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The proposed release of the yellow fever mosquito, *Aedes aegypti* containing a naturally occurring strain of *Wolbachia pipientis*, a question of regulatory responsibility

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Abstract In 2010 a proposal to release the yellow fever mosquito, *Aedes aegypti*, containing an intracellular symbiotic bacterium, *Wolbachia*, as a means of reducing the severity of outbreaks of dengue fever was lodged in Australia. The mosquito was infected with *Wolbachia* through embryonic microinjection. This proposal uncovered a gap in the regulatory process normally used to assess the release of species into Australia. Firstly, while the association between the mosquito and the bacterium was new, both species naturally occurred in Australia and so legislation governing the introduction of new species into Australia was ruled not relevant. Secondly, the infection of the mosquito with *Wolbachia* did not involve gene technology and so was not subject to legislation governing the approval of genetically modified organisms. The solution came through the decision to use existing legislation to regulate *Wolbachia* as a veterinary chemical product. This was a good outcome as it overcame the barrier that a lack of regulatory oversight may have posed to field trials taking place. Furthermore, the approach taken demonstrated a very high level of scrutiny with regard to biosafety. This case is an example of how science is leading to advances that outstrip existing regulatory frameworks. An acceptable regulatory solution has been found, but the novelty of the science is such that the appropriateness of the regulatory process now needs to be reviewed to ensure that it is no more onerous for both the proponents and the regulators than it needs to be.

Keywords Biological control · Dengue · Arboviruses · Genetically modified organism · Legislation · *Wolbachia* · *Aedes aegypti* · Risk assessment

1 Introduction

The introduction of species into a country is usually a regulated process. However, there are instances where the release request falls outside normal regulatory pathways. In such instances, the proponent is placed in a regulatory 'no man's land'. One response could be to simply go ahead with the release, but this leaves the proponent vulnerable should the introduction lead to adverse consequences. On the other hand, they can attempt to cobble together a 'work around' that enables regulators to bring the request under their jurisdiction. An example is the 2009/2010 request to release into Australia a mosquito that had been infected with a symbiotic bacterium, *Wolbachia pipientis*, as a means of reducing the incidence of dengue fever. The complication was that both the mosquito and the *Wolbachia* used occur naturally in Australia, but not as a mosquito containing the bacterium. The challenge was to find a regulatory process that could assess the release proposal.

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1.1 Background

The Eliminate Dengue Program (http://www.eliminatedengue.com/en/HOME.aspx) is a large multi-institution research program involving Australia, Thailand, Vietnam and the USA and is led by the University of Queensland. It aims to control mosquito-borne diseases such as dengue, yellow fever and Chikungunya.

Dengue fever and its more extreme form dengue haemorrhagic fever are major global human health problems estimated to cause between 50–100 million cases annually (http://www.who.int/mediacentre/factsheets/fs117/en/). Both the geographic spread and severity of outbreaks are increasing. In Australia, the vector, *Aedes aegypti*, occurs in the northern parts of the country (Russell et al. 2009). Dengue is not endemic in Australia but is continually reintroduced by means of infected travellers (Gould and Solomon 2008). As in other parts of the world, outbreaks in Australia are becoming more frequent. The most recent occurred in 2009 in Cairns and resulted in over 1,000 reported cases (Anonymous 2009a, b). Currently, there is no cure or vaccine for dengue and control is targeted at reducing vector abundance.

The Eliminate Dengue Program is using the naturally occurring obligate intracellular insect bacterium *Wolbachia pipientis* to suppress transmission of dengue viruses (Moreira et al. 2009). Insects are naturally infected with a range of intracellular bacteria such as *Wolbachia*. These bacteria are not transmitted infectiously between individuals, but are instead transmitted vertically, from mother to offspring, through the eggs of an infected female. *Wolbachia* are extremely common, infecting more than 20 % of all insect species (Werren et al. 1995; Jeyaprakash and Hoy 2000; Hilgenboecker et al. 2008). Currently, *Wolbachia* does not naturally infect *A. aegypti* although there is evidence to suggest that in the past it may once have been infected (Klasson et al. 2009, Woolfit et al. 2009). The *Wolbachia* used to infect *A. aegypti* was isolated from *Drosophila melanogaster*. It is known as *wMel* and is present in numerous species of Diptera in Australia. *Aedes aegypti* was infected via microinjection into mosquito eggs (McMeniman et al. 2009). Subsequently, all eggs produced by infected females contain *Wolbachia*.

