infected insects in food products are harmful to humans nor are there any adverse effects reported from people being bitten by insects containing *Wolbachia*. In fact, as part of this project, human volunteers have been providing bloodmeals to *Wolbachia*-infected mosquitoes by using their own legs or arms, receiving thousands of bites without adverse consequences. This number is far higher than the number of potential bites people in the release areas would receive following the release of *Wolbachia*-infected mosquitoes.

Ethics

The work reported in this manuscript used human volunteers for mosquito feeding as approved by the University of Queensland Human Ethical Committee - approval 2007001379. Written consent was obtained from each participant used for blood-feeding.

Can *Wolbachia* affect/be transferred to humans? Experimental assessment

Despite the evidence against negative effects of *Wolbachia* against humans, a number of experiments were undertaken to assess its potential transfer to humans and the potential development of an immune response specific to *Wolbachia*.

Transfer to humans? - One general concern is whether *Wolbachia* would be injected into the human body via the insect saliva during blood-feeding (D McNaughton, unpublished observations). In order to detect the presence of *Wolbachia*) polymerase chain reaction (PCR) analysis of the *Ae. aegypti* mosquito saliva has been performed (Moreira et al. 2009b). PCR amplification of the *Wolbachia wsp* gene and the mosquito apyrase gene showed that despite *Wolbachia* is present in salivary glands, it is absent in the saliva (Moreira et al. 2009b). Even when the transposable element IS5, present in at least 13 copies within the bacterial genome (Wu et al. 2004), was used as a very sensitive PCR target, no amplification was obtained. These negative PCR results are supported by the size of the intracellular *Wolbachia* (around 1-2 μm in diameter) and the diameter of mosquito salivary ducts (less than 1 μm), which indicate that even if *Wolbachia* was present in the secreted salivary fluid it would be unlikely to move freely through the salivary ducts (Moreira et al. 2009b). Nevertheless, *Wolbachia* antigens or residues could be injected into humans during biting which could then potentially induce an immune response.

Development of an immune response specific to Wolbachia? - We therefore investigated the antibody response specific to *Wolbachia* in the human volunteers that regularly feed mosquito colonies. Sera from human volunteers that blood-fed repeatedly the *Wolbachia*-infected mosqui-

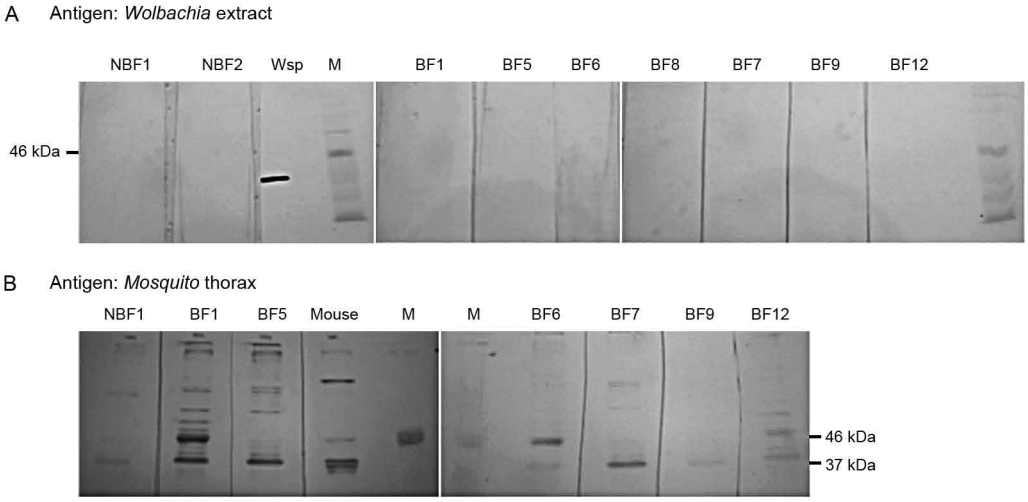

Fig. 1: Western blot analysis to detect any potential anti-*Wolbachia* antibody in human sera. *Wolbachia* (A) or mosquito thorax extracts (B) were run in a 12% SDS-PAGE and incubated with either human sera or anti-WSP (*Wolbachia* surface protein) antibody (A) or with human sera or mouse anti-mosquito saliva serum (B). Non blood-feeder (NBF)1 and NBF2 are sera from NBF volunteers. Blood-feeder (BF)1, BF5, BF6, BF7, BF8, BF9, BF12 are sera from BF volunteers. M: Kaleidoscope prestained standards (Bio-Rad).

to lab colonies being exposed to many thousands of bites over a four-year time frame were collected [blood-feeder (BF), n = 17] for immunological analysis. Sera from humans who never blood-fed any mosquito lab colony were also collected as negative controls [non blood-feeder (NBF) n = 5]. Both Western blot and enzyme-linked immunosorbent assays (ELISA), classically used in combination for accurate diagnostics of human diseases such as human immunodeficiency virus (Sax et al. 2010), were performed for immunological detection of any possible human antibody specific to *Wolbachia* antigens.

