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Commentary: The Cartagena Protocol in the context of recent releases of transgenic and *Wolbachia*-infected mosquitoes

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Abstract. The Cartagena Protocol on Biosafety is the fundamental document of the United Nations on the safe transfer, handling and use of living modified (LM) organisms. Progress is being made in the development of specific guidelines for LM mosquitoes; however, several issues relating to LM mosquitoes with invasive gene drive systems remain unresolved. Recent releases of LM sterile mosquitoes and mosquitoes infected with a non-transgenic strain of *Wolbachia* allow us to assess the suitability of the Protocol to LM mosquitoes, and to highlight weaknesses of the Protocol that should be addressed prior to an open release of mosquitoes engineered with invasive gene drive systems. One weakness, highlighted by recent exports of LM mosquito eggs from the United Kingdom, is that the Advance Informed Agreement procedure does not apply to LM mosquitoes being considered for release following laboratory studies and/or cage trials in the receiving country. This means that, under the most likely release scenario for any LM mosquito, the exporting country is not required to perform and finance a risk assessment. Another weakness, highlighted by the release of self-propagating *Wolbachia*-infected mosquitoes by Australia, is that several countries are not signatories to the Protocol and may not feel obliged to abide by terms they did not agree to. Releases in the Cayman Islands also highlight confusion over the applicability of the Protocol to transboundary movements between Parties and non-Parties. Lessons learned from these releases should guide the Protocol to addresses the biosafety concerns posed by self-limiting and self-propagating varieties of LM mosquitoes.

Keywords: Advance Informed Agreement, *Aedes aegypti*, Dengue fever, Living modified mosquito, *Medea*, RIDL, transboundary movement, *Wolbachia*, X-shredder.

INTRODUCTION

The Cartagena Protocol on Biosafety is a multilateral environmental agreement that governs the safe transfer, handling and use of living modified (LM) organisms with possible adverse effects on biodiversity or human health (Secretariat of the Convention on Biological Diversity, 2000). The Protocol focuses on the international movement of LM organisms, and outlines methodological steps to consider so that an initial environmental release may be based on a scientifically-sound risk assessment (Annex III of the Protocol). While the Protocol was written primarily with concerns over the safety and trade of LM crops in mind, progress is being made in the development of specific guidelines for LM mosquitoes (Fontes, 2009). These can generally be divided into two categories: self-limiting LM mosquitoes, whose transgenes only persist in the environment for a limited time; and self-propagating LM mosquitoes, which are capable of propagating transgenes across generations. A Sub-Working Group on LM Mosquitoes has been assigned by the Ad Hoc Technical Expert Group (AHTEG) on Risk Assessment and Risk Management, and its first guidance document (AHTEG, 2010) was presented to the Fifth Meeting of the Conference of the Parties Serving as the Meeting of the Parties to the Cartagena Protocol on Biosafety, which took place in Nagoya, Japan in October 2010. This document outlines a number of potential risks that must be considered prior to an open release of LM mosquitoes; however, issues relating to self-propagating LM mosquitoes capable of spreading transgenes beyond their release site are inadequately addressed and should be resolved in future guidance documents (Marshall, 2010).

Recent releases of LM *Aedes aegypti* mosquitoes carrying a dominant lethal gene, also known as RIDL (Release of Insects carrying a Dominant Lethal, Oxitec Limited, United Kingdom), allow us to assess the suitability of the Protocol to a self-limiting strain of LM mosquito. This strain (OX513A) has been engineered with a repressible sterile phenotype that affects both male and female offspring of transgenic individuals (Phuc *et al*., 2007). Large-scale releases of transgenic males are expected to reduce dengue

