Genetically Modified Insect Factories: A New Source of Superbugs?

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Antibiotic resistance is recognised to be a major problem for human and animal health as it makes treatments for many bacterial infections ineffective. Bacteria that are resistant to multiple antibiotics are sometimes known as “superbugs”.

Genetically modified (GM) mosquitoes and agricultural pests have been developed which are bred using the common antibiotic tetracycline in their feed. This report explores concerns that mass-breeding and repeated large-scale open releases of such GM insects could spread antibiotic resistance into the environment, potentially creating more superbugs.

Mass production of GM insects in factories, using tetracyclines as an additive in their feed, could lead to drug resistance in their microbiota, in the same way that treating bees with tetracyclines has selected for antibiotic resistance. Oxitec’s GM insects may then disseminate antibiotic resistance when released into the environment in the repeated, large-scale releases needed to vastly outnumber wild pest insect numbers.

Large-scale experimental releases of tens of millions of such insects are already taking place in some countries, without considering this important risk. If commercial uses were to take place in the future, many billions of GM insects would be released year after year in attempts to suppress wild pest insect populations over vast areas and perhaps whole countries. If such GM insects became a new source of antibiotic resistance worldwide, this could have serious adverse impacts on human and animal health.

This report highlights evidence that:

- Exposure to antibiotics of the tetracycline class in feed during mass production is likely to lead to the development of antibiotic resistant bacteria in the GM insects’ guts;
- Repeated releases of tens of millions of GM insects will disseminate these bacteria and their resistance genes widely in the environment;
- In many insect species, bacteria and antibiotic resistance genes may spread through the insect population from the mainly male GM insects that are released to their wild mates and offspring;
- Horizontal gene transfer may lead to transfer of antibiotic resistance to bacteria causing food- and water-borne diseases, such as *E. coli*, via larvae that develop in the food (agricultural pests) or water (mosquitoes);
- Direct routes of transfer to humans or animals include swallowing GM agricultural pests at the larval stage when they will contaminate fruit and vegetable supplies, swallowing GM mosquito larvae via contaminated water, or swallowing adult GM insects during mass releases, when they vastly outnumber the wild population;
- Disposal of tetracycline-contaminated water from GM insect factories may also pose a risk and antibiotic resistance might also be transferred to workers in these factories;
- The potential for antibiotic resistance to be transferred to other bacteria in the longer term via indirect routes, such as being spread by rodents eating GM larvae on crops to the bacteria carried by ticks which cause Lyme disease, needs further

investigation, although horizontal gene transfer is much rarer in these species of bacteria.

- Mass-breeding of GM insects using tetracycline breaches existing policies and regulations, which advise against indiscriminate feeding of antibiotics to animals worldwide and which ban this practice in some countries.

Background: Oxitec's GM insects

The UK company Oxitec has created genetically modified (GM) insects - including mosquitoes, flies and moths - for open release in very large numbers into the environment, swamping the wild population by an order of magnitude or more. The mainly male GM insects that are released are intended to mate with wild insects and to reduce the wild population because many of the offspring are genetically programmed to die before they are old enough to reproduce.

Oxitec's technology has been developed for use in mosquitoes and agricultural pests. Oxitec is a spin-out company from Oxford University[1], with close links to multinational seed and agrochemical firm Syngenta. Ex-Syngenta staff who joined Oxitec since 2006 Oxitec include Oxitec's CEO, Chief Scientific Officer, Regulatory Affairs Manager and Head of Business Development.[2] Oxitec's Chair is also a former employee of Syngenta. Oxitec consultants include Colin Ruscoe, former site manager at Syngenta Crop Protection, who is Chairman of the British Crop Production Council.[3] Oxitec's former Head of Business Development (from 2006 to 2010), Ann Kramer, is another ex-Syngenta employee who continues to act as a consultant for the company.[4] From March 2009 to June 2011, Oxitec received research funding directly from Syngenta for genetic transformation of *Lepidoptera*. This order of insects includes the diamondback moth, which Oxitec has made an application to release in New York State.[5]

Oxitec's GM insects can be bred in a laboratory for research purposes but a large-scale production facility or insect farm is needed to produce the numbers needed to perform open release experiments or to use the insects on a commercial scale. For open release, the GM insects are sorted into males and females and, with the intention that only the males are then released (although some females are inadvertently released due to limitations in the sorting process). In order to allow breeding in the factory, the genetic killing mechanism can be switched off using a common antibiotic (tetracycline) in the insects' feed, allowing multiple generations to survive and breed in the laboratory.[6] The use of tetracycline in the insects' feed during mass production raises concerns that antibiotic resistant bacteria might develop in the insects' guts and then be spread into the environment upon release. Further, mass production will give rise to large quantities of tetracycline-contaminated feed which may also spread antibiotic resistant bacteria during its disposal.

Oxitec's patented technique for genetically modifying insects is known as RIDL (Release of Insects carrying a Dominant Lethal genetic system). Currently, all the company's open field experiments involve its OX513A strain of the *Aedes aegypti* mosquito. This is genetically engineered to contain a red fluorescent marker and the RIDL 'conditional lethality' trait which kills the offspring of the mosquitoes at the larval stage, in the absence of the tetracycline antidote. Experimental releases of GM mosquitoes using this approach are ongoing in Brazil and Panama and being considered in the USA (Florida Keys). Commercial releases of GM mosquitoes are being considered in Brazil: they have been approved by Brazil's genetic technology regulator, CTNBio, but not yet by the Brazilian Health Surveillance Agency, ANVISA. Other countries (Malaysia and the Cayman Islands) have conducted smaller-scale experiments which have now been discontinued.

Experimental releases of Oxitec's GM fruit flies have also been approved for fruit orchards in Brazil, although these experiments have not yet taken place, and experimental releases of GM moths are being considered in fields of brassica crops (such as broccoli, cabbages and oil seed rape/canola) in the USA (New York State). These GM agricultural pests are female-killing only i.e. only the female offspring are genetically programmed to die in the absence of the tetracycline antidote (the company calls this female-sex RIDL or fsRIDL). [7, 8,9,10,11]

Oxitec's aim is to establish a new method of pest control, involving large-scale releases of GM insects worldwide (including mosquitoes and agricultural pests). Repeated very large-scale releases of GM insects are required to suppress wild insect populations and it is not yet clear whether this form of pest control can be successful.

Although Oxitec claims to have achieved temporary suppression of mosquito populations during its experiments in Brazil, it has yet to publish any evidence of this or to show any reduction in the tropical disease dengue fever which it is targeting.[12,13] In fact, a dengue emergency has been declared in one of the areas that Oxitec has been conducting its experiments.[14] Many concerns have been raised about the release of large numbers of GM insects into the environment, and about the poor quality of the risk assessments that have been conducted. [15,16,17,18,19,20,21,22]. However, the focus of this report is on the issue of antibiotic resistance only. This important issue has not been considered in any of the environmental risk assessments for GM insects conducted prior to their release into the environment.

Risk assessments undertaken prior to experimental releases of GM mosquitoes in Brazil, Panama, Malaysia and the Cayman Islands have failed to consider antibiotic resistance. Releases of GM Medfly into fruit orchards in Brazil have also been approved without considering the potential for GM Medfly to spread antibiotic resistance into the environment and the human food chain (these releases have not yet taken place). The US Animal and Plant Health Inspection Service (APHIS) is considering approving the mass production of GM diamondback moths fed on tetracycline as part of Oxitec's proposed experiments in New York State, again without any consideration of antibiotic resistance in the risk assessment published for public consultation. In addition, regulators at the FDA are reportedly considering approving mass releases of GM mosquitoes fed on tetracycline in the Florida Keys.