Wolbachia can confer a range of beneficial, neutral and pathogenic phenotypic characteristics on hosts (McGraw and O'Neill 2004), including cytoplasmic incompatibility (CI). This reproductive trait provides a mechanism by which *Wolbachia* is able to invade an interbreeding insect population by reducing the reproductive output of female insects that do not carry *Wolbachia* if they mate with a *Wolbachia* infected male (Hoffmann and Turelli 1988). In addition to CI, *Wolbachia* in *A. aegypti* greatly reduces the replication of dengue and other human pathogens in the mosquito (Kambris et al. 2009; Moreira et al. 2009; Bian et al. 2010). *Wolbachia* can also shorten the lifespan of *A. aegypti* (McMeniman et al. 2009) and causes a 'bendy proboscis' phenomenon in ageing *A. aegypti* females whereby they cannot penetrate human skin for blood feeding (Turley et al. 2009).

1.2 Horizontal transfer

Wolbachia is known to transfer between unrelated species in a process known as horizontal transfer, but this is a seemingly rare event and the mechanism is unknown. Experimental attempts to achieve horizontal transfer have been largely unsuccessful (Heath et al. 1999; Huigens et al. 2004). This poses the interesting question of how *Wolbachia* establishes in insect hosts naturally when 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 effort, the mechanisms of natural transfer remain unknown. The only two recorded examples of natural horizontal transfer have been between different parasitoids superinfecting the same insect host (Heath et al. 1999; Huigens et al. 2004). It should also be noted that the scientific community researching *Wolbachia* is quite large and there is a significant body of literature on *Wolbachia* biology, with over 1,500 peer reviewed papers published. Currently, approximately two scientific papers on *Wolbachia* are being published every week—so there is a considerable appreciation of the biology and natural history of *Wolbachia*. The general consensus of the scientific community is that natural horizontal transfer events are extremely rare. However, 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 has allowed time for numerous rare events to accumulate.

1.3 Production of Wolbachia-infected *Aedes aegypti*

The process by which *Wolbachia* was successfully transferred to *A. aegypti* was complex. *Wolbachia* are routinely cultured in insect cell lines (Dobson et al. 2002; Furukawa et al. 2008; Jin et al. 2009; O'Neill

et al. 1997; Xi and Dobson 2005) and the principal means of transfer to novel hosts is by microinjection, which is both technically challenging and unpredictable in terms of success (McMeniman et al. 2008). The key to the successful transfer was believed to be due to the pre-adaptation of *Wolbachia* in mosquito cell cultures of *Aedes* species. Here, the *wMel* strain used to infect *A. aegypti* was maintained in an *A. albopictus* cell line for ~240 passages (about 2.5 years) before being transferred to an *A. aegypti* cell line and cultured for another 60 passages. Subsequent genomic analysis of this strain after serial passages revealed a number of genetic changes that occurred in the *Wolbachia* genome. It is believed that the genetic changes incurred during cell culture were critical elements of the adaptation process required for successful transfer. Stable infection in live *A. aegypti* was then achieved by embryonic microinjection (McMeniman et al. 2008, 2009). The mosquito containing the *Wolbachia* is known as *wMel A. aegypti*, a wild mosquito containing a naturally occurring *Wolbachia*.

1.4 Release aim

The aim of the release was to seed the wild mosquito population with *Wolbachia* infected individuals and to then use the CI conferred by the *Wolbachia* to enable the bacteria to introgress into the wild population. The net result would be a mosquito population with a greatly diminished capacity to act as a vector for this virus. The proposed release involves establishing small populations of *Wolbachia*-infected *A. aegypti* to produce a self-sustaining population.

