Movement of genetically modified insects for research purposes

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Summary

Insects play a crucial role in research. Many laboratories are developing technologies to control insect vectors or agricultural pests by using genetic modifications that either reduce insect reproduction or increase refractoriness to disease transmission. Those tools include gene drive elements that may spread such genetic traits in a self-sustaining and cost-effective manner. Since international research collaborations are nowadays routine, movement of genetically modified insects between laboratories of different regulatory jurisdictions is very common. This article describes the requirements and guidelines for transportation of genetically modified insects for research and the experience gained by an Italian laboratory as a research centre involved in several shipments of wild and modified mosquitoes within an international research consortium aimed at developing sustainable tools for malaria control.

Keywords

Arthropod containment – Gene drive – Genetically modified insects – Transportation.

Introduction

It is difficult to overestimate the importance insects play in research. Since the times of Thomas Hunt Morgan in the 'fly room' at Columbia University at the beginning of the 20th Century, just to cite the most famous insect model organism, the fruit fly *Drosophila melanogaster*, insects have filled many laboratories around the world. The sharing and movement of strains of laboratory insects, such as the thousands of

Drosophila strains and mutants, is a very common practice between researchers, as reported by Cook and Parks (1), this issue. With the advent of transgenic technologies, the number of different mutants and genetically modified strains has dramatically increased, and thus the need for movement of strains between laboratories, in a spirit of shared knowledge, transparency and collaboration. In the last two decades, the number of insect species which have been genetically modified extends beyond the *Drosophila* world and there are now several classes of arthropods routinely modified. Several agricultural pests are reared and transformed in laboratories, including the Mediterranean fruit fly, the Diamondback moth, the Pink bollworm, and the Spotted wing Drosophila fly. Public health research aimed at developing tools to control insect vectors of human or animal diseases are focused on *Anopheles* and *Aedes* mosquitoes, the former being responsible for transmitting malaria and filariasis while the latter is a vector for dengue, Zika, yellow fever, and chikungunya viruses.

Gene editing and genetic manipulation of insects are efficient tools that have increased the understanding of the molecular and cellular basis of many biological, evolutionary or developmental processes. In the last decade innovative genetic technologies also have opened the possibility of providing solutions to certain medical or agricultural problems caused by pest insects. The novel properties of these insects require precautionary containment in laboratories while biosafety studies are conducted. In addition, rearing and maintenance of potential disease vectors must be preceded by an evaluation of the risks of their escape from containment, in order to inform any other precautions that must be taken in their handling, rearing and especially shipping between laboratories.

Genetically modified (GM) insects are defined as organisms in which genetic material has been purposely altered through genetic engineering in a way that does not occur naturally. This includes both stable germline transformation that targets the nuclei of developing germ cells for DNA introduction or gene editing applications, such as generation of mutations by the use of specific nucleases such as the CRISPR/Cas system (2). There are several methods for introducing foreign DNA into

the developing embryo (or into adult ovaries), with the most effective being microinjection into individual embryos. Once injected into the embryo, DNA must recombine in the target genome by the use of transposable elements, site-specific integration systems, virus-mediated recombination or by homologous recombination. Identification and selection of stable germline transformation usually relies on phenotypical markers, such as eye or body colour mutants or the use of fluorescent proteins.

One class of genetic modifications that needs a special mention is gene drives. Gene drives refer to a phenomenon whereby heritable elements (a gene) bias their own inheritance with respect to the rest of the genome, thereby increasing in frequency ('driving') in the population over generations (3). Gene drives are still at a research stage in laboratories and none have been released into the environment yet. Gene drives offer great potential as a tool to control insect vectors of diseases because their invasive nature allows them to spread and modify entire populations in a limited timeframe, making them attractive for cost-effective and self-sustaining public health applications (4). Because gene drives are designed to be highly effective at establishing in wild population, guidelines and recommendation are in place to ensure laboratories conducting research on gene drives meet containment standards to minimize risks of accidental release of gene drive carrying organisms (5, 6, 7).

Shipment of genetically modified mosquitoes

Import and export of live mosquitoes for research

The Polo d'Innovazione Genomica Genetica e Biologia (Polo GGB) is an Italian research and development laboratory and a service facility for genomics, bioinformatics, genetics, and ecology studies. Polo GGB is also a member of Target Malaria, an international research consortium aimed at developing genetic technologies for the control of *Anopheles* mosquitoes with the aim to reduce the burden of malaria in Africa (targetmalaria.org). The technology is based on the use of genetically modified mosquitoes with the goal to reduce the populations of human malaria mosquitoes, and as a consequence reduce malaria transmission.