Antibiotic resistance – a major public health concern

Antibiotic resistance occurs when micro-organisms, usually bacteria, become resistant to antibiotic drugs. Antibiotics are commonly used to treat infections in humans and animals, so if bacteria become resistant to them these diseases require different treatments. These second-line treatments are often less effective, more likely to cause side effects, or may be more expensive. If there are no second-line treatments for an infection, or if the bacteria causing the disease become resistant to all treatments (when they are sometimes known as "superbugs"), an infection may become untreatable.

There is a growing recognition that antibiotic resistance poses a serious, worldwide threat to public health. The World Health Organisation (WHO) has highlighted resistance in many common bacterial pathogens and warned of a future in which common infections and minor injuries could kill.[23] Very high rates of resistance have been observed in bacteria that cause common health-care associated and community-acquired infections (such as urinary tract infections, pneumonia, wound and blood stream infections and diarrhoea) in all regions of the world and there are also threats to disease-specific programmes such as treatments for tuberculosis.

The annual cost to the US health care system from antibiotic-resistant infections is estimated to be between $21 and $34 billion[24] and it is estimated that 2 million people become infected with bacteria that are resistant to antibiotics and at least 23,000 people die as a direct result in the United States each year[25]. A recent UK report estimates that at least 700,000 people die globally each year as a result of antimicrobial resistance (AMR).[26] This report predicts that there will be 300 million premature deaths and up to $100 trillion (£64 trillion) lost to the global economy by 2050, if the problem is not tackled. Malaria resistance is expected to lead to the greatest numbers of fatalities, while *E. coli* is the largest detractor from GDP, accounting for almost half the total economic impact.

In low-income countries or poorer communities in mid-income countries, drinking water is a major source of bacterial infections and antibiotic resistance in such infections can be a major problem. Bacteria such as *Escherichia coli* (*E. coli*) can cause diarrhoea of varying severity, and some types (notably shiga-toxin producing *E coli*, or enterohaemorrhagic *E. coli*, EHEC) sometimes lead to death. A number of studies have found resistant *E. coli* bacteria in drinking water supplies, most likely due to contamination with sewage.[27,28,29]

Antimicrobial resistance also exists in food-producing-animals and the food chain, but major gaps exist in information about its extent and how it spreads. The largest non-human use of antimicrobial agents is in food animal production, and most of this is in healthy animals in order to increase growth (particularly in the USA) or prevent diseases in the crowded conditions in which livestock and poultry are typically raised under industrial production.[30] This selects for antibiotic resistance genes: for example such genes increase in abundance and diversity in medicated pigs and can be detected on pig farms. [31,32]

There is now considerable evidence that transfer of antimicrobial resistance from food-producing animals to humans directly via the food chain is a likely route of spread, although the complex routes through which resistance spreads are poorly understood.[33] For example transfer can occur by direct handling or close contact between infected animals and humans, transmission via contaminated animal products (including food), and transmission via contamination of the environment, which may be direct from animals (excretions and secretions) or indirect, via, for example, the spreading of slurry or the contamination of watercourses. These mechanisms can lead to development of antibiotic resistant food-borne pathogens, making such diseases harder to treat. Food products containing antibiotic resistant bacteria include meats, fish and dairy products (milk, cheese and eggs). In addition, bacteria on fruits and vegetables often originate from fertilisers (including manures and slurries) and irrigation water, which may contain resistant bacteria.

One important example is MRSA (Methicillin-resistant *Staphylococcus aureus*), a bacteria responsible for several hospital-acquired infections in humans. MRSA is resistant to beta-lactam antibiotics, which include penicillin, and in some cases it also carries resistance to tetracycline: this is known as multidrug-resistant *Staphylococcus aureus* (MDRSA). A direct contamination route has been demonstrated from antibiotic use on pig farms to the spread of resistant bacteria via agricultural workers and veterinarians.[34,35,36,37,38]

An additional route of spread is the transfer and exchange of genetic information between bacteria, for example in the guts of animals or humans. Bacteria become resistant to antibiotics by acquiring genetic mutations which create resistance genes. These mutations can occur randomly or can be acquired, usually from other bacteria, through a process known as horizontal gene transfer (HGT). Exposure to an antibiotic naturally selects for the survival of the organisms with the genes for resistance. In this way, a gene for antibiotic resistance may readily spread through bacteria. The development of resistance is a normal

evolutionary process for microorganisms, but it is accelerated by the selective pressure exerted by widespread use of antibacterial drugs in humans and animals. Resistant strains are able to propagate and spread more quickly where there is over-use or misuse of antibiotics.[39] In environmental samples where antibiotics are present at high concentrations, resistance genes are both diverse and highly enriched in parallel with transposases (highly mobile gene capture systems known to be associated with resistance genes).[40]

Many antibiotic resistance genes occur on plasmids. Plasmids are small circular, double-stranded DNA molecules, which commonly occur in bacteria and can replicate independently of the main DNA sequence in the chromosomes of the cell. Plasmids can be transmitted from one bacterium to another via horizontal gene transfer (HGT) and hence antibiotic resistance can spread, even to bacteria of other species. HGT has been a major contributing factor to the rapid spread of antibiotic resistance amongst pathogenic bacteria in the last 50 years, including multi-drug resistance (creating "superbugs").[41,42]

Use of an antimicrobial agent such as tetracycline selects for growth of a bacterial strain that has a gene expressing resistance to the agent. It also selects for the assembly and evolution of complex genetic vectors encoding, expressing, linking, and spreading that and other resistance genes. Once evolved, a competitive construct of such genetic elements may spread widely through the world's bacterial populations. The levels of resistance at any time and place may therefore reflect in part the total number of bacteria in the world exposed to antimicrobials up until then.[43]

There are diverse mechanisms by which bacteria obtain resistance to tetracyclines, ranging from efflux (a mechanism responsible for moving antibiotics out of the cell), drug modification, target mutation and the employment of specialized ribosome protection proteins (RPPs).[44] Ribosomes are micro-machines for making proteins in the cell and are the targets for these antibiotics. There are 28 known efflux mechanisms that confer resistance to tetracycline, of which TetA is one of the commonest, and 12 different classes of RPPs, of which the best characterized are TetO and TetM. A recent study has shown that administration of tetracyclines to human volunteers can have a temporary effect of selecting *E. coli* showing co-resistance to multiple antibiotics, some of which can persist within the gut for up to a year.[45]

Measures to prevent the spread of antibiotic resistance

The World Health Organisation (WHO) recommends a package of measures to combat antimicrobial resistance, including regulating and promoting rational use of medicines, including in animal husbandry, and reducing use of antimicrobials in food-producing animals.[46]

The European Union (EU) banned the use of all antibiotics as growth promoters in animal feed in 2006.[47] The EU had already banned antibiotics used in human medicine from being added to animal feed and the 2006 ban was the final step in the phasing out of antibiotics used for non-medicinal purposes. The ban is implemented via Regulation (EC) No. 1831/2003.[48]

Some antibiotics, including tetracyclines, were also banned as additives to animal feed in Brazil in 1998.[49,50,51]

The US Food and Drug Administration (FDA) has acknowledged that developing strategies for reducing antimicrobial resistance is critically important for protecting both public and animal health. In its Guidance for Industry (GFI) #209 "The Judicious Use of Medically

Important Antimicrobial Drugs in Food-Producing Animals"[52], issued in 2012, the FDA discusses its concerns regarding the development of antimicrobial resistance in human and animal bacterial pathogens when medically important antimicrobial drugs are used in food-producing animals in an injudicious manner. In addition, the Judicious Use Guidance provides two recommended principles regarding the appropriate or judicious use of medically important antimicrobial drugs:
(1) Limit medically important antimicrobial drugs to uses in animals that are considered necessary for assuring animal health, and
(2) Limit medically important antimicrobial drugs to uses in animals that include veterinary oversight or consultation.
FDA considers all antimicrobial drugs listed in Appendix A to GFI #152 (Appendix A) to be "medically important", including tetracycline. The Guidance is clear that the use of medically important antimicrobial drugs for production purposes in food-producing animals does not represent a judicious use of these drugs. Guidance for Industry #209 also highlights that "*the administration of medically important antimicrobial drug to entire herds or flocks of food-producing animals would represent a use that poses qualitatively higher risk to public health than the administration of such drug to individual animals or targeted group of animals*".