For Western blots *Wolbachia* extracts were first loaded on SDS-PAGE gels and after blotting, membranes were incubated with either a rabbit anti-WSP (*Wolbachia* surface protein) antibody or with the different human sera from volunteers (Fig. 1). A single band with the right size for the *Wolbachia* WSP protein (26 kDa) (Braig et al. 1998) was detected only when the blot was incubated with an anti-WSP antibody (Fig. 1A). This same band was not detected when blots were incubated with the human sera from either BFs (which would recognize potential *Wolbachia* antigens) or non BFs. As a control, extracts from *Wolbachia*-uninfected mosquitoes were loaded on gels and the blots were incubated with human sera or with a mouse anti-mosquito saliva antibody [developed as shown by Drahota et al. (2009)] to detect whether volunteers were responding to mosquito bites (Fig. 1B). As previously shown (Orlandi-Pradines et al. 2007), most of the sera of exposed human subjects (BFs) recognized an *Ae. aegypti* protein of 45 kDa. Also, the majority of BFs responded to a smaller size band of around 37 kDa, which was previously named D7 (James et al. 1991) and has been shown to be recognized by exposed humans (Peng et al. 1998). These data show that human volunteers that have been exposed to many thousands of bites from *Wolbachia*-infected mosquitoes over prolonged periods of time have antibody responses to mosquito saliva but have not developed IgG antibodies specific to *Wolbachia* that could be detected by Western blot.

ELISA was also carried out on the individual sera to detect and measure the IgG antibodies specific to *Wolbachia* antigens to complement and verify the Western blots. *Wolbachia* were purified and homogenized from either *Ae. aegypti* cells or *Drosophila* flies using previously described protocol (McMeniman et al. 2008). The same purification protocol was applied to *Wolbachia*-uninfected *Ae. aegypti* cells and *Drosophila* to evaluate the IgG response specific to possible remaining insect antigens [background optical density (ODn)]. ELISA plates were coated with antigens and individual sera were added and incubated overnight. IgG detection was performed using a goat anti-human IgG antibody. Individual IgG levels specific to *Wolbachia* were expressed as difference of OD (ΔOD) values and calculated according to the formula ΔOD = ODw - ODn, where ODw represents the individual measure of OD for each purified *Wolbachia* samples.

Antigens coated on the ELISA plates were either *Wolbachia* extracts purified from *Ae. aegypti* cells (each well coated with purified *Wolbachia* from 3 x 10^4 cells) or *Wolbachia* extracts purified from *Drosophila* flies (each well coated with purified *Wolbachia* from 1 fly), along with their respective uninfected controls. For the two host backgrounds (either *Ae. aegypti* cells) (Fig. 2B) or *Drosophila* flies (Fig. 2C), no difference in the level of IgG specific to *Wolbachia* was detected between BFs and NBFs. As a control, *Wolbachia*-uninfected *Ae. aegypti* salivary gland extracts were coated on the ELISA plates (each well coated with 1/10th of a single salivary gland extract) and incubated with human sera to detect any IgG response specific to mosquitoes (Fig. 2A). BFs presented significantly higher IgG level specific to *A. aegypti* salivary gland extracts compared to NBFs (p = 0.0129), confirming the development of human antibody against mosquitoes as shown by the Western blot.

Both immunological investigations, Western blots and ELISA, show that humans repeatedly bitten by *Wolbachia*-infected *Ae. aegypti* develop antibodies against mosquitoes as already described for exposed humans, but do not develop IgG antibody against *Wolbachia*. All those results indicate that *Wolbachia* antigens are not injected into humans during the mosquito bloodmeal and therefore do not initiate an immune response in human host.