As part of the development pathway of the technology, Polo GGB has been involved in several shipments of live mosquitoes between partner laboratories for research purposes. Typical shipments for this purpose usually involve 1000-5000 mosquito eggs with a total transit time of around 24-72 hours. This includes imports of GM mosquito strains from partner laboratories (for instance from Imperial College London) where the GM strains are generated by germline transformation. Also, there are imports of wild strains (from colonies established using field caught mosquitoes) from partners in African countries (for instance the Institut de Recherche en Sciences de la Santé, IRSS, in Burkina Faso). These are imported in order to test, in laboratory cages, the capabilities of the GM strains introgressed into the genetic background of wild mosquitoes from a potential future field site. This information is valuable in risk assessments and in planning for future field studies.

The pathway for technology development and testing undertaken by Polo GGB for Target Malaria, based on international recommendations for the use and testing of genetically modified insects for population control (8) follows a step-wise approach for efficacy and safety tests at first in contained laboratories in regions where the target organisms are not endemic. As a consequence, movement of both wild and GM strains between different partner laboratories is necessary to perform initial regulatory studies, before importing the GM strains into contained use facilities in destination countries for further regulatory studies.

The Polo GGB exports both wild-type and GM strains to collaborators and to partner laboratories in endemic countries, subject to specific permits being obtained from the relevant national authorities and intellectual property agreements set by the research partners. This may include export of insects from wild-type strains that were originally collected in the field or GM mosquitoes based on those same background genotypes after they are approved to be tested in confined settings or eventually released in the field in the endemic country.

Documentation required for shipment

Shipment of live GM insects is subjected to international standards as well as national regulations that are specific for each country. The

documents required for shipping insects (e.g., mosquitoes) for research purposes distinguish whether the insects are considered an 'infectious substance' (or are infected and can transmit a pathogen). If the GM insect is also classed as an infectious substance, this has priority over it being genetically modified, and those more rigorous requirements should be followed. Insects carrying pathogenic agents are subjected to special rules and permits that will not be addressed in this article, as our experience is based on pathogen-free insects. Also, permits for deliberate release of GM insects into the environment are addressed by different regulations and are not discussed here.

Shipment of live insects requires liaising with competent authorities (see below) that are responsible to authorise the importation and transit of such material. No transport of the material can occur unless it is accompanied by a complete set of documents: the commercial document (Pro Forma Invoice), Import Permit, and Free Export Declaration.

A commercial document is required for all shipments and gives a complete and detailed description of the material shipped, including insect species of origin, category and quantity (in grams), the place of origin, reason for sending, HS code (or Harmonized System Tariff code) and the complete name and address of parties involved, including country of sender and receiver parties - also known as Consignor and the Consignee/User (9). Value of product should always be included in the commercial document. Customs rules in each country require valuation of the material and no country accepts a zero value. If the material has no commercial value, a symbolic amount is indicated (usually € 1 or US$ 1), with indication that the material is for research purposes only.

Requisite for shipping GM insects is a permit of import and Free Export Declaration. The Import Permit is requested by the receiver institute and obtained from the competent authority of the destination country, which also approves the import, transit, use and disposal of research samples under conditions that ensure the control of risks to public and animal health (9). The competent authority designated to regulate GM

organisms differs from country to country. In Italy Import Permits are issued by the Ministry of Health, in the US by the Animal and Plant Health Inspection Service (APHIS) from the United States Department of Agriculture, whereas in other countries it is often by the Ministry of Environment (or equivalent). Movement of insects within institutions in the same country or between European Member States does not require an Import Permit.

The Free Export Declaration ensures that the goods included in the shipment are not subjected to export restrictions in accordance with current national regulations and international agreements. The shipper certifies that no illicit or prohibited items are included in the package. In addition to Free Export Declaration, an Export Permit could be requested to ensure that material exported by an Institute complies with the provisions of international agreements. An Export Permit is issued by the competent authority of the country from where the material is shipped.

Since insects, especially mosquitoes, could serve as vectors for diseases and could be infected with human or animal pathogens, a Sanitary (Health) Certificate might be required at the discretion of the competent authority of the destination country to certify the non-contagious and non-infectious status of the shipped material. The Sanitary Certificate includes the information that the eggs (or other life-stage for shipping) are free of known human or animal pathogens. There is some debate around the use of Sanitary Certificates for this purpose (as discussed in Wohlfarter *et al.* [10] and Quinlan *et al.* [11], this issue); ultimately it is the responsibility of the sender institute to certify the non-infectious status. GM insects for research purposes that have been reared and bred in laboratories can be declared pathogen-free in the same way as wild type insects, and thus there are no further restrictions or documentation needed for the movement of GM insects compared to non-GM insects of the same species, unless imposed by the countries involved in the trade.