In December 2013, the FDA announced the implementation of a plan to help phase out the use of medically important antimicrobials in food animals for food production purposes.[53,54] The FDA issued further industry guidance and published a draft Veterinary Feed Directive,[55] designed to reduce the regulatory burden for use of Veterinary Feed Directive (VFD) drugs. VFD drugs are new animal drugs intended for use in or on animal feed which are limited to use under the professional supervision of a licensed veterinarian in the course of the veterinarian's professional practice. Guidance for Industry #213[56] contains non-binding recommendations for sponsors of products containing antimicrobial new animal drugs of importance to human medicine that are administered in medicated feed or drinking water of food-producing animals. This Guidance is intended to facilitate voluntary changes to the drugs' conditions of use consistent with FDA's recommendations included in Guidance for Industry #209. The policy in GFI #209 and GFI #213 applies to all three tiers of medically important antimicrobial drugs: this includes tetracyclines. In March 2014, the FDA announced that all but one animal drug company had committed in writing to seek withdrawal of approvals for any production uses of affected drug applications and change the remaining therapeutic uses of their products from over-the-counter (OTC) to use by Veterinary Feed Directive (VFD) or prescription.[57] Once approvals have been withdrawn, continued use for production purposes will become illegal.

Some food producers in the USA are also moving to limit the routine use of antibiotics in livestock production in response to consumer pressure.[58]

In 2005, the Codex Alimentarius Commission (Codex), which develops international food standards adopted a "Code of Practice to Minimize and Contain Antimicrobial Resistance".[59] The Code of Practice makes a number of recommendations regarding the responsible use of antimicrobials in food-producing animals. For example, the document recommends that responsible use 1) should be controlled by the veterinary profession or other parties with the requisite expertise, and 2) does not include the use for growth promotion of veterinary antimicrobial drugs that belong to or are able to cause cross-resistance to classes of antimicrobial agents used in humans (or submitted for approval for use in humans) in the absence of an appropriate risk analysis. Codex argues that the successful containment of antimicrobial resistance requires the collaboration of a wide range of stakeholders, working together to protect consumer health by ensuring the safety of food products of animal origin.[60]

In addition to the use of antibiotics in animal production, antibiotic resistant traits are sometimes included in GM plants as a tool for researchers to identify which plants have successfully taken up another genetic trait: these are known as antibiotic resistant marker genes (ARMGs). The international food safety body Codex Alimentarius states that alternative transformation technologies that do not result in antibiotic resistance marker genes in foods should be used if available and safe, and antibiotic resistance genes that encode resistance to clinically used antibiotics should not be present in foods.[61] The FDA has also warned against using ARMGs for oxytetracycline because of its use in agriculture. [62] The EU directive on the deliberate release into the environment of genetically modified organisms (2001/18/EC) requires that antibiotic resistance marker genes are monitored and used with caution[63] and in 2004, the European Food Safety Authority (EFSA) classified the tetA gene conferring resistance to tetracyclines as a Group 3 ARMG. [64] Group 3 contains antibiotic resistance genes which confer resistance to antibiotics highly relevant for human therapy. EFSA stated that irrespective of considerations about the realistic importance of the health threat, these genes should be avoided in the genome of transgenic plants and should not be present in GM plants placed on the market in the EU or in plants used for experimental field trials.

Below, we consider evidence that GM insect releases could prove to be a new source of antibiotic resistance in the environment.

Mass breeding and release of GM insects using tetracycline

Oxitec's 'conditional lethality trait' is created by genetically engineering insects to express a protein called tTA (tetracycline-controlled transactivator). High level expression of tTA kills the insects at the larval stage, although the mechanism for this is not fully understood. Tetracycline (an antibiotic which is used commonly in agriculture and medicine) binds to tTA and prevents it leading to the expression of more tTA, allowing the insects to survive to adulthood.

Thus, Oxitec's GM insects have been genetically engineered to require the antibiotic tetracycline, or one of a group of related antibiotics, in their feed during mass production. Tetracycline acts as a kind of antidote to the genetic killing mechanism. It does this by inhibiting the expression of the protein tTA, which otherwise kills most of the offspring of the insects at the larval stage (in the case of Oxitec's RIDL GM *Aedes aegypti* mosquitoes) or most of the female offspring (in the case of Oxitec's fsRIDL GM agricultural pests). Including tetracycline in the feed allows very large numbers of insects to be bred to the adult stage, before sorting to produce mainly male adults for release.

Oxitec uses a diet supplemented with 30 µg/ml of tetracycline to breed its GM *Aedes aegypti* mosquitoes in the lab.[65] Its diamondback moths are reared on an artificial diet containing 100 µg/mL of tetracycline[66] or chlortetracycline (CTC, a tetracycline analogue)[67] and its olive flies[68] and Mediterranean Fruit flies[69,70] (Medfly) are also reared on 100 µg/mL of tetracycline.

To date, Oxitec has conducted experimental open releases of GM mosquitoes, but has not made commercial releases. Many millions of GM mosquitoes have already been released in trials, mainly in Brazil, and the numbers could increase to billions if Oxitec's GM mosquitoes are to be used commercially. Oxitec's approach requires repeated, ongoing large-scale releases to suppress wild insect populations, potentially over very large areas, including perhaps whole countries in the future.

One paper, which fits a simple computer model of mosquito populations to Oxitec's Cayman Islands data, predicts that releases of 7 million GM mosquitoes a week, in an initial phase, would be needed to suppress a population of only 20,000 wild mosquitoes, followed by releases of 1.9 million GM mosquitoes a week for long-term suppression, if a mixture of pupal and adult releases are used, or 2.8 million a week if only adults are released.[71] Brazil's Oxitec's new factory in Campinas, Brazil has an initial capacity to produce 2 million *Aedes aegypti* GM mosquitoes a week, with plans for larger production units in the future.[72]

Oxitec produces about 250 GM mosquito larvae in one breeding tray, creating 220 adults or 110 adult males at an 88% survival rate. Each tray contains one litre of tetracycline water (at 30 µg/ml concentration) and this water may require replacement once a week during the 7 to 10 day development of the eggs to pupae.[73] This means that to produce 2 million male GM mosquitoes a week, Oxitec requires about 18 to 36 thousand litres of tetracycline water, which then requires disposal.

No figures are available for production of Oxitec's GM agricultural pests as the company has not yet released these GM insects out into the environment. However, the technology is only likely to work if wild insects are outnumbered by GM males by an order of magnitude or more, meaning it is likely that releases of billions of GM insects over a wide area would be needed for commercial scale use. A related approach involving the mass release of pest insects sterilised by irradiation has been used in several pest control programmes. For example, since 2006, between 1.7 and 2.1 billion irradiated pink bollworms were released per year in Arizona, in a successful effort to control this pest.[74]

Thus, Oxitec's approach involves the mass production and proposed ongoing and repeated release of billions of GM insects mass-reared on diets containing tetracycline, including both mosquitoes and agricultural pests. In the process of mass production, very large quantities of tetracycline contaminated water will require disposal.