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transmission because wild females that mate with transgenic males produce no viable offspring, thus reducing the disease vector population size. A project by the Cayman Mosquito Research and Control Unit (MRCU) evaluated an Oxitec Ltd. strain in the first open field trial of an LM mosquito in November-December 2009 in the Cayman Islands to assess the competitiveness of transgenic males in the field. This was followed by a large-scale release of transgenic males in May-October 2010 to test for population suppression (Wilson, 2009). Results from these trials are not yet published; however a presentation by Luke Alphey at the 59th Annual Meeting of the American Society for Tropical Medicine and Hygiene suggests an up to 80% reduction in the local *Ae. aegypti* population ~11 weeks following commencement of the trial which was sustained until the trial ended (Subbaraman, 2011). More recently, the Institute for Medical Research (IMR) in Malaysia carried out another open field trial in December 2010-January 2011 using the same Oxitec Ltd. strain. This took place in an uninhabited area of Bentong to assess the dispersal and longevity of transgenic males in the field (AFP, 2011). Another self-limiting strain engineered with a female-specific flightless phenotype (Fu *et al*., 2010) has undergone successful contained trials and is being evaluated.

Recent releases of *Ae. aegypti* mosquitoes infected with the *wMel* strain of *Wolbachia* – a maternally-inherited intracellular bacterium – highlight questions of relevance to self-propagating varieties of LM mosquitoes. The *wMel* strain of *Wolbachia* is non-transgenic (McMeniman *et al*., 2009), and hence does not fall within the remit of the Cartagena Protocol; however, it does have several properties in common with self-propagating LM mosquitoes – the infection is associated with physiological changes that are beneficial for disease control, and it is capable of manipulating its host's reproductive biology in a manner that promotes its spread through a population (Moreira *et al*., 2009a). As the *Wolbachia* infection spreads through a population, it is hoped that its anti-disease properties – reduced dengue viral load, reduced mosquito lifespan, and reduced ability to obtain blood meals with age (Moreira *et al*., 2009b) – will be conferred to the population as a whole, resulting in reduced dengue transmission to humans. The first open field trial of *Wolbachia*-infected *Ae. aegypti* (henceforth referred to as *wAe. aegypti*) began in January 2011 in two isolated communities in Queensland, Australia, and is showing initial signs of success (McCutcheon, 2010; Ryan and Sexton-McGrath, 2011).

In this paper, we discuss the implications that these releases have for the applicability of the Cartagena Protocol to self-limiting and self-propagating varieties of LM mosquitoes. This leads us to highlight weaknesses of the Protocol that should be addressed prior to an open release of LM mosquitoes with invasive gene drive systems such as *Medea* or homing endonuclease genes.

RELEASES OF STERILE LM MOSQUITOES

As Benedict and Robinson (2003) have argued, it is appropriate that the first releases of transgenic mosquitoes were of genetically sterile males. Male mosquitoes do not bite, reducing the risks to human health, and sterile males produce unviable offspring, thus reducing the population of disease-transmitting female mosquitoes in subsequent generations. Furthermore, because the released transgene encodes sterility, it is only expected to persist in the wild for a few generations following a release. This minimizes biosafety issues because, provided that the LM mosquitoes are not released at the border between two countries, they will remain confined to the country of release. The Cartagena Protocol then applies to the import of LM mosquitoes intended for release into the environment, and requires that Parties make decisions on this import based on a scientifically-sound risk assessment (Article 15 of the Protocol).

In terms of the sterile LM mosquitoes developed by Oxitec Ltd., a risk assessment was conducted for the strain released into the Cayman Islands and Malaysia following a UNDP-sponsored Workshop on the Risk Assessment of Transgenic Insects in November 2008 in Malaysia (Beech *et al*., 2009). This was a scientifically-sound and transparent exercise that assessed 31 risks of a hypothetical open field release of the bi-sex lethal LM mosquito strain (OX513A), and has been expanded upon by Patil *et al*. (2010), who identified an additional two risks for this strain, and another eight risks for a strain engineered with a repressible female-specific flightless phenotype (Fu *et al*., 2010). All of the potential risks were determined to be of either low or negligible magnitude. One of the most serious concerns was that suppression of *Ae. aegypti* populations could lead to replacement with *Aedes albopictus* and a consequent increase in chikungunya transmission; however, this risk was considered of low magnitude because releases of sterile *Ae. aegypti* are not expected to result in population extinction, and the long-term strategy would be to suppress both *Ae. aegypti* and *Ae. albopictus* populations (Beech *et al*., 2009). This risk assessment satisfies the requirement, mandated by the Advance Informed Agreement (AIA) procedure (Articles 8 to 10 and 12 of the Protocol), that the exporting country perform a risk assessment at its own expense, if requested by the importing country, prior to the first transboundary movement of an LM organism intended for environmental release.

