Options for Containment of Genetically Modified Mobile Arthropods

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Options for containment of genetically modified mobile arthropods

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Preface

Special attention is required when performing experiments (or other activities) with genetically modified (GM) arthropods to ensure that arthropods do not escape containment. The current Dutch GMO legislation contains details on containment measures (physical containment and working procedures) when working with GM micro-organisms, plants or animals. The containment measures that are prescribed for GM animals are, however, not specifically written for GM arthropods. Due to the specific characteristics of arthropods (their ability to move, their size etc.) specific containment measures are often necessary when performing experiments with GM arthropods.

The GMO office and the Netherlands Commission on Genetic Modification (COGEM) have commissioned a research project to gain insight in the containment measures that ensure optimal containment of GM arthropods.

This report is the result of this project and lists the aspects of physical containment facilities and general procedures that are important when working with GM arthropods. Arthropod containment levels are defined and for each level the requirements are listed.

The report provides an overview of those properties of an arthropod that are important when deciding which physical containment facilities, equipment and working procedures are necessary to keep the GM arthropod of interest in containment. It mentions additional containment measures that could be used to ensure containment of species that have a higher escape potential (e.g. small but strong flying insects).

For several groups of GM arthropods (fruit flies, mosquitoes, mites, midges, aphids, etc.) the biological characteristics that are important when determining containment measures are presented, and details on the necessary containment facilities, equipment and working procedures are given.

Overall, the research report provides an excellent overview of all aspects that are of concern when determining the measures that are necessary to ensure containment of GM arthropods. It is a report worth reading!

Marjan Bovers
Chair of the advisory committee

Summary

The use of genetic modification is increasing rapidly. Basic regulations for containment of genetically modified arthropods already exist. The very diverse properties and related risks of different species, however, may require additional containment measures to warrant biosafety. This is most relevant for species that pose more risk when escaped (disease vectors and exotic species) and those that are more difficult to contain e.g. because they are highly mobile or tiny and easily overlooked.

This report summarizes the relevant properties of different arthropod groups to be taken into account, the arthropod containment levels that have been used (inter)nationally and provide information that may be helpful for researchers, biosafety officers and inspectors to design safe containment facilities and working procedures.

It is concluded that for several species current regulation on containment based on permits for handling GM *Drosophila melanogaster* can be sufficient. However, there are several arthropod groups for which additional measures and working protocols are necessary for optimal containment. For these arthropods working protocols and possibly containment facilities should be adapted to the specific species properties. These include for example thrips, midges, mites, and ticks, in particular when pathogens are associated. Training and creating awareness among researchers about safety and containment principles is a basic requirement to warrant protocols to be effective. In addition, applicants should be aware that other legal containment requirements besides those for GM arthropods may be relevant. For a number of arthropod groups possible containment measures have been listed in this report.

Samenvatting

Het gebruik van genetische modificatie bij onderzoek aan arthropoden neemt snel toe. Inperking bij het gebruik van GM insecten is geregeld via de regelgeving voor dierverblijven (Regeling GGO bijlage 4). Daarbij is in de verwachte nieuwe Regeling een extra algemeen protocol in voorbereiding voor inperking van arthropoden, met name gebaseerd op het protocol dat uitgewerkt is voor Drosophila melanogaster. De grote variatie in het gedrag van verschillende soorten arthropoden maakt het echter noodzakelijk om naar soort-specifieke risico's te kijken wanneer het erom gaat optimale inperkingsmaatregelen te treffen. Dit is vooral van belang voor vliegende, snel-bewegende soorten of kleine soorten die gemakkelijk aan de aandacht ontsnappen. Ook wanneer het exotische soorten of vectoren van pathogenen betreft is maatwerk noodzakelijk.

Dit rapport geeft een overzicht van voor inperking relevante eigenschappen van verschillende groepen arthropoden, en de inperkingsmaatregelen die zowel nationaal en als internationaal voor deze groepen gebruikt worden. Het gaat daarbij vooral om de inrichting en het gebruik van faciliteiten voor ingeperkt gebruikt. Deze informatie kan gebruikt worden door onderzoekers en overheidsfunctionarissen betrokken bij de ggo vergunningverlening en naleving van de regelgeving.

Het rapport is bedoeld voor vergunning-aanvragers en voor betrokkenen bij de vergunningverlening en handhaving en geeft bruikbare handvatten en toetsingspunten om te bepalen welke aanvullende maatregelen getroffen moeten worden om te voldoen aan de voorgeschreven regels voor een optimale inperking van genetisch gemodificeerde organismen.

Deze zijn toepasbaar op alle geleedpotigen, op inperkingsmaatregelen voor bepaalde groepen zoals onder andere voor kleine vliegende insecten zoals tripsen en knutten maar ook voor mijten en teken die zich gemakkelijk en onopvallend hechten aan huid en kleding wordt in het rapport nader ingegaan. Ook wanneer het vectoren van ziekteverwekkers betreft zijn veelal extra maatregelen gewenst. In al deze gevallen is het nodig de werkprotocollen en inrichting van faciliteiten af te stemmen op de specifieke eigenschappen van de soort. Om de inperkingsmaat-regelen effectief te laten zijn is het noodzakelijk onderzoekpersoneel grondig te instrueren en zich bewust te maken van de eigenschappen van het organisme waarmee gewerkt wordt en de noodzaak maatregelen te nemen om veilig te kunnen werken.