In the longer term, Oxitec plans to genetically engineer a much larger number of species so that its technology becomes a major new tool for tackling insect pests. Oxitec holds a licence to import pests for R&D to its Oxfordshire laboratory including other species of fruit fly, boll weevils, Red Palm weevil, owlet moths, Peach Twig borer, Pink bollworm, Tomato Leafminer, False Codling moth, Oriental Fruit moth (Peach moth), and Codling moth.[75]

Tetracycline and antibiotic resistance

The tetracycline class of antibiotics used by Oxitec is one of the most commonly used therapeutics in human and veterinary medicine. This class includes tetracycline and its derivatives (including oxytetracycline, OTC; doxycycline, DOX; and minocycline). In humans, tetracyclines are used to treat many different bacterial infections, such as *Helicobacter pylori* (which causes ulcers) and many others.[76,77] Because of this widespread use, tetracycline resistance genes have frequently been found in bacteria in wastewater treatment plants.[78] The tetracycline class of antibiotics is also widely used to treat infections in farm animals such as poultry, pigs, sheep and cattle, as well as fish, domestic pets, and (in some countries) bees.

The US FDA Guidance for Industry (GFI) #152 "Evaluating the Safety of Antimicrobial New Animal Drugs with Regard to their Microbiological Effects on Bacteria of Human Health Concern," includes an appendix that ranks antimicrobial drugs into three tiers, "critically important," "highly important," or "important," in regard to their human medical importance.[79] All the drugs in Appendix A are considered "medically important" by the FDA. Amongst these, the tetracyclines (tetracycline, chlortetracycline, demeclocycline, doxycycline and

minocycline) are listed as “highly important”. Their use as the sole therapy for Rickettsial disease and Anthrax therapy and prevention are highlighted.

Tetracyclines are on the World Health Organisation (WHO) Model Lists of Essential Medicines[80] and are categorised as “Highly Important” for human medicine worldwide, and as “Critically Important” in areas of the world where *Brucellosis* (a disease which mainly affects food workers and causes long-lasting flu-like symptoms[81]) is still likely to be transmitted to humans from food production animals (including South and Central America).[82] The WHO’s list is published to help formulate and prioritize risk assessment and risk management strategies for containing antimicrobial resistance due to human and non-human antimicrobial use.

The World Organisation for Animal Health (OIE) classes tetracyclines as Veterinary Critically Important Antimicrobials worldwide because they are very important in the treatment of many bacterial and chlamydial diseases in a broad range of animal species.[83] Few economical alternatives are available to treat many of these diseases. There are no alternatives to tetracyclines in the treatment of animals against heartwater (*Ehrlichia ruminantium*, a tick-borne disease, common in Africa and the Caribbean, that affects cattle, sheep, goats, antelope and buffalo) and anaplasmosis (*Anaplasma marginale*, the most prevalent tick-borne, livestock pathogen worldwide). Anaplasmosis results in significant morbidity and mortality of US cattle population, which affects the exportation of beef.[84] Biting flies can also transmit the disease, but are less efficient vectors than ticks.

There is little doubt that the spread of tetracycline-resistance can be extremely harmful. For example, the use of tetracycline from 1948 onwards is thought to have led to antibiotic resistant bacteria became the leading cause of bacterial infections in newborn babies in Europe and North America during the 1960s. As a result of the widespread use of tetracycline, a diverse population of the bacteria GBS (Group B *Streptococcus*) (which cause newborn bacterial infections) was replaced by only a few tetracycline resistant clones.[85]

The US CDC lists the sexually transmitted disease gonorrhoea as an infection where there is already a high degree of resistance to tetracycline (estimated at 23%). Group A Streptococcus (GAS) (which causes many illnesses, including pharyngitis (strep throat), streptococcal toxic shock syndrome, necrotizing fasciitis (“flesh-eating” disease), scarlet fever, rheumatic fever, and skin infections such as impetigo) has also developed resistance to tetracycline in the United States.[86]

Tetracycline resistant strains of food-borne bacteria such as *Salmonella*, *Listeria, Campylobacter*, *E. coli* and *Enterococci* have been identified in pork and/or chicken products in many countries and are thought to result from antibiotic use in meat production.[87,88,89,90,91,92,93,94] Tetracycline resistant *Vibro cholerae* bacteria, which cause cholera through their presence in contaminated water supplies, have also been reported in many countries, including Bangladesh[95], Ecuador[96], India[97], Madagascar[98], Mozambique[99] and Tanzania[100], causing serious problems with treatment in some cases. Tetracyclines (especially doxycycline) are the drugs of choice for effective cholera treatment in severely ill patients.[101]

Some forms of doxycycline are used to treat or prevent anthrax, or to treat infections caused by mites, ticks, or lice.[102] Doxycycline is the standard treatment for Lyme disease (caused by infection with the bacteria *Borrelia burgdorferi*) which is spreading throughout New York State, where Oxitec has proposed releasing GM diamondback moths.[103] Rickettsial diseases are usually treated using doxycycline. These diseases include the spotted fever or typhus groups and are caused by a group of pathogens called *Ricksettia* which often transmitted by

fleas, lice, mites, and ticks.[104] The bacteria *anaplasma* and *ehrlichia*, which cause the tick-borne animal diseases anaplasmosis and ehrlichiosis, are also *Ricksettia*. Human ehrlichioses can also occur.[105]

Tetracyclines are widely used in veterinary medicine mainly for the treatment of gastrointestinal, respiratory and skin bacterial infections, infectious diseases of locomotive organs and of genito-urinary tract as well as systemic infections and sepsis. A wide variety of tetracycline resistance determinants have been discovered in various relevant microorganisms.[106]

When considering the transfer of antibiotic resistance, it is important to be aware of the difference between facultative and obligate intracellular bacteria. Facultative intracellular bacteria are capable of living and reproducing either inside or outside cells. Examples include bacteria responsible for foodborne diseases such as *Salmonella* and *Listeria*, *V. cholerae* (which causes cholera), *Brucella* (which cause brucellosis) and the parasite that causes plague, *Yersinia pestis*. Obligate intracellular bacteria cannot reproduce outside their host cell and include *Chlamydia*, *Borrelia burgdorfero* (which causes Lyme disease) and *Rickettsia*.

Horizontal gene transfer (HGT) to facultative intracellular bacteria is common and resistance to tetracyclines is widely observed, as illustrated by some of the examples above. However, HGT is rare in obligate intracellular bacteria and most remain sensitive to tetracycline, suggesting resistance does not develop easily.[107,108] Nevertheless, some researchers have raised concerns about the development of tetracycline-resistant *Chlamydia suis* in pigs and the potential for transfer to *Chlamydia trachomatis*, responsible for human infection, although HGT in this bacteria is rare.[109,110,111,112] Other research has identified a large fraction of mobile genetic elements, including plasmids, in *Rickettsia* and found that HGT in *Rickettsia* may be more common than previously thought.[113,114] Insensitivity to tetracycline has been reported in some cases of Scrub typhus (the most common severe rickettsial disease, caused by infection with *Orientia tsutsugamushi*).[115] As a result, some Scrub typhus patients in Thailand responded poorly to treatment, due to infection with resistant strains, and some strains from southern India also appear to be resistant. A possible mechanism appears to be the expression of ribosome protection proteins (RPPs) in the resistant strains. Several antibiotic-resistant mutants of *Borrelia burgdorferi*, which causes Lyme disease, have been isolated and circular plasmids can be transduced to other cells, contributing to horizontal gene transfer (HGT).[116] These findings highlight the possibility that tetracycline resistance could also develop in obligate intracellular bacteria in the longer term, potentially posing problems for treatment of some serious diseases.