Depending on the nature of the insect movement and the relationship between sending and receiving institutes, a Material Transfer

Agreement (MTA) or documentation of responsibilities and liabilities might be needed. MTAs are contracts that govern the relationship between the laboratories – including the rights and obligations, of the recipient when it comes to the use and potential commercial exploitation of materials. Those agreements are of profound political importance in the context of forging greater collaborations in malaria control research (and other research), particularly when it comes to mediating economic and power imbalances between laboratories in the North and the South. For example, an MTA could indicate that the receiving institute does not have the right to transfer the material further, or to register intellectual property from the material, or other such restrictions (see Sreerama Kumar *et al.* [12], this issue)

It is recommended, especially when shipping transgenic insects, to accompany the material with a Certificate of Analysis to ensure the recipient laboratories are informed on the nature of the modifications. The certificate should include an exhaustive description of the genetic modifications and their origin, instructions on how to rear and maintain the strain, and include any protocols or other instructions to detect and verify the transgene (PCR assay, fluorescent markers, phenotype).

Other documents might be included on the list of shipment paperwork to give additional information on the ecological and health status of the material (Declaration of Sensitive Goods or Declaration of Non-Dangerous Goods) or to reduce the risk of material damage (X-Ray Check Exemption).

A shipment may be stopped by Customs officials due to incorrect or incomplete shipping paperwork, leading to delays in shipments that can compromise the survival of material and its usefulness in the destination country.

Shipping live mosquito eggs is always challenging and requires co-ordination between the sender, the logistics providers, the courier, and the recipient to ensure correct transport and timely arrival. Due to its complexity, it is suggested to rely on specialized logistics companies (e.g. World Courier, AmerisourceBergen) that can guide the parties

involved through all the paperwork preparation, including Customs regulations, and that are sensitive to the importance of transit time.

Packaging

Transport of GM insects (or GMOs in general) is subjected to strict national and international regulations. These regulations describe the safety standards and packaging requirements, which ensure that the contents of the package must not escape or be dispersed into the environment during handling or transportation: International Air Transport Association (IATA) and International Civil Aviation Organization (ICAO) for transport by air; Agreement concerning the International Carriage of Dangerous Goods by Road (ADR) and by Rail (RID) for transport by road or rail in Europe and neighbouring countries; International Maritime Organization (IMO) for international shipping by sea. The package must also prevent exposure of the parcel content to handlers in the shipment chain or any member of the public who can come into contact with the package. This is not only for safety, but to ensure the integrity of the material.

GM organisms, as a group, are classified as dangerous goods when transported by air. Therefore, the applicable requirements are IATA Dangerous Goods Regulations, classified as UN 3245 (Miscellaneous dangerous substances and articles, including environmentally hazardous substances) and Packing Instruction P904 (ICAO/IATA PI959 – shipping containment as Class 9). GMOs also need to be packaged to maintain any biosecurity containment requirements during ordinary handling in transportation. This requires a triple packaging system (Fig.1): i) Primary receptacle, which is a leak proof sealing bag that contains the mosquito eggs; ii) secondary container, a leakproof large plastic tube that is used to enclose and protect the primary receptacle; and iii) rigid outer packaging, in which the secondary container is placed with suitable cushioning material. Outer packaging protects their contents from external effects, such as physical damage. Additional precautions can be requested, for example adding a Parafilm or appropriate tape around the lid of the container to prevent any leakage. The material to be transported must be labelled in such a

manner as to notify any handler that the item to be transported contains GMOs. For this reason, the label UN3245 must be displayed on opposite sides of the outer packaging on a background of a contrasting colour and must be clearly visible and legible.

Each layer of packaging must be of such construction and sturdiness to independently prevent the release of the material under normal conditions during transport (temperature, humidity, pressure), and each layer must be independently closable or sealable.

It is recommended to ship mosquitoes as eggs or non-flying stages. This is because movement of reproductively viable stages of insects, especially to countries where pathogens of diseases are present and known to be vectored by such insects, might pose health risks. Handling and shipment of non-motile life stages reduces the risk of escapees and also increases survival during transportation, since eggs are usually more resistant than adults. However, the non-motile eggs need to be protected from extreme heat, cold, and desiccation during transport, therefore insulated or adequate containers are necessary to maintain insect viability. If transportation is anticipated to be lengthy, the package may additionally include a cold pack to help regulate temperature. Most insects do not require large containers, feeding or added water during transportation (see the case described by Denton *et al.* [13], this issue, as an exception). However, depending on the species of mosquito (e.g. Anopheline), eggs might need to be kept wet for survival. Wrapping eggs in damp filter paper inside a sealed bag is usually sufficient and no free water should be used to minimize the risk of leaking.