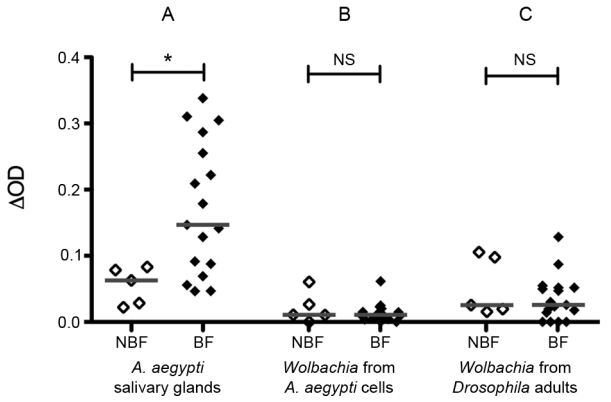

Fig. 2: ELISA analysis to detect IgG antibody levels specific to *Aedes aegypti* salivary glands or to *Wolbachia* antigens in human sera. Individuals difference of optical density (ΔOD) values are shown for blood-feeders (BF) and for non-blood-feeders (NBF) and bars indicate the median value. Results are presented according to antigens: *Wolbachia*-uninfected *Ae. aegypti* salivary gland extracts (A), *Wolbachia* extracts purified from *Ae. aegypti* cell culture (B) and *Wolbachia* extracts purified from *Drosophila* flies (C). NS: non significant. Asterisk means statistical differences between groups (non parametric Mann Whitney test).

Can *Wolbachia* be transferred into the environment? Baseline data

Another major concern from the public is whether the release of *Wolbachia* infected mosquitoes would negatively affect the environment, that is whether *Wolbachia* could be transferred to other organisms or become established in the soil (D McNaughton, unpublished observations).

As for the previous question, a large piece of evidence indicates that this risk is negligible. *Wolbachia* is not infectious and is transmitted only vertically through the eggs from one generation to another (Werren et al. 2008). *Wolbachia* bacteria are obligate endosymbionts, that is, they

can only live inside the cytoplasm of their host's cells and are not able to survive outside their host organism, and as such, they are not expected to persist in the environment outside the mosquitoes that carry them. *Wolbachia* will degrade together with mosquito bodies when they die and the residues will be indistinguishable from natural organic components and of no toxicological significance.

Transferring *Wolbachia* from flies into mosquitoes was a daunting task in the laboratory, which took several years. The recent success in obtaining transinfection of *Ae. aegypti* mosquitoes with the *w*MelPop strain (McMeniman et al. 2009) is believed to be due to the pre-adaptation of *Wolbachia* in *Aedes* tissue culture (McMeniman et al. 2008). This strain, initially isolated from *D. melanogaster* flies, was maintained for three years in tissue culture cells and only after this passaging scheme a stable infection in *Ae. aegypti* was successfully established by embryonic microinjection (McMeniman et al. 2009). Subsequent analysis of this strain after serial passage has revealed a number of genetic changes that occurred during the adaptation process (unpublished observations) and that were probably critical for the establishment of *Wolbachia*-infected mosquitoes. For some strains, the introduction of *Wolbachia* into a new host can be done without adaptation in cell line. Nevertheless, we believe that a successful introduction will still require hundreds to thousands of microinjections before obtaining a stable infection, at least in mosquito species (T Walker, unpublished observations). The difficulty of generating *Wolbachia* infections in new species indicates that the risk of horizontal transfer of *Wolbachia* to other lower flies is negligible.

Additional indirect evidence comes from the fact that *Wolbachia*-infected and uninfected species can coexist in the same habitat or predate on each other without acquiring the infection. In Australia, *Ae. aegypti* inhabits the same larval habitat as its close relative *Aedes notoscriptus,* which is naturally infected with *Wolbachia* (unpublished observations). However, extensive surveys of *Ae. aegypti* in Australia and around the world have determined that *Ae. aegypti* is not naturally infected with *Wolbachia* (Kittayapong et al. 2000, Ricci et al. 2002, Rasgon & Scott 2004). Despite the opportunity for *Wolbachia* to transfer naturally from *Ae. notoscriptus* to *Ae. aegypti*, which has been possible for the entire time that *Ae. aegypti* has been present in the Australian environment, that transfer has never happened.

An even larger natural experiment has been running in south east Asia where *Ae. aegypti* occurs in sympatry with *Ae. albopictus*. Again *Ae. albopictus* is naturally infected with two *Wolbachia* strains (Sinkins et al. 1995, Zhou et al. 1998, Dobson et al. 2001), often inhabits the same larval containers as *Ae. aegypti* and it is known that both species will ingest smaller instars of the other species. Again despite this close contact no natural transfer of *Wolbachia* has ever been reported from *Ae. albopictus* to *Ae. aegypti*. All these observations show that horizontal transmission does not occur easily or at a high frequency. These types of natural experiments do not provide evidence to support the case for frequent horizontal transfer.