Doxycycline is also used to prevent malaria in chloroquine-resistant areas and can be used along with quinine to treat malaria.[117] Malaria is endemic in some of the same regions as dengue fever (which Oxitec proposes tackling by releasing GM *Aedes aegypti* mosquitoes). Malaria is caused by parasitic protozoans (a type of single cell microorganism) of the type known as *Plasmodium*, and resistance mechanisms are likely to be different from those in the bacteria discussed above. Malaria also appears not to have developed resistance to doxycycline yet, suggesting that the parasite may not acquire resistance easily.[118] HGT can occur from bacteria to *Plasmodium* parasites[119], however it is unclear whether antibiotic resistance could be transferred from bacteria to *Plasmodium* in practice.

Oxytetracycline is also used in fruit production in some countries to treat bacterial diseases of plants, especially fire blight in pear and apple and bacterial spot in peach and nectarine.[120] Whilst this is a relatively minor use compared to livestock production, it may be important in the context of Oxitec's GM insects, which include GM fruit flies and other agricultural pests.

Oxytetracycline is used to treat fire blight and bacterial spot on fruit trees in the USA and is used in Mexico and Central America to control fire blight in apple and diseases caused by a number of other pathogens on several vegetable crops. Oxytetracycline is also the only antibiotic that can be used internally in plants: it may be injected into the trunks of palm and elm trees to treat lethal yellowing diseases and other high-value plants to treat other diseases caused by phytoplasmas. However, this is an expensive and labour intensive treatment and is rarely used. According to Oxitec, in Brazil, tetracyclines are permitted for use on tomato, potato, beans, cucumber, coffee, peach, plum, passion fruit and pepper.[121]

Development and spread of antibiotic resistance in insects' gut bacteria

Insect guts are reservoirs for antibiotic resistance genes which they can spread between rural and urban environments, including kitchens and hospitals.[122] Insects commonly associated with food animals (such as houseflies and cockroaches) likely represent a direct and important link between animal farms and urban communities for antibiotic resistance traits. Research has shown (i) the association of multidrug-resistant bacterial strains of food animal origin with flies and cockroaches, (ii) bacterial proliferation and horizontal transfer of antibiotic resistance genes in the insect digestive tract, and (iii) the potential of these insects to transmit multidrug-resistant bacteria from food animals to the urban environment. For example, houseflies in food-handling and -serving facilities carry antibiotic-resistant (including tetracycline-resistant) and potentially virulent gut bacteria (*enterococci*) that have the capacity for horizontal transfer of antibiotic resistance genes to other bacteria.[123] One study has shown that a tetracycline resistance gene on a plasmid can be frequently transferred between strains of the bacteria *Enterococcus faecalis* in the mouth parts and digestive tracts of houseflies.[124] Resistant bacteria have also been found in bedbugs, cockroaches, oil flies and Mexican fruit flies; and antibiotic resistance genes, including tetracycline resistance genes, have been found in bacteria in the gut of the gypsy moth, an invasive species which consumes a wide range of host plant species.[125]

Horizontal gene transfer (HGT) can occur in insect guts, potentially transferring antibiotic resistance from relatively harmless to harmful bacteria. For example, antibiotic resistance genes (including tetracycline resistance genes) can transfer from *Escherichia coli* bacteria to *Yersinia pestis*, a parasite that alternates between mammals (usually rodents) and fleas and is the causal agent of the Black Plague. This transfer is caused by horizontal gene transfer within the digestive tract of fleas, and this may be the source of antibiotic-resistant *Y. pestis* strains isolated from plague patients in Madagascar.[126,127] Prophylactic antibiotic treatment is critical for persons exposed to bubonic or pneumonic plague, which re-emerged in Madagascar in the 1990s.

Antibiotic resistance genes, including tetracycline resistance genes, have been found in bacteria in the guts of bees.[128,129] This research shows that long-term exposure to antibiotics has caused accumulation of resistance determinants in micro-organisms in the guts of honeybees. For decades, American beekeepers have routinely treated colonies with oxytetracycline for control of larval pathogens, and these bees have multiple tetracycline resistance genes. Tetracycline resistance genes in these bees are consistently associated with mobile elements, such as transposons and plasmids, suggesting they are newly acquired. In contrast, very limited resistance occurs in bees from countries not using antibiotics in beekeeping and in wild bees. Thus, 50 years of using antibiotics in beekeeping in the United States has resulted in extensive tetracycline resistance in (non-disease-causing) bacteria in the guts of bees. The resistance genes can move between bacterial hosts via mobile genetic elements and the bees can pick up and physically transport tetracycline-resistant bacteria among themselves. Ampicillin resistance was also detected among the honeybee gut flora in this study, although they had not been treated with it,

confirming other studies showing that showing that prolonged use of a single antibiotic can lead to multidrug resistance. While no disease was described in this study, tetracycline resistant foulbrood pathogens (the most virulent bacterial disease of honeybees) have been identified in other research.[130] In this research the tetracycline resistance gene was also shown to be transferrable to other bacteria, with the potential to cause widespread tetracycline resistance.

Implications for Oxitec's technology

Mass production of GM insects in factories, using tetracyclines as an additive in their feed, could lead to drug resistance in their microbiota, in the same way that treating bees with tetracyclines has selected for antibiotic resistance. Oxitec's GM insects may then disseminate antibiotic resistance when released into the environment in the repeated, large-scale releases needed to vastly outnumber wild pest insect numbers.

Releases of GM insects into the environment therefore raise important questions about the potential spread of antibiotic resistant bacteria. In addition, mass production raises concerns about disposal of large quantities of larval insect feed containing tetracycline and there may also be issues of worker safety in relation to exposure to tetracycline over long periods.

The species Oxitec is currently producing include genetically modified *Aedes Aegypti* mosquitoes (currently being released in experiments in Brazil and Panama and proposed for experimental release in the USA), Mediterranean Fruit flies (Medfly, *Ceratitis capitata*, approved for experimental release in Brazil), diamondback moths (*Plutella xylostella*, proposed for experimental release in the USA) and olive flies (*Bactrocera oleae*, which Oxitec claims it will reapply to release in Spain, having withdrawn its original application).

For agricultural pests, the most likely direct route for antibiotic resistant bacteria present in the gut to spread to humans is via consumption of contaminated vegetables or fruit. Consumption is likely because the female larvae are genetically programmed to die at the larval stage, when they will mostly be inside the fruit (in the case of Mediterranean Fruit flies and olive flies) or on the leaves of brassicas (e.g. broccoli, cabbage) in the case of diamondback moths. In these species, bacteria are likely to be transferred to the larvae from the released adult males, via mating and egg laying. For *Aedes aegypti* mosquitoes, antibiotic resistant bacteria present in the gut could potentially spread from GM mosquitoes directly to humans via by being swallowed during the releases, when very large numbers are released in inhabited areas, or potentially through biting by the small proportion of female GM mosquitoes that are inadvertently released or that survive to adulthood in future generations. Antibiotic resistant bacteria might also spread via drinking water (where the wild females lay their eggs) if antibiotic resistance genes pass from the guts of the released males to their mates and thence to their eggs and larval offspring. For both mosquitoes and agricultural pests, antibiotic resistance genes might also move indirectly to humans or livestock through the environment, for example via predators or through the soil.

These possibilities are considered in more detail below, followed by some discussion of the potential consequences for human, animal and plant health. The disposal of tetracycline-contaminated feed is also considered.

GM Aedes aegypti mosquitoes: potential resistance gene transmission routes

Journalists have reported that in Brazil "…*it's impossible to talk during the liberation sessions without accidentally swallowing a few*…" of Oxitec's adult GM mosquitoes due to the very large numbers being released to try to swamp the wild population.[131] Thus, antibiotic

resistant bacteria that have developed in the guts of GM mosquitoes could be ingested directly by humans or animals swallowing the adults. In this way, antibiotic resistance genes could pass directly from the released adult males, bred on a tetracycline diet, into human or animal guts, where medically important bacteria could acquire resistance.