2 Regulatory environment

The release of a species into Australia is primarily governed by four pieces of legislation: Quarantine Act 1908, Biological Control Act 1984, Environment Protection and Biodiversity Conservation Act 1999 and Gene Technology Act 2000. The release of *Wolbachia*-infected *A. aegypti* (hereafter *wA. aegypti*) raised a principal regulatory problem, to find a regulator that had the jurisdiction to approve the release. A further complication is that in Australia, mosquitoes are regarded as a human/public health problem and so sit within the jurisdiction of health departments; in the national context this is the Department of Health and Ageing. This Department has no direct responsibility for the Quarantine, Biological Control or Environment Protection and Biodiversity Conservation Acts; it does have responsibility under the Gene Technology Act.

2.1 Biological control agent

Initially it was thought that *wA. aegypti* could be considered an inoculatively released biological control agent, one where the agent is released in small numbers, establishes, increases in numbers and then spreads of its own accord. While Australia has specific legislation governing the introduction and release of biological control agents, the Biological Control Act 1984, in most cases this legislation is not used. The reason is that the legislation deals primarily with a specific situation, one where there are conflicting interests regarding whether the release should or should not occur. The legislation provides a mechanism whereby the potential cost of the release (for example, loss of income) incurred by one sector is weighed against the benefits gained by another.

In most cases though, the release of a biological control agent does not have a sector that identifies a potential for a negative impact on livelihood. In these cases, agents are introduced under the combined use of the Quarantine Act 1908 and the Environment Protection and Biodiversity Conservation Act 1999. These pieces of legislation fall under the joint jurisdictional responsibilities of the Australian Government Departments of Agriculture, Forestry and Fisheries (DAFF) (Quarantine Act 1908) and Sustainability, Environment, Water, Population and Communities (SEWPAC) (Environment Protection and Biodiversity Conservation Act 1999), respectively. These pieces of legislation govern the regulatory process whereby decisions to allow or prevent the introduction of new species are made. In the case of the proposed release of *wA. aegypti*, the key question was whether the release involved the introduction of a new species. In this situation, both the *A. aegypti* and the *Wolbachia* being used were already in Australia so in both cases the species were considered to be in Australia. The fact that the two species were being incorporated to produce a 'novel organism association' with considerable changes to its biology was not relevant. As a consequence neither law provided the avenue for DAFF and/or SEWPAC to take regulatory responsibility for the release.

2.2 Genetically modified organism

The next question was whether *wA. aegypti* could be considered a genetically modified organism (GMO). In Australia, GMOs are regulated under the Gene

Technology Act 2000. The objective of the legislation "is to protect the health and safety of people, and the environment, by identifying risks posed by or as a result of gene technology, and by managing those risks through regulating certain dealings with genetically modified organisms" (Gene Technology Act 2000). Under the legislation a genetically modified organism is defined as:

(a) an organism that has been modified by gene technology; or
(b) an organism that has inherited particular traits from an organism (the initial organism), being traits that occurred in the initial organism because of gene technology; or
(c) anything declared by the regulations to be a genetically modified organism, or that belongs to a class of things declared by the regulations to be genetically modified organisms; but does not include:
(d) a human being, if the human being is covered by paragraph (a) only because the human being has undergone somatic cell gene therapy; or
(e) an organism declared by the regulations not to be a genetically modified organism, or that belongs to a class of organisms declared by the regulations not to be genetically modified organisms.