This poses the interesting question of how does *Wolbachia* establish in insect hosts naturally if it is so hard to establish infections in the laboratory? This has been a central question for the *Wolbachia* research community for many years and despite considerable energy trying to answer this question by numerous research groups, mechanisms of natural transfer remain unknown. The only recorded examples of natural horizontal transfers have been between different parasitoids superinfecting the same insect host (Heath et al. 1999, Huigens et al. 2000, 2004). However this seems to be an unlikely explanation for a general mechanism. Furthermore parasitoids are not significant parasites of mosquitoes. The general consensus of the scientific community is that natural horizontal transfer events are extremely rare and the wide distribution of *Wolbachia* among insects is explained by the many millions of years that *Wolbachia* is believed to have been associated with insects, which in turn has allowed time for numerous rare events to accumulate.

Can *Wolbachia* be transferred into the environment? Experimental assessment

However, despite this body of evidence, and because of the concerns expressed by the community in the social research, a number of studies were conducted to evaluate if *Wolbachia* could be transferred from mosquitoes to other species. The possible horizontal transfer to mosquito predators and non-predator species or environments in the vicinity of the mosquitoes were evaluated.

Transfer to mosquito predators

In order to test the potential transfer of *Wolbachia* from infected mosquitoes into their natural predators, we designed predation experiments using two different spider species (*Menemerus bivittatus* and *Pholcus phalangioides*), commonly found in the Australian environment.

TABLE I

Screening for *Wolbachia* transfer from mosquitoes to their predators

Species	*Wolbachia* detection[a]	Mosquito detection[b]
Menemerus bivittatus	0/80	NA
Pholcus phalangioides	2/440	2/2

a: *Wolbachia* detection performed by polymerase chain reaction (PCR) targeting the specific IS5 sequence. Primer sequences: IS5-F (5′-GTATCCAACAGATCTAAGC-3') and IS5-R (5'-ATAACCCTACTCATAGCTAG-3′). PCR conditions: 95ºC for 3 min followed by 35 cycles of 95ºC for 30 sec, 55ºC for 30 sec, 72ºC for 1 min and a final extension at 72ºC for 10 min; *b*: whenever a sample was positive for *Wolbachia* DNA, it was also screened for the presence of mosquito DNA through PCR targeting the ribosomal protein gene RpS17. Primer sequences: RpS17F (5'-CTGGAGATTTTCCGTTGTCA-3') and RpS17R (5'-GACACTTCCGGCACGTAGTT-3'). PCR conditions: 95ºC for 3 min followed by 35 cycles of 95ºC for 30 sec, 60ºC for 30 sec, 72ºC for 1 min and a final extension at 72ºC for 10 min.

Although some populations of these spiders contain *Wolbachia* infections (Rowley et al. 2004), the populations used in the experiments were not naturally infected, as determined by PCR using *Wolbachia* specific primers.

A first experiment was done using 80 jumping spiders (*M. bivittatus*, Salticidae) captured in Brisbane QLD, Australia. They were kept in individual containers and fed for four weeks with *Wolbachia*-infected mosquitoes (10 mosquitoes/spider/week) followed by two weeks of feeding with uninfected mosquitoes to clear their guts from possible remaining undigested infected mosquitoes. Spiders were then killed by freezing and stored at -20ºC until DNA extraction and PCR screening.

A second experiment was done on daddy long-legs spiders (*P. phalangioides*, Pholcidae). The experimental set up was similar to the previous one but it was extended for a longer period and a higher number of individuals were used. Twenty specimens were captured in Brisbane QLD and placed in five cages (30 x 30 x 30 cm). All cages contained males and at least one female in order to allow breeding. The spiders were fed with *Wolbachia*-infected mosquitoes during 16 weeks using up to 1,600 mosquitoes/cage/week as the population of spiders increased rapidly in all the cages. This feeding regime was followed by three weeks of feeding with uninfected mosquitoes to clear the spider guts from possible remaining undigested infected mosquitoes. The 412 spiders obtained after breeding and the 28 egg sacs present on females were then killed by freezing and stored at -20ºC prior to DNA extraction and PCR screening.

No jumping spiders were found positive for *Wolbachia* at the end of the first experiment. Similarly, after being fed as the sole food supply with thousands of *Wolbachia*-infected mosquitoes for weeks, and breeding in those conditions, only two daddy long-legs were positive for *Wolbachia* sequences. However, those two spiders were also positive for mosquito DNA, indicating that residual undigested infected mosquitoes were present within the spiders (Table I).