Biting by female mosquitoes is another mechanism through which antibiotic resistance might be transferred, although more research is needed to establish whether this is in fact the case. Oxitec's approach relies on the repeated release of very large numbers of adult male GM mosquitoes, which do not bite, however some biting GM females will inevitably be released accidentally. Oxitec has published figures on the number of biting female GM mosquitoes that are inadvertently released.[132] They report that female contamination is on average 0.02%. If correct, this would mean that 200 biting female GM mosquitoes are released in every million males. Current production of Oxitec's GM mosquitoes in Brazil is 4 million a week, but much greater numbers would be needed for use on a commercial scale. In the Cayman Islands, mechanical sorting was less effective, leading to about 5,000 biting female mosquitoes in every million males (additional sorting was then performed by hand).[133] If these biting females carry antibiotic resistant bacteria, it may be possible that they can be transferred directly to the human bloodstream via the mosquitoes' saliva when people are bitten. Biting of humans can also occur in the workplace, as well as during open release. Bacteria have been detected in the salivary glands and reproductive systems of several mosquito species including *Ae. aegypti*.[134] However, it is unclear whether these would develop antibiotic resistance during feeding and, if they do so, whether they could be transferred to humans through a bite. Unlike the gut, blood is normally clean from bacteria but this is not the case if the person's blood is carrying an infection.[135] If a bitten human's blood is infected with a bacterial disease, resistance might be transferred to these bacteria. However, even if it does occur, this route is likely to be less important than ingestion, because of the much higher numbers of bacteria in the human gut.

A third mechanism through which antibiotic resistance might be transferred to human gut bacteria is via drinking water supplies. The *Aedes aegypti* mosquito lays its eggs in water containers in and around human dwellings, where the larvae develop, and dengue is particularly prevalent in poorer communities which have no running water and are dependent on water butts for supplies. The majority of the GM offspring produced by mating with wild mosquitoes are expected to die in these water containers at the larval stage. If resistance genes can be transferred from adult males (and the smaller number of females) released from the GM mosquito factory to the next generation via the female's eggs, and then to bacteria in water supplies, they could rapidly spread to humans. Bacteria in malarial mosquito populations are known to spread through several different mechanisms: co-feeding, sexual mating, and maternal and paternal transmission to progeny.[136] However, there is less information about the extent to which bacteria, or their antibiotic resistance genes, in the released *Aedes aegypti* males will be passed to the next-generation larvae in this species.

A number of bacteria have been identified in the guts of *Aedes Aegypti*. In one study, *Serrattia odorifera* was the only microbe commonly associated in the midguts of all pupae and adults studied, suggesting it remains with the mosquito from the larval stage.[137] Another study highlights the ability of midgut bacteria to adapt to their host.[138] Very sparse information is available on the nature of the microbial community associated with *Aedes aegypti* mosquito larvae in domestic water containers in and around human dwellings. A study of bacteria associated with the larvae of *Aedes Aegypti* was published for the first time in 2014.[139] Hence many uncertainties remain.

Antibiotic resistance genes from the guts of GM mosquitoes might also reach humans or animals (including pets or livestock) indirectly, for example via predators. A wide variety of mosquito predators and parasites might take up antibiotic resistant bacteria through ingestion of GM adults or larvae and spread antibiotic resistance genes further through the environment. Several species of small crustaceans called copepods consume larvae in pools and ponds,[140] as do several species of fish,[141] tadpoles and aquatic insects,[142] some of which specialise in consuming particular species of mosquitoes.[143] Predators of adult mosquitoes include bats, birds, dragonflies and frogs. Specific predator species and their relative abundance and interactions vary considerably in different ecosystems. Thus one potential indirect route for transfer of antibiotic resistance genes might be via consumption of fish that have eaten GM mosquito larvae; another might be via contact with bat or bird droppings. Indirect routes for transfer of antibiotic resistance genes are considered in more detail below for the case of agricultural pests.

As well as being a vector for a group of viruses such as chikungunya, dengue fever virus, and yellow fever virus, *Aedes aegypti* mosquitoes also carry the protozoan parasite, *Plasmodium gallinaceum* (which causes malaria in poultry[144]). It has been demonstrated that strains of *Aedes aegypti* also support the complete development of the filarial roundworms (nematodes) *Brugia malayi* (which causes elephantiasis in humans), *Brugia pahangi* (which infects the lymph vessels of domestic cats and wild animals), and *Dirofilaria immitis* (heartworm, which infects dogs and other animals).[145] Thus, another possibility that would require further investigation is whether antibiotic resistance might be spread from the GM mosquitoes via these parasites or roundworms. Bacterial genes can transfer to nematodes through HGT, although the rate of transfer is relatively low compared to that between bacteria.[146]

GM agricultural pests: potential resistance gene transmission routes

As with GM mosquitoes, antibiotic resistant bacteria that have developed in the guts of GM agricultural pests could be ingested directly by humans or animals swallowing the adults during mass releases of the mainly male GM insects. However, a more important route is likely to be via eating the larval offspring of the released GM insects in or on fruit or vegetables. If antibiotic resistance genes are passed to wild females via mating and then to their eggs, these will normally be laid inside the fruit (in the case of fruit flies or olive flies) or on the leaves of the plants which the larvae consume (such as broccoli, in the case of diamondback moths). As most of the female offspring die at the larval stage, many will die inside the fruit or on the leaves of crops destined for human or animal consumption.

Any female agricultural pests which are inadvertently released with antibiotic resistant bacteria in their guts could transfer antibiotic resistance directly into fruit or onto vegetables. Female fruit flies pierce the fruit to lay their eggs inside it, as do olive flies with olives.

There is limited information about transfer of antibiotic resistance genes from released adult GM males to their wild female mates, or to their larvae, which will grow in or on the fruit and vegetables. However, parental-to-offspring transmission of microbiotas does occur in vertebrates and may promote the co-evolution of whole communities with their host species.[147] The evidence for transfer of bacteria from adults to larvae for Medfly, Olive flies and Diamondback moths is considered below.

Insects vary widely in their gut bacteria and many lack mechanisms for direct transfer of gut bacteria, with the exceptions of social insects (such as ants and bees) and insects that have evolved specific mechanisms for transfer of bacteria to progeny such as egg-smearing or egg capsules.[148] Nevertheless, gut bacteria in many insect species have been shown to

contribute to diverse functions such as nutrition, protection from parasites and pathogens, modulation of immune responses, and communication. Whilst many or most bacteria are acquired each generation from the outside environment, others are transmitted between hosts. Maternally transmitted symbiotic bacteria (i.e. bacteria with a mutually dependent relationship with their insect hosts) are widespread in insects. In some cases these bacteria are essential for the host insect to survive (known as obligate endosymbionts), but in other cases they are not essential and can be transmitted horizontally as well as maternally (these are known as facultative endosymbionts). Co-adaption between the bacteria and the host insect is most pronounced when there are reliable mechanisms for the direct transmission of symbionts from one host insect to another, especially to progeny.