One weakness of the Cartagena Protocol, highlighted by exports of LM mosquito eggs by Oxitec Ltd. to the Cayman Islands (a British Overseas Territory), Malaysia and several other destinations, is that the AIA procedure does not apply to LM organisms destined for contained use (Article 7 of the Protocol). In almost all cases, LM mosquitoes are first exported for careful analysis in laboratory studies and cage trials in the receiving country, and the importing country is not entitled to request the exporting country to perform a risk assessment under these circumstances. Therefore, although risk assessments were performed for the LM mos-

quito strains developed by Oxitec Ltd. (Beech *et al.*, 2009; Patil *et al.*, 2010), it is not clear that these were required for exports to Malaysia, Brazil, India, Vietnam, Singapore, France and the United States. Initial containment effectively sidesteps the risk assessment requirement because, once the LM mosquitoes have been received by the importing country, the country is free to release them into the environment in accordance with their own national regulations.

One argument for the inapplicability of the AIA procedure to LM organisms imported for contained use is that the AIA procedure requires an evaluation of the receiving environment as part of the risk assessment (Annex III of the Protocol); however, for strains sent for initial laboratory analysis or cage trials, release sites may not be identified and open releases may not even be planned. One solution, rather than waiving the AIA procedure entirely, would be to waive the requirement of assessing the receiving environment for LM organisms destined for contained use, perhaps only requiring a generic assessment at the national scale. Many countries will require a risk assessment along these lines on their own accord; however, the current inapplicability of the AIA procedure essentially leads to this section of the Protocol becoming a non-binding set of guidelines in these cases.

Releases of Oxitec Ltd. LM mosquitoes by the MRCU in the Cayman Islands also highlight confusion over the applicability of the Cartagena Protocol to transboundary movements between Parties to the Protocol and their overseas territories. As an overseas territory of the United Kingdom (UK), the Cayman Islands are not a member state of the United Nations (UN) and hence are unable to ratify UN protocols independently of the UK. Discussions in the UK parliament suggest that overseas territories are not considered to be the same Party as the UK, but are encouraged to become a Party to the UK's instrument of ratification of the Protocol (UK Parliament, 2010). The Cayman Islands have not done this, and hence are considered to be a non-Party. Parliamentary discussions further suggest that the provisions of the Protocol do not apply to transboundary movements of LM mosquitoes from the UK (a Party) to the Cayman Islands (a non-Party) (UK Parliament, 2010); however, this is in contradiction to Article 24 of the Protocol, which states that "transboundary movements of LM organisms between Parties and non-Parties shall be consistent with the objective of this Protocol."

Regardless of the applicability of the Cartagena Protocol, the export of LM mosquitoes to the Cayman Islands was subject to a similar European Commission regulation (EC Regulation 1946/2003), which requires that the exporter notify the competent authority in the importing country of the first transboundary movement of an LM organism, and to await its consent to proceed (UK Parliament, 2010). The decision to release the LM mosquitoes into the environment is then subject to local legislation. Parties to the Protocol are required to notify the Biosafety Clearing-House (BCH) of decisions to import or release LM organisms into the environment (Article 20 of the Protocol), and Malaysia has done this; however, since the Cayman Islands are a non-Party to the Protocol, they are not required to notify the BCH.

The release of LM mosquitoes into the environment in the Cayman Islands and Malaysia was greeted with some controversy (Nightingale, 2010; Enserink, 2010a, 2011; Subbaraman, 2011; AFP, 2011); however, the major criticisms concerned the manner in which information about the trials was disseminated – an issue which is beyond the scope of the Cartagena Protocol. In both cases, the degree of community engagement was questioned, and several groups complained that they had not been informed about the details of the releases in advance. Additionally, for the Cayman Islands, a video was posted on YouTube describing the trial (GIS Marketing and Communications, 2010), but this did not mention that the released mosquitoes were genetically modified. Despite this, the releases did abide by national regulations in both cases. In the Cayman Islands, the trial abided by a draft biosafety bill that had yet to become law, the MRCU obtained a permit from the Cayman Islands Department of Agriculture, and a risk analysis and environmental impact assessment were carried out (UK Parliament, 2010). In terms of community engagement, elected political representatives were educated, information about the trial was sent to local newspapers, and flyers were distributed among the local population (Enserink, 2010a).