1. Introduction

When animals (including GM arthropods) are used for scientific research, requirements for research facilities, equipment and working procedures should ensure the safety for researchers, the public and the environment. For this reason containment levels are continuously adapted and improved in parallel with developments in research.

The designation of a genetically modified organism (GMO) for this report is the definition as has been laid down in the EU Directive 2009/41/EC on the contained use of genetically modified micro-organisms. This definition equally applies to animals, including arthropods. GMOs are defined as organisms, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination.

The use of genetic modification of insects and other arthropods in both fundamental and applied research is rapidly increasing (Beech *et al.*, 2012) in a wide variety of species. As experimental settings and applications are getting more diverse, current containment procedures and guidelines may need improvements to warrant biosafety in research and to minimise risk levels.

According to Dutch law, regulations have been made for the contained use (housing and activities) of genetically modified animals. These are based on the EU Directive 2009/41/EC, and are called 'Besluit genetisch gemodificeerde organismen milieubeheer' (the GMO decree) and 'Regeling genetisch gemodificeerde organismen' (BGGO 2009).

Current practice is that license applications for activities with new GM arthropods are judged case by case. Activities with GM *Drosophila melanogaster* have been reviewed and approved on several occasions and therefore form a solid and safe base that can be used as a starting point for other arthropods. In this report the standard 'animal housing' rules, i.e., room design and operation procedures, for activities with genetically modified *Drosophila melanogaster* are the starting point for the considerations for other arthropods. In this report the specific risks and containment measures for some groups will be discussed. To cover all future species with their typical behaviour and specific risks that require additional containment measures would not be feasible. This report is not aimed at changing current procedures or regulations but will focus on additional options for safety in case the species to be studied is likely to generate additional risks due to its biological characteristics. It is felt that experiments with arthropods such as insects, mites and ticks often need extra attention as these creatures can more easily spread by walking or flying than species such as fruit flies or are more harmful when escaped. This report focuses on these more risky arthropod groups.

Containment systems have already been developed since the early 1980s when research became more and more aware of the risk of escapes from laboratories in particular when working with quarantine organisms and exotic vectors of diseases. Principles for containment of genetically modified insects elaborated on these earlier containment systems and are used in many different but also similar ways at science centres all over world (see Appendix III). The guidelines for working with genetically modified plants and micro-organisms are overlapping and complementary with those of arthropods and measures from one system are helpful to improve containment in other systems. But the active mobility of arthropods requires additional specific measures.

This report focuses on measures to ensure containment of mobile or small arthropods. In particular because these organisms may spread rapidly when they escape. Containment experience is limited to only a few model species thus far (predominantly mosquitoes and some quarantine insects). More generic and specific measures and guidelines are wanted to cover a broader range of insects. Such guidelines will be helpful to research institutions, risk managers and biosafety officers to perform a risk analysis on research proposals and to develop optimal protocols for containment.

This report focuses on containment options for indoor environments such as laboratories, climate rooms, and greenhouses. For field testing of GM insects (in particular for sterile male techniques, mainly outside the EU) which is not covered by this report we refer to Beech *et al.* (2012).

2. Containment principles for experiments with GM Arthropods

There are several arguments to develop specific guidelines for working with genetically modified arthropods. Although the guidelines for GM arthropods are overlapping with guidelines for GM micro-organisms. Guidelines cannot be copied from microbiological research, not only because work on arthropods differs from microbiological work, but also because many arthropods species are moving actively, and some find ways to overcome barriers such as sealing. Therefore extra care could to prevent potentially escape from experimental places.

First, in contrast to micro-organisms most arthropods are visible and easily recognized even though some can be very tiny. In many cases they can also be individually handled. This leads to the impression that they are more manageable than micro-organisms. Traditionally, entomologists are not trained used to work in sterile conditions or to follow strict safety procedures. They tend to work with cages and boxes rather than with Petri dishes. For some of them contamination prevention is not a daily routine to take care of. Where safety and cleanliness is basic in microbiological laboratories this is less common in entomology. Traditionally, only in case of quarantine species and vectors of human or animal diseases there is more safety consciousness. Of course, a new generation of researchers is entering modern laboratories, but training entomologists may need extra attention when working with GM insects.

The second, and more obvious issue for insects and other creeping arthropods such as mites and ticks, is that they actively move by walking and flying and hence have more potential to escape from experimental and breeding units in case of insufficient containment measures. They can actively search a place to hide and respond to environmental signals such as light, (plant) odours and moisture gradients. They can also actively adhere to materials, clothing, plants, skin and hair. In this way and by using air flows and air streams they can be easily displaced.