Overall, the experiments were done on more than 500 spiders and more than 900 eggs (through the analysis of 28 daddy long-legs egg sacs) from the two different species (jumping spiders and daddy long-legs). Those results show that the transmission of *Wolbachia* from mosquitoes to their spider predators is not a common event and was not observed in this experimental survey.

TABLE II

Screening for *Wolbachia* dissemination from mosquitoes to the environment through polymerase chain reaction targeting the specific IS5 sequence

Sample type	*Wolbachia* detection
Soil	0/40
Plants	0/40
Earthworms-millipedes	0/30

A last experiment was then performed to verify that *Wolbachia* did not disseminate in the environment surrounding large numbers of infected mosquitoes. Samples were collected in the Mosquito Research Facility (Cairns), a semi-field fully enclosed outdoor greenhouse style laboratory designed and constructed specifically for this project (www.mosquitoage.org/en/HOME.aspx.) in which thousands of *Wolbachia*-infected mosquitoes were bred for several months. The different samples collected were meant to represent a full variety of species or "environment" in which *Wolbachia* could have disseminated. Samples comprised soil samples, plant leaves and roots, earthworms and millipedes, all collected from inside the enclosure. DNA extraction from all the samples was then performed with appropriate protocols and the presence of *Wolbachia* in the samples was assessed by PCR targeting the specific IS5 *Wolbachia* genes.

The results of the screening done for the possible dissemination of *Wolbachia* to non-predator species and environmental samples show that no *Wolbachia* was detected in any of the samples collected in the semi-field cages where *Wolbachia*-infected mosquitoes were bred for months, neither in plants, soil, nor arthropods living in the cages (Table II).

All together, the results of these experiments show that there is no transmission of the bacteria to any of the environmental samples studied. These results are in fact not surprising and are consistent with the obligate intracellular status of *Wolbachia* and its maternal vertical transmission. One could say that the sample sizes of these experiments are such that very rare horizontal transmission events would not have been detected. However we can be confident from these experiments that horizontal transfer of *Wolbachia* to non-targets does not occur at high frequency.

Even if transfers did occur between individuals, the nature of *Wolbachia* invasions into insect populations is such that a critical threshold frequency of infection must be overcome before the *Wolbachia* infection can spread into a population. This threshold frequency relates directly to the fitness cost imposed by the *Wolbachia* infection on the host (Hoffmann & Turelli 1997, Turelli 2010) and in the case of the *Wolbachia* strains we have been working with, the threshold is estimated to fall between 0.2-0.45. That means that *Wolbachia* infection rate must exceed a local frequency of 20-45% before it can spread. The theory behind this prediction is explained in detail in the literature (Hoffmann & Turelli 1997, Turelli 2010).

Finally the consequence of any horizontal transmission events that might establish in foreign species needs to be considered. It is known that *Wolbachia* infections are quite common in the natural environment in a range of insect species, more than 60% of insects are estimated to be infected by *Wolbachia* (Hilgenboecker et al. 2008), including mosquitoes that commonly bite people such as *Ae. notoscriptus*, *Ae. albopictus*, *Cx. quinquefasciatus* and stored grain pests such as *Tribolium* (Wade & Chang 1995) *Cadra* and *Ephestia* (Ikeda et al. 2003), which leave residues in human food and even iconic species such as the protected Cairns Birdwing butter-

fly. Furthermore, within houses people host the fruit fly *D. melanogaster*, which is infected worldwide with the *w*Mel strain of *Wolbachia,* one of the strains used in this project (Zhou et al. 1998). Considering the wide distribution of *Wolbachia* and no indications that it is impacting negatively on infected insect populations we would contend that the consequences of any harm arising from low probability transfer events would be negligible.

To implement or not to implement?

In conclusion, as it is our responsibility to insure that the "remedy will cause no harm", and as community concerns were centred upon safety and the possibility of *Wolbachia* transfer, a number of experiments were conducted to verify that the *Wolbachia*-based strategy to control mosquito-borne disease is safe for people, other organisms and the environment. The results presented in this paper show that no experimental evidence of any negative impact of *Wolbachia*-infected mosquitoes was obtained.

Assessing experimentally the potential consequences that could happen over a long-term period and large geographic scale could be a daunting task. Many questions related to long-term consequences can only be assessed once the release is done. Questions such as the evolution of the virus in response to the presence of *Wolbachia*, or the persistence of the virus blocking phenotype after generations in natural population are examples of key concerns for the scientific project and for the community but indeed require *a priori* the release to be answered.

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