Furthermore, under the legislation, 'gene technology' is defined as

"Any technique for the modification of genes or other genetic material, but does not include:

- sexual reproduction; or
- homologous recombination; or
- any other technique specified in the regulations." (Gene Technology Act 2000, Division 2, Definitions, p. 6)

The Office of the Gene Technology Regulator (OGTR), established within the Australian Government Department of Health and Ageing to provide administrative support to the Gene Technology Regulator in the performance of his functions under the Gene Technology Act 2000, is the body responsible under the legislation to regulate the testing and release of GMOs. They further describe 'gene technology' as: "Biotechnology is a broad term that covers the practical use of biological systems to produce goods and services. It encompasses the transformation of materials by micro-organisms (e.g. fermentation), methods of propagation, such as plant cloning or grafting, and may involve genetic alteration through methods such as selective breeding. Recent advances in biotechnology provide ways of introducing very precise changes to genetic material that allow, for the first time, the transfer of properties of a single gene from one organism to another.

These new techniques, commonly referred to as "gene technology", involve the modification of organisms by the direct incorporation (or deletion) of one or more genes to introduce or alter a specific characteristic or characteristics. Organisms created using gene technology techniques are commonly referred to as 'genetically modified organisms' (GMOs)." (http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/pubfactsheets-3/$FILE/factwhatis.pdf).

This interpretation is similar to the one under the Cartegena Protocol which uses the term 'modern biotechnology' defined as, "the application of in vitro nucleic acid techniques, or fusion of cells beyond the taxonomic family, that overcome natural physiological reproductive or recombination barriers and are not techniques used in traditional breeding and selection." (http://bch.cbd.int/protocol/).

The current interpretation of 'gene technology' is therefore a process that involves recombinant DNA methodologies to introduce DNA into the organism. A key element is that in GMOs the chromosomal content of the organism is changed in an irreversible manner; this is not the case for *Wolbachia*-infected mosquitoes as infections can be lost from the host (e.g. Keller et al. 2004). As a consequence, the OGTR ruled that the process by which *A. aegypti* was infected with *Wolbachia* and the way in which the *Wolbachia* was prepared for transfer into *A. aegypti* did not, in either case, involve gene technology. As a result *wA. aegypti* was not a GMO and so regulation under the Gene Technology Act was not appropriate.

2.3 A regulatory 'no man's land'

This resulted in a situation where we had a 'novel organism association'; an organism composed of two wild, naturally occurring species that were already in Australia and not considered to be a GMO. The consequence was that none of the legislation normally used to regulate species introductions into Australia was considered relevant to the proposed release.

The absence of a regulator was a source of concern for the Eliminate Dengue Program. The State of Queensland, through the agency Biosecurity Queensland, was supportive of the release and a decision was taken to present a submission to the Primary Industries Ministerial Council (PIMC) seeking support for the release. This was not ideal given the

mandate of PIMC, but its formal role in matters relating to biosecurity meant that it was perhaps the best option through which to achieve formal support for the release. PIMC consists of the Australian Federal State/Territory and New Zealand Ministers responsible for agriculture, fisheries/aquaculture, food and forestry and is the peak government forum for consultation, coordination and, where appropriate, integration of action by governments on primary industries issues.

The Council's terms of reference are to:

(a) "develop, implement and review policies and strategies for achieving agreed national approaches to the development of sustainable primary and related food industries;
(b) actively liaise with other Ministerial Councils and other bodies on matters relevant to the activities of the Council; and
(c) direct the work of and consider matters submitted by its Standing Committee" (http://www.mincos.gov.au/about_pimc).

On the surface, the choice of PIMC is unusual in that neither dengue nor *A. aegypti* are issues affecting the sustainable primary and related food industries. However, both are issues relevant to biosecurity and so fall under the purview of the National Biosecurity Committee (NBC) which reports directly to PIMC. The role of the NBC is to provide strategic leadership in managing national approaches to emerging and ongoing biosecurity policy issues across jurisdictions and sectors. The NBC takes an overarching, cross-sectoral approach to national biosecurity policy in order to achieve national policy objectives for biosecurity in Australia (http://www.daff.gov.au/animal-plant-health/pihc). So even though dengue and *A. aegypti* fall under the aegis of the National Arbovirus and Malaria Advisory Committee (http://www.health.gov.au/internet/main/publishing.nsf/Content/health-arbovirus-namac-overview.htm) which sits within the Federal Department of Health and Ageing, the link to biosecurity was sufficient to enable the connection to PIMC. As part of the due diligence process that proceeds a submission to PIMC, the proposal to release *wA. aegypti* was circulated through various Federal agencies and, therein a regulatory solution was found through the Australian Pesticides and Veterinary Medicines Authority (APVMA). The APVMA is an Australian Federal government statutory authority established in 1993 to centralise the registration of all agricultural and veterinary chemical products.