In Malaysia, Oxitec Ltd. and the IMR worked closely with the Malaysian Government in assessing risk factors examined by the Genetic Modifications Advisory Committee (National Biosafety Board, 2010). The Natural Resources and Environment Ministry placed advertisements about the trial in local newspapers on August 5th and 9th 2010 (The Star Online, 2011), and nine Non-Governmental Organizations (NGOs) were invited to provide feedback during a one month public feedback period from August 5th until September 4th 2010 (Fong, 2011). The terms of the trial were publicized on the IMR's website for a month, and the IMR put up notices in the trial area three weeks in advance (The Star Online, 2011). Permission was obtained from local authorities, and the IMR held two public talks with local people – one in collaboration with the Bentong Municipal Council, and another with the Bentong Malaysian Chinese Association (Fong, 2011). Community engagement requirements for the first release were reduced because the trial was carried out in an uninhabited area. Despite this, negative reactions were encountered, particularly from NGOs and the media. Lessons should be learned from their criticisms, while acknowledging that the first use of a transgenic technology will always be greeted with some opposition.

RELEASES OF *WOLBACHIA*-INFECTED MOSQUITOES

Releases of *Wolbachia*-infected mosquitoes in Queensland, Australia in January 2011 were greeted with much less controversy than releases of genetically sterile mosquitoes, likely because the *Wolbachia* infection did not involve genetic

modification. This also meant that there were fewer regulatory hurdles to overcome prior to releasing the *wAe. aegypti* mosquitoes into the environment (Enserink, 2010b), making them an attractive alternative to self-propagating LM mosquitoes engineered with gene drive systems. The Cartagena Protocol does not apply to mosquitoes infected with non-transgenic strains of *Wolbachia* because these do not fall under the definition of an LM organism (Article 4 of the Protocol), which is defined as a living organism containing a novel combination of genetic material obtained through either "*in vitro* nucleic acid techniques" or "fusion of cells beyond the taxonomic family" (Article 3 of the Protocol). Therefore, while the Australian release was subject to national regulations, it was not subject to international regulations regarding subsequent potential transboundary movements.

When the Eliminate Dengue Program at the University of Queensland sought approval to release *wAe. aegypti* into the environment, the situation was described by regulators as a "regulatory no man's land" (De Barro *et al*., 2011). Both *Wolbachia* and *Ae. aegypti* were already present in Australia, so the release did not constitute the introduction of a new species. Furthermore, the Office of the Gene Technology Regulator ruled that *wAe. aegypti* is not a genetically modified organism (GMO), and so could not be regulated under the Gene Technology Act. In the end, a submission was presented to the Primary Industries Ministerial Council, following which it was concluded that the Australian Pesticides and Veterinary Medicines Authority (APVMA) provided the most appropriate regulatory framework. Therein, *wAe. aegypti* was considered to be a "veterinary chemical product" on the basis that *Wolbachia* is a "substance that is used for application to an animal… as a way of directly or indirectly modifying the physiology of the animal so as to alter its natural development or reproductive capacity" (Australian Government, 1994). A decision was taken to approve the trial based on the results of a prior risk assessment undertaken by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) (Murphy *et al*., 2010) and a risk assessment focusing on environmental impact undertaken by the APVMA with support from the Federal Commonwealth Government's Department of Environment, Water, Heritage and the Arts. The trial is currently being monitored by the APVMA.