Movement of insect carrying gene drives

There are no regulations specific to transport and movement of insects (or organisms, in general) carrying gene drive elements, and the documentation for gene drive organisms is not different from those for non-driving GM strains (14). However, recommendations and guidelines for movement of gene drive organisms are in place to ensure additional precautions are observed (5, 6, 7). Those include the recommendations to avoid shipping reproductively viable life stages,

the use of rigorous packaging approved for shipment of hazardous organisms to maintain the containment level and with documentation of responsibility and liabilities between partners, as would be the case for other GM organisms. Contained use approvals would specify that the receiving laboratories recognise the invasive nature of the transferred insects and ensure the destination laboratory is suited to receive and maintain GM organisms carrying gene drives. A certificate of analysis for the strain should be included with the shipment of gene drive strains, and biosafety officers at the receiving institute should verify containment conditions and authentication processes are adequate and ready to receive shipments (5, 6).

Conclusions

Exchange of live insects between institutions for research purposes is a very common practice, involving both wild-type and transgenic strains.

Strict regulations and packaging requirements are in place to ensure that live GM organisms are not accidentally dispersed into the environment and to protect handlers that could come in contact with the package. Despite these requirements, risk management for shipment of GM mosquitoes differs little from those associated with the same species unmodified. Transport of insects that can transmit human or animal diseases, such as mosquitoes, requires rigorous packaging, careful handling and appropriate documentation, especially to those countries where pathogens are present that could be vectored by the transported insects. The same is true for species that could establish in new environments, without authorisation for release.

When shipping mosquitoes carrying gene drive elements it has also been recommended to validate the strain using molecular procedures both before and after transport to ensure there are no unintended genotypes linked to the drive trait.

Regulations for import and transit of GM insects for research differ from country to country, and recommendations currently exist to guide the researcher to ensure proper transportation between laboratories. A

key factor is that these shipments are destined for laboratory studies, not field releases, which have different requirements and ramifications.

Glossary

Genetic modification: The process of altering the genetic composition of an organisms by use of genetic engineering in a way that does not occur naturally.

Gene drive: i) It is a process by which a genetic element (natural or synthetic) biases its own inheritance in respect to the rest of the genome, even in the presence of some fitness cost. ii) A gene drive is also the genetic elements that can cause the process of biased inheritance in its favour. iii) Gene drive refers also to the tool or technology to achieve a goal, for instance when referring to the use of gene drive mosquitoes to control a population.

Introgression (or introgressive hybridization): it is the process of transferring a genetic trait (for instance alleles, a gene or a transgene) from one species to the genetic pool of another by a series of consecutive backcrossing of the interspecific hybrid to one of the parental species. It is often used to change the genetic background of a transgenic strain from one species to another.

PCR: Polymerase Chain Reaction (PCR) is a method to amplify a specific fragment of DNA in order to obtain sufficient quantity to be easily detected and visualized. It is used in a wide range of applications (medical, diagnostic, forensic, DNA sequencing, etc) including the identification of a specific genetic modification in a genome.

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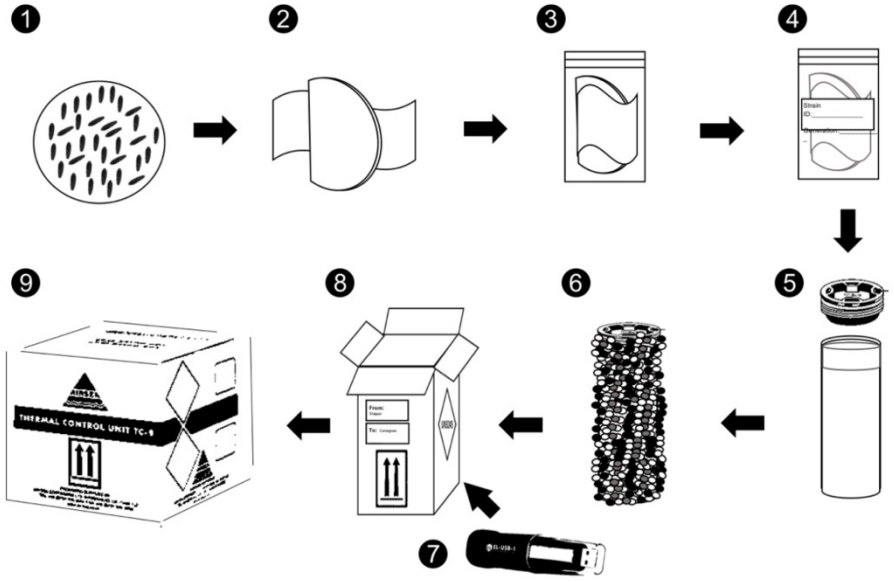

Fig. 1

Shipment of live *Anopheles* mosquitoes. Non-motile forms such as eggs or non-flying stages (1) are wrapped into wet filter paper (2) and sealed into a leak-proof primary container (3) properly labelled (4). A secondary insulated container (5) is used to protect the primary container. Outer packing (6) protects the secondary container which is inserted into a rigid outer packaging (8), labelled according to the type of biological material included. An environmental data logger could be also added (7) to monitor the conditions during transport. The material and all required documentation are finally added to the parcel (9).