2.4 The Australian pesticides and veterinary medicines authority

The APVMA assessed the proposed release of *wA. aegypti* to determine whether it could be permitted for the release as a "Veterinary Chemical Product". On the surface this seems somewhat counter-intuitive, but a reading of the relevant legislation provides the basis for the decision. The legal advice (John Owusu, APVMA pers. comm.) given to the APVMA focused on the relevant subsection under the Agriculture and Veterinary Chemicals Code Act 1994 (AgVet Code), section 5(2) paragraph (c). Here, a veterinary chemical product is defined as, (2) a *substance* that is used for *application* to an *animal by any means*, as a way of directly or indirectly; (c) *modifying the physiology of the animal;* so as to *alter its natural development or reproductive capacity* (http://www.comlaw.gov.au/ComLaw/Legislation/ActCompilation1.nsf/0/6D3FCE09FD5175C1CA257788008025FE/$file/AgrVetChemCode1994_WD02.pdf see pp. 37–38), the terms in italics are those that needed to be established as applying for the purpose of allowing the definition of veterinary chemical product to be met.

The advice concluded that *Wolbachia* was a "substance" in accordance with the definition contained at section 3 of the AgVet Code as it was either 'an organism', 'part of an organism', 'material that is produced from an organism' or 'matter whose production involves the use of an organism'. The injection of *Wolbachia* into the mosquito complied with "application… by any means". Furthermore, *A. aegypti* was an "animal" within both the ordinary meaning of that term and within the definition of "animal" contained at section 3 of the AgVet Code which expands on, rather than confines, that ordinary meaning, "animal means any animal (other than a human being), whether vertebrate or not, and whether a food producing species or not, and includes:… (c) any other prescribed form of animal life, whether prescribed by reference to a species or in any other way."

Finally, the advice considered whether the application of *Wolbachia* into *A. aegypti* "modifies its physiology" so as to "alter its natural development or reproductive capacity". In considering this, two interpretations as to the operation of section 5 (2) (c) were addressed,

- Firstly, that modification of the physiology of the animal will automatically be satisfied by evidence of an alteration to the animal's natural

development, productivity, quality or reproductive capacity, or
- secondly, that modification of the physiology of the animal is a separate requirement to be satisfied and, once satisfied, it needs to be established that the modification in physiology has resulted in an alteration to the animal's natural development, productivity, quality or reproductive capacity.

The advice considered that the first interpretation was reasonably open because of the use of the words "so as to" in section 5 (2) (c) rather than the use of the word "and" which would have more clearly established that two separate requirements needed to be satisfied.

If the first interpretation was applied, there was sufficient evidence that insertion of *Wolbachia* into the mosquito resulted in modification of the physiology of the mosquito that led to alteration of the animal's natural development (i.e. shortening of the lifespan) or reproductive capacity (i.e. inhibit reproduction as a consequence of crosses between male *Wolbachia* mosquitoes and uninfected females).

If the second interpretation was applied, inhibiting the reproduction of uninfected females and the lifespan shortening would need to be evidence of modification of the physiology of the mosquito in order for section 5 (2) (c) to be satisfied. The term "physiology" is not defined in the AgVet Code, but the Macquarie Dictionary, Revised Edition defines it as "the science dealing with the functioning of living organisms or their parts". Applying this ordinary meaning, it followed that "modification of the physiology of an animal" would involve modification to the functioning of the mosquito or its bodily parts. The advice considered that inhibiting the reproductive systems and/or lifespan shortening would appear to involve modification to the functioning of the mosquito in its entirety or, at least, a modification to the functioning of the mosquito's bodily parts.