The common Mediterranean fruit fly (Medfly) is a potential vector of human pathogens, such as *E. coli*, to fruits, since it is able to transfer bacteria from animal excreta (one typical feeding site) directly into the fruits where females lay their eggs.[149] Species of gut bacteria in the Medfly have been characterised.[150] Some major components of the Medfly's gut bacterial community are vertically transmitted from the female parent to its offspring, some within the egg and others on the surface of the egg.[151] During oviposition bacteria are transferred to the fruit and establish and proliferate within it, causing its decay and perpetuating the Medfly-associated bacterial community. An initial, egg-borne, diverse community of bacteria expands throughout the fly's life cycle.[152] Thus, it is likely that antibiotic resistance genes can spread from the guts of released GM insects through at least two mechanisms: horizontal gene transfer to bacteria such as *E. Coli* encountered during feeding on excreta and spread through the environment, including onto fruit; or parental transmission via wild female mates or the smaller number of released GM females and thence into the fruit and the larvae which grow inside it.

Studies have identified several species of bacteria in the digestive tracts of wild olive flies.[153,154] In adult olive flies, bacteria densely colonize the midgut and the association extends via the egg and continues in the larval stage where bacteria heavily populate the midgut during the entire larval development within the olive fruit. This suggests antibiotic resistance genes in the guts of released GM males are likely to pass from the released adult male GM olive flies to the eggs of wild females and hence into the GM larvae which develop inside the olives. The majority of the GM female larvae are expected to die within the fruit and therefore be consumed by any human or animal which eats the olives.

One study has evaluated the effects of five antibiotics, including tetracycline, on the diversity of the gut bacteria of Diamondback moths.[155] All the antibiotics were toxic to some bacteria and reduced bacterial diversity and larval growth and development. However, the development of antibiotic resistance was not studied. Another study reveals differences in gut bacteria related to exposure to insecticides in current and subsequent (unexposed) generations of Diamondback moths.[156] There is also some evidence that *Lepidoptera* (the group of insects which includes diamondback moths) can transmit their immune status, influenced by their midgut microbiota, to future generations.[157] It is therefore plausible that antibiotic resistance genes might pass from released males to their mates and thence to next-generation larvae, although more research may be needed to establish this. Female larvae containing antibiotic resistant bacteria will mostly die within the crop and antibiotic resistance genes could then be transferred to human or animal guts by direct consumption.

Another possible route of transmission of antibiotic resistance genes from adult GM insects to their offspring, and thus into the water supplies or food where the larvae develop, is via symbiotic bacteria such as *wolbachia*. *Wolbachia* are intracellular bacteria that manipulate the reproduction of their arthropod hosts. They are predominantly transmitted vertically from mother to offspring but also occasionally horizontally between species. Around 40% of

arthropod species are estimated to be infected with *wolbachia*.[158] Reports of *wolbachia* infected species include Medfly[159] and diamondback moth.[160] *Aedes aegypti* has no native *wolbachia* infection, but alternative methods of control are being investigated which involve infecting them with *wolbachia* from other insects.[161] Research would be needed to establish whether *wolbachia* or other symbiotic bacteria could acquire antibiotic resistance genes during feeding of the GM insect host with tetracycline, or via HGT from other bacteria, and then spread them into the environment. Some *wolbachia* strains do appear to be resistant to oxytetracycline, although the mechanisms for antibiotic resistance to develop in these bacteria are not fully understood.[162,163]

Indirect routes of transfer via wildlife may also be important for the spread of antibiotic resistance genes. The intestines of wild animals can act as reservoirs of antibiotic resistance genes, including genes resistant to tetracyclines.[164,165,166] It is therefore possible that wild animals that consume GM insects will acquire antibiotic resistance from them via horizontal gene transfer into their own gut bacteria.

For diamondback moths, predators include various species of beetles, syrphid flies, lacewings and several families of spiders and there are also parasitoids, including wasps.[167] Small mammals, including rodents, reptiles and birds are also likely to consume GM diamondback moths at release sites.[168] Rodents provide an important route for the spread of a number of infectious diseases: the potential for antibiotic resistance to spread via rats and mice is therefore considered further below. Brassica crops can also be used as feed for cattle and sheep, providing a route for transfer of antibiotic resistance genes to the guts of livestock.[169]

Many generalist predators of insects, such as ants, spiders, mantis, and assassin bugs will attack fruit flies. Birds will attack the larvae as they emerge from fruit, and some soil nematodes attack the pupea.[170] Parasatoids are also important.[171] Ants and other ground-based insects consume Medfly and olive fly pupae.[172,173] Fruit-eating (frugivorous) birds consume olives and other fruits.[174] For example, some species of parrot forage for fruit in orchards in Brazil.[175] Small mammals such as tayras also raid fruit orchards in Brazil. Thus there are many species which might consume olive flies or fruit flies as adults, larvae or pupae and spread any antibiotic resistance genes they contain into the wider environment.

Earthworms can move bacteria such as *E. coli* through the soil.[176] Newly hatched Medfly and Olive fly larvae exit the olive or fruit to pupate in the soil and olive fly can overwinter in the soil.[177,178] Thus, GM olive flies or Medfly which survive beyond the larval stage (including most males) may transfer pupae containing antibiotic resistance genes to soil and potentially to medically relevant bacteria.

Potential health implications of transmission routes for humans, animals and plants

Swallowing GM insects, or water or food contaminated by them, could lead to antibiotic resistance genes from the bacteria in the guts of the GM insects reaching the human gut. Horizontal gene transfer of antibiotic resistance genes can then occur into bacteria, including those causing infectious diseases, creating a source of antibiotic resistant pathogens. These pathogens can cause infections via the faecal-oral route or through hospital acquired infections.[179] Antibiotic resistance genes that reach the human gut, through swallowing adults or larvae, including via contaminated food or water, can thus spread resistance to tetracycline in tetracycline-treated diseases such as food and water-borne diseases, urinary tract infections and stomach ulcers. In the case of Medfly, which feed on animal faeces and can transfer bacteria into the human food chain, antibiotic resistance may easily spread to bacteria such as *E. coli*, which cause diarrhoea. Similarly, direct consumption of GM insects

by animals could transfer antibiotic resistant bacteria to animal guts and hence to pathogens of veterinary significance. For example, if cattle consume brassicas contaminated with antibiotic resistance genes from the gut bacteria of GM insects, antibiotic resistance might be transferred to bacteria in the animals' guts.

It is less clear whether being bitten by female GM *Aedes aegypti* mosquitoes (some of which will inevitably be released) could lead to antibiotic resistance genes reaching the human bloodstream via mosquito saliva.

If antibiotic resistance genes are transferred to small mammals such as mice, through eating contaminated GM larvae (such as diamondback moth larvae on brassicas), resistance genes may be transferred to pathogens inside the mice through horizontal gene transfer and this might offer another route for circulation of antibiotic resistant bacteria in the environment.

Tetracycline resistance in facultative intracellular bacteria, which cause many food- and water-borne diseases, is common and transfer of antibiotic resistance to these species is therefore very likely. The potential for resistance to develop in obligate intracellular bacteria, such as *Rickettsias* or *Borrelia burgdorferi*, which causes Lyme disease, is less well understood and will be less likely because the frequency of horizontal gene transfer is lower. However, if it does occur, the implications would be serious because many such diseases are reliant on tetracyclines for treatment.

The tick-borne parasite *Borrelia burgdorferi* is transmitted to humans from a natural reservoir among rodents and other small mammals by ticks that feed on both sets of hosts.[180,181] This raises the possibility that antibiotic resistance could be transferred to humans via ticks and rodents, affecting treatment for Lyme Disease. A similar mechanism might spread resistance to treatment for typhus fever from *Rickettsia prowazekii* bacterial infection, which can be spread by flying squirrels via lice.[182,183]

Disposal of tetracycline contaminated water from production facilities

As described above, a single small-scale GM mosquito suppression programme using Oxitec's GM mosquitoes is likely to require tens of thousands of litres of tetracycline water every week, with larger quantities required for commercial-scale production. Similar quantities are likely to be required for mass production of agricultural pests, although no data is available for these.