As pointed out by De Barro *et al*. (2011), the Australian regulatory framework is rigorous; however, a major weakness is that, if the trial is successful, *wAe. aegypti* is predicted to spread throughout Australia and possibly beyond, while only Australia was considered in the regulatory approval. The same weakness is reflected in the risk assessment conducted by the CSIRO (Murphy *et al*., 2010). Here, a total of 50 risks were identified following community engagement exercises, a workshop and email solicitation with a dengue consultation group. The CSIRO acknowledges that, if successful, "*Wolbachia Ae. aegypti* will be self-sustaining after the inoculative release and… will be driven into the Australia *Ae. aegypti* populations…" (Murphy *et al*., 2010); however, the possibility that the strain may spread into other countries is not considered (Vasan, 2010). It may be argued that, since the release is judged safe by Australia's rigorous standards, then it will also be safe for other countries; however, this assumes that the standards of one country apply to another, which is not necessarily the case. Nevertheless, there are no international regulations that apply to non-transgenic *Wolbachia*-infected mosquitoes, and so, even if a release is expected to have international implications, the decision to release is a national one.

Furthermore, even if *wAe. aegypti* was considered to be an LM organism and hence within the remit of the Cartagena Protocol, the significance of the Protocol would be undermined by the fact that Australia is not a signatory. Like many countries with strong biotech industries, such as Argentina and the United States, Australia is weary of signing the Protocol because it may restrict trade, partly because a strong interpretation of the precautionary principle may allow economic protectionism to masquerade as environmental protection (Cosbey and Burgiel, 2000). Article 24 of the Protocol states that the Protocol also applies to "transboundary movements of LM organisms between Parties and non-Parties," and a release of *wAe. aegypti* in Australia could conceivably spread into any number of Parties to the Protocol (Papua New Guinea, for instance); however, it seems overly optimistic that a non-Party will feel obliged to abide by a Protocol that it did not agree to. The Nagoya-Kuala Lumpur Supplementary Protocol on Liability and Redress (Secretariat of the Convention on Biological Diversity, 2011) applies to "damage resulting from transboundary movements of LM organisms," and includes movements originating from non-Parties in its scope (Article 3 of the Supplementary Protocol); however, it is unclear whether the liability procedures (Article 12 of the Supplementary Protocol) would provide an adequate incentive to prevent a non-Party from releasing a self-propagating LM organism on their own accord. In essence, even if the Protocol did apply to *wAe. aegypti*, the Australian release may still have occurred.

SELF-PROPAGATING LM MOSQUITOES

The regulatory approaches that governed the releases of genetically sterile and *Wolbachia*-infected mosquitoes have significant implications for LM mosquitoes engineered with gene drive systems capable of propagating transgenes across national borders. Of particular concern, the AIA procedure of the Cartagena Protocol is not expected to apply to many imports of self-propagating LM mosquitoes because the first mosquitoes will likely undergo cage trials in the receiving country to assess their behavior in the ambient environment in a location that the species naturally inhabits (Benedict *et al*., 2008). Under these circumstances, the importing country will not be entitled to request the exporting country to perform a risk assessment at their own expense, which is a concern for developing countries with a lack of resources to conduct a comprehensive risk assessment of their own (Mar-

shall, 2010). As was the case for the sterile LM mosquitoes developed by Oxitec Ltd., it is likely that a risk assessment for self-propagating LM mosquitoes will be conducted anyway, either voluntarily or as required by national or regional regulations in the exporting country; however, it is concerning that a risk assessment is not mandated by the Cartagena Protocol under the most likely scenario in which a release follows contained study.

Another concern is that, although a release of LM mosquitoes with invasive gene drive systems could propagate transgenes over entire continents, the Cartagena Protocol may not be strong enough to prevent some countries from acting unilaterally in a decision to release them. A strict interpretation of the Protocol suggests that a release of LM mosquitoes with invasive gene drive systems would require a multilateral agreement between all affected nations – a difficult task considering the potential scale of spread (Marshall, 2010) – however, the release of *wAe. aegypti* mosquitoes, which are also expected to propagate across national borders, based on a unilateral decision by Australia, a non-Party to the Protocol, highlights the possibility that LM mosquitoes with invasive gene drive systems could also be released in the absence of an international agreement. Such a release could occur because a non-Party may not feel obliged to abide by the terms of a protocol it did not agree to, the likelihood of damage to a Party resulting from an unintentional transboundary movement may be considered minimal, and the liability measures outlined in the Supplementary Protocol may be inadequate to dissuade a non-Party (or even a Party) from conducting a release on their own accord. Several disease-endemic countries, such as Cote d'Ivoire, Sao Tome and Principe, Equatorial Guinea, Sierra Leone and Vanuatu, are yet to sign the Protocol, in addition to the countries with strong biotech industries mentioned earlier.