The legal advice therefore concluded that either interpretation resulted in *Wolbachia* meeting the definition of a veterinary chemical product. Furthermore, the current convention is that organisms below the level of nematode are subject to consideration for regulation by APVMA.

The subsequent evaluation drew heavily on the results of the risk analysis of the pending release of the *wA. aegypti*. In the risk analysis, expert knowledge was incorporated into Bayesian belief networks to obtain the risk measure for the release. The risk analysis concluded that there was a negligible risk that the release of *wA. aegypti* would result in more harm than that currently caused by naturally occurring *A. aegypti* over a 30 years timeframe (Murphy et al. 2010, http://www.eliminatedengue.org/LinkClick.aspx?fileticket=nMtZNaIayzw%3dandtabid=3911). In addition, APVMA with support from the Federal Commonwealth Government's Department of Sustainability, Environment, Water, Population and Communities, undertook a further risk assessment with a particular focus on possible environmental impacts. The decision to support trial releases has now been made.

This is a good outcome. It enables research on an issue of enormous public good to be taken to the field and overcomes the barrier that a lack of regulatory oversight may have posed to the field trials taking place. Furthermore, the approach taken directly addresses the concerns raised by Marshall (2010). Marshall (2010) raised the concern that because *Wolbachia*-infected mosquitoes were not genetically modified and so beyond the scope of the Cartagena Protocol (and related protocols such as that regulated through Australia's OGTR) the issue of biosafety would not be handled to the same level of rigour, would not be legally binding and so were by implication weaker and by further implication, posed a higher level of risk. In reality this concern is unfounded. The field trials planned in Australia (the first time this technology has been tested in the field anywhere in the world) for *wA. aegypti* have been subject to considerable external scrutiny. Firstly, an independent risk analysis of the release was undertaken (Murphy et al. 2010, http://www.eliminatedengue.org/LinkClick.aspx?fileticket=nMtZNaIayzw%3dandtabid=3911) and has since been made publically available. Subsequently, APVMA has undertaken its own assessment and found the technology to be sufficiently safe to allow field trials. These trials will be undertaken under the scrutiny of the APVMA and will adhere to the measures that the APVMA has imposed and as such are legally binding. In all, this demonstrates a very high level of scrutiny with regard to biosafety and clearly demonstrates that simply because a technology is beyond the scope of regulation covering GMOs, that the focus on biosafety is no less rigorous.

3 Conclusion

This case is an example of how science is leading to advances that outstrip existing regulatory frameworks and underscores the need for researchers to engage proactively with regulators to inform them of

the implications their science has for existing policy, legislation and regulation. This is in fact what the University of Queensland did. They proactively engaged with a range of regulatory authorities and fully opened their research to external scrutiny with the result that a regulatory solution was found which enabled their research to progress.

This case does raise a new precedent and it is an example of how a new science-driven technology might challenge existing regulatory processes. The process of engagement achieved a regulatory solution, but the question is whether this solution can now be refined to ensure that the level of scrutiny placed on this technology is only as onerous as it needs to be in order to achieve an equivalent level of biosafety as that offered by existing regulatory processes. Key issues for consideration are whether regulation as a veterinary chemical product is ultimately the best way of handling these types of 'novel organism associations' especially when the ultimate aim is to achieve establishment in the environment and the delivery of self-sustaining control without the need for constant releases.

The appropriateness of different legislation to regulate the release of 'novel organism associations' needs to be considered more fully as the science is new and as such the regulatory implications have yet to be fully considered. It is likely that as knowledge of how to manipulate *Wolbachia* and other types of intracellular symbionts increases, there will be further need to review the release of other such 'novel organism associations'. It is important to now consider fully how this new technology should be regulated so as to ensure that the process is no more onerous for both the proponents and the regulators than it needs to be.

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