Tetracyclines can be found in human waste treatment plants (i.e. in sewage) and tetracycline resistance genes have been detected in such plants and in septic tanks and near confined animal feeding operations.[184,185,186,187] Disposal of tetracycline-contaminated water from GM insect production factories might therefore also contribute to the spread of antibiotic resistant bacteria in the environment.

Workers at GM insect production sites will also be exposed to tetracycline and to GM insects which may have developed antibiotic resistant gut bacteria.

As discussed further below, GM insects might also survive to adulthood in tetracycline-contaminated water in the environment. Some GM insects might also be inadvertently released in tetracycline-contaminated waste water disposed of from production sites.

Unintentional survival of GM mosquitoes could increase dispersal

Unintentional survival of Oxitec's GM insects can occur due to failure of the genetic killing mechanism. This can occur if resistance develops to the trait or if the insects encounter sufficient levels of the antibiotic tetracycline, or its derivatives, to inactive the killing mechanism. The possibility of unintentional survival of the GM insects is considered here because it makes it more likely that antibiotic resistance is spread widely in the environment.

Some of Oxitec's GM insects are expected to survive to adulthood (including all GM males in the case of Oxitec's GM agricultural pests), even in the absence of tetracycline. However, contamination with tetracycline and related antibiotics is widespread in the environment and could lead to significantly increased survival rates. Environmental contamination with tetracycline or the tetracycline derivatives oxytetracycline (OTC) and doxycycline (DOX) could allow much greater numbers of GM mosquitoes to survive and breed.

In the laboratory, 3% of the offspring of Oxitec's GM mosquitoes survive to adulthood, even in the absence of the antidote tetracycline.[188] Oxitec's GM diamond back moths had female survival rates to adulthood in the absence of chlortetracycline (CTC, a tetracycline analogue) of 1%, 0% and 5%, relative to wild moths, for the three different GM strains the company has developed, all of which it wants to test out in the open. [189] For all Oxitec's GM agricultural pests, the males are not affected by the killing mechanism and are expected to survive and breed for many generations.

When Oxitec's GM mosquitoes were fed cat food containing industrially farmed chicken, which contains the antibiotic tetracycline, their survival rate increased to 15-18%. Oxitec originally hid this information[190] but later admitted to an 18% survival rate of larvae fed on cat food in a published paper.[191] Oxitec claims that this survival rate will not happen in the wild because the GM larvae will breed only in clean water. However, a number of studies have found that *Aedes aegypti* mosquitoes can breed in septic tanks where there can be high levels of contamination with antibiotics such as tetracycline. [192,193,194,195,196,197] *Ae. aegypti* also commonly live in areas where discarded takeaways are likely to contain meat contaminated with tetracycline. When fed on tetracycline at 0.1µg/ml (i.e. 100µg/l), approximately 55% of Oxitec's *Aedes aegypti* mosquito larvae survive to adulthood in the laboratory.[198] In one study, levels of tetracycline from beef carcasses at a slaughterhouse in Iran were 131.0 µg/kg in meat, 254.9 µg/kg in liver and 409.1 µg/kg in kidney.[199] This suggests that tetracycline levels in industrially-farmed meat are likely to be high enough for a high percentage of the GM larvae to survive to adulthood if they come into contact with such meat, as the accidental survival of the larvae when fed cat food demonstrated.

For Oxitec's diamondback moths, at chlortetracycline (CTC) concentrations of 0.1 µg/mL and 1 µg/mL, female survival to adulthood was around 15% and 55% respectively, relative to wild-type.[200] GM agricultural pests are likely to encounter high levels of tetracyclines in animal manure and slurry. Oxytetracycline can be found at concentrations above 500 µg/g in animal manure and doxycycline at up to 78.5 µg/g dry weight in broiler manure.[201,202] A global review reports lower but still relevant concentrations of tetracyclines of up to 0.88 µg/g in pig manure, 11.9 µg/g in poultry manure and 0.208 µg/g in cattle manure.[203] These concentrations are likely to be more than enough to partially or totally inactivate the killing mechanism in Oxitec's GM insects if the larvae come into direct contact with contaminated manure.

Oxytetracycline is also used directly to treat fireblight and bacterial spot in fruit trees, as described above, and this is another mechanism through which GM Medfly is likely to encounter concentrations sufficient for the larvae to survive. For example, in Brazil, where releases of GM Medfly have been approved, oxytetracyline is used on peach, plum and

passion fruit. The maximum residue level (MRL) is 0.25mg/kg for all commodities except for plum where the MRL is 0.7mg/kg.[204]

The percentage of surviving GM insects could also increase if resistance to the genetic killing mechanism evolves over time.[205] Like mutations in bacteria, genetic mutations in the insects themselves which allow the GM insects to survive and breed successfully could be rapidly selected for during mass production.[206]

Spread of GM insects

The GM *Aedes aegypti* mosquitoes being released by Oxitec in Brazil have eggs which can survive several months under dry conditions in a dormancy state: the GM mosquito eggs are then easily dispersed. Mosquitoes are easily transported anywhere where there are movements of people - on boats, trucks or trains - and have spread worldwide via breeding sites in tyres.

Similarly, agricultural pests are widely dispersed in fruit and vegetables, including through global trade. As noted above, if Oxitec's GM pests are used in agriculture, fruit and vegetables are likely to contain large numbers of dead female larvae, due to the action of the genetic killing mechanism at this stage, plus smaller numbers of live GM males, which can survive or multiple generations. Although many pests fly relatively short distances in normal circumstances, they can be dispersed further by the wind, or move *en masse* to new fields or orchards in particular conditions.[207,208]

Thus, in addition to the mechanisms described above for transport through the local environment (e.g. via animals, water and soil) there is potential for worldwide dispersal of antibiotic resistance genes from GM insects, including via the human food chain.

Conclusions

UK company Oxitec aims to establish a new method of pest control, involving the repeated, large-scale release of billions of GM insects (including mosquitoes and agricultural pests) into the environment.

However, mass-breeding and repeated release of large numbers of GM insects using the widely-used antibiotic tetracycline in their feed poses a risk of spreading antibiotic resistance in bacteria inside the insects' guts. Failure to include this issue in risk assessments is a major concern, because antibiotic resistance is a serious threat to human and animal health.

The evidence in this review suggests that Oxitec's use of tetracycline poses unnecessary risks to health. There are plausible routes for resistance genes to develop in the guts of GM insects fed on tetracycline and to circulate widely in the environment. This may increase the difficulty of treatment of some serious human diseases. For example, antibiotic resistant genes could be spread to *E.coli* bacteria and into fruit by GM Mediterranean Fruit Flies and then enter the human food chain. GM olive flies and diamondback moths may also spread antibiotic resistance genes into the food chain via olives or brassica crops, affecting humans or animals. Other species, such as rodents, flying squirrels or earthworms could also play a role in disseminating antibiotic resistance, perhaps into diseases that they carry. Antibiotic resistance genes in the guts of GM mosquitoes may be transferred to humans or animals directly if they are swallowed as adults, or passed on to mates and larvae and thus to water supplies.

The disposal of large volumes of tetracycline-contaminated water from GM insect production factories could also spread antibiotic resistant bacteria in the environment.

Mass-breeding of GM insects using tetracycline breaches existing policies, which advise against indiscriminate feeding of antibiotics to animals (in the USA and worldwide via the WHO) and in some cases ban this practice (e.g. in the EU and Brazil). Allowing the repeated large-scale release of genetically modified organisms (GMOs) which may spread bacterial resistance to tetracycline also contradicts policies on GM plants, where the use of antibiotic resistant marker genes has been restricted to antibiotics which are not in common use.

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