Issues relating to self-propagating LM mosquitoes should be taken seriously, as work is currently ongoing to create *Medea* elements capable of driving disease-refractory genes (e.g. Ito *et al.*, 2002; Franz *et al.*, 2006) into mosquito populations, with the goal of rendering entire vector populations refractory to disease (Chen *et al.*, 2007). Work is also ongoing towards the development of an X-shredder which utilizes a homing endonuclease to cleave the X chromosome during spermatogenesis (Windbichler *et al.*, 2008), thus creating a bias towards Y-bearing spermatozoa and eventually leading to an all-male population crash (Burt, 2003). Both systems are capable of spreading across national borders, with the latter strategy inducing a cascade of population crashes in its wake. Interestingly, the goal of creating an X-shredder in *Anopheles gambiae*, the main African malaria vector, has led to the intermediate creation of an LM sterile male (Windbichler *et al.*, 2008), which could be used for self-limiting population suppression.

Finally, work is ongoing towards the creation of gene drive systems that will only spread into a population if they exceed a critical population frequency (e.g. Davis *et al.*, 2001; Marshall *et al.*, 2011). These systems have three desirable features for biosafety when the goal is local population replacement: accidentally-released mosquitoes are unlikely to persist in the wild because they will inevitably be present at sub-threshold levels; mosquitoes released at super-threshold frequencies at an isolated release site are expected to spread transgenes locally while remaining at sub-threshold levels at nearby locations; and transgenes can be eliminated from the release site through a sustained release of wild mosquitoes diluting transgenes to sub-threshold levels. These desirable features are acknowledged in the first guidance document of the Sub-Working Group on LM Mosquitoes (AHTEG, 2010), although a proper ecological assessment will be required on a case-by-case basis when such a release is considered.

CONCLUSION

Ostera and Gostin (2011) have argued for a new international treaty governing the environmental release of genetically or biologically-modified arthropod disease vectors. A dedicated treaty would certainly be able to address the unique biosafety concerns posed by LM and *Wolbachia*-infected mosquitoes; however, any treaty will inevitably be faced by the problem that there will be non-signatories who may choose to release self-propagating modified mosquitoes on their own accord. Given that releases of modified mosquitoes have already begun, an adaptation of the Cartagena Protocol that quickly addresses the pressing needs of LM mosquitoes provides a better solution, particularly considering that the Protocol already has 160 signatories – a total which has taken more than a decade to achieve.

The exemption from the AIA procedure of LM mosquitoes being considered for release following initial laboratory studies and/or cage trials is one major weakness of the Protocol. This issue is important to address prior to the development of LM mosquitoes with invasive gene drive systems because their risks are magnified by the ability of transgenes to spread globally, however a risk assessment is not currently mandated under the most likely release scenario. Another weakness is that it may currently be impossible to release LM mosquitoes with invasive gene drive systems in a manner that is consistent with the Protocol if one of the countries into which they may spread is fundamentally opposed to GMOs (Marshall, 2010). A mechanism for independent review of LM organisms with international implications for biodiversity and/or human health should be considered in these cases (Ostera and Gostin, 2011), since a paralytic Protocol is less likely to be abided by. Such a review should consider both the risks and benefits of LM mosquitoes (Morris, 2011), and the promise that LM mosquitoes have for reducing the global burden of vector-borne diseases.

It will be difficult to encourage non-Parties to abide by the terms of the Protocol no matter what changes are made; however, clarification should be provided to Parties of the Protocol that the Protocol does apply to exports of LM organisms to non-Parties. Clarification should also be provid-

ed that the Protocol does apply to mosquitoes infected with transgenic strains of *Wolbachia*, if such strains prove useful. Further discussions will be required to guide the evolution of the Cartagena Protocol, and lessons learned from recent releases will ensure that the Protocol moves in the right direction to address the unique biosafety concerns posed by self-limiting and self-propagating LM mosquitoes.

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