Above mentioned differences between insects and micro-organisms and research characteristics necessitates a set of special guidelines for genetically modified insects.

There are three insect groups that have a tradition in biosafety measures (not necessarily related to genetic modification) and which containment principles can be applied to many other insects and other arthropods. These are:

1. *Mosquitoes that transmit malaria parasites*. Much work has been done with strains that have been manipulated, including genetic modification. Apart from experimental conditions in the laboratory also semi-field studies have generated containment principles that can be used in laboratories and greenhouses. Rapid developments take place in a GM approach of the sterile male technique in mosquito control (Benedict *et al.*, 2010).
2. *Quarantine insects*. Although there are a few examples of studies on GM quarantine species (e.g. Mediterranean fruit fly, see 3), quarantine species are relevant for this report because they are often subjected to the same procedures as GM arthropods. They have often been kept and studied in areas where they are not indigenous. Due to their obvious risk and economic impact, containment has always been an important issue here.
3. *Fruit fly strains*. *Drosophila* (mainly *D. melanogaster*) has been a model species for decades. In many cases where modification is applied, wingless forms are used for easy handling and safety. Apart from *Drosophila* several other fruit flies are currently modified for sterile male technique approaches (Beech *et al.*, 2012). The Sterile Insect Technique (SIT) involving area-wide release of mass-reared and sterilized pest insects has proven successful to reduce, control and eradicate economically important pest species, such as the Mediterranean fruit fly (medfly) (Scolari *et al.,* 2008). The development and introduction of new transgenic strains of *Ceratitis capitata* able to produce male-only progeny following heat-shock treatments is now feasible and a concrete possibility (Saccone *et al.*, 2007).

For these groups containment strategies have been developed which also have been used to formulate 'arthropod containment levels' (Scott 2005). In the new version of the 'Regeling GGO' containment measures for GM *Drosophila melanogaster* will be included in addition to the Dutch rules for GM animal housing (including) these containment strategies can form a basis for other arthropods species for which risk is regarded as substantial, but it should be determined case by case whether changes and/or additions are necessary according to the general risk questions given below.

When strains are not genetically modified and naturally occurring a basic arthropod containment is often sufficient in those cases. As soon as there is an increased risk or a wish to reduce risk due to the species properties (disease vector, invasive, genetic modification) higher arthropod containment levels should be applied. Such additional measures should be determined case by case when a more strict prevention of escape is needed based on risk assessment. Such measures and related protocols obviously are group or species specific.

In particular this may be needed with species that are highly mobile, very small, hard to detect or to recapture, and those that easily survive and spread in case of escape. Numerous possible measures are mentioned by Scott (2005), the USDA guidelines for containment (2003), and by the Canadian Food Inspection Agency (2012).

3. Current practices based on risk

Requirements for containment levels are generally based on the biosafety levels needed for the organisms to be worked with. A description of those international biosafety levels can be easily found on the internet sources such as from the World Health Organization (http://whqlibdoc.who.int/publications/2004/9241546506_partI.pdf).

Hence, arthropod containment levels or, more general, arthropod containment levels (ACL's) are in many aspects similar to or based on the biosafety levels for plants or micro-organisms. But they are adapted to the specific risk factors associated with arthropod properties. This applies to different facilities such as laboratories, greenhouses or (semi)field experiments.

Obviously, all containment levels are risk based. A multitude of risk assessment criteria is evaluated to establish the necessary containment levels for genetically modified arthropods. Risk is related to potential impact of escape on human or animal health, on plant production and on natural ecosystems. For genetically modified arthropods, the containment is mandatory. When considering the necessary containment level / measures, the characteristics of the species, and the character of the modified genetic trait are important, but it is also important whether the species is a pathogen vector or a quarantine species.

In order to decide on containment measures a number of questions are relevant:

- Is the arthropod species naturally occurring (native) in the area of the experiments?
- If the species is exotic, how likely is it that the species is able to settle and survive given the climatic and biotic environment? This not only counts for the natural or crop environment, but may also count for human dwellings (ants, cockroaches and so on).
- Does the species affect human, animal or plant health? And if so, how likely is it that any modification would change its spread and impact?
- Does the species transmit any pathogen and is genetic modification affecting its transmission potential?
- Is it feasible to monitor, control and eradicate the species in case of escape? This can be difficult, but successful cases are known.
- Would genetically modified strains be able to survive, reproduce, compete and interbreed with naturally occurring populations? Are they potentially more fit or is it likely that the modification changes its ecological behaviour? This also counts for improved beneficial arthropods.
- Is the ecology, and -if relevant- the vector biology of non-modified strains, sufficiently known to evaluate impact shifts of genetically modified strains?

Risk factors are an important driving force to decide to what extent additional containment measures should be applied in addition to those legally required. The aim is always to make sure that the GM arthropods are contained in the laboratory or greenhouse.

4. General Arthropod Containment Levels

In the last two decades containment principles for arthropods (genetically modified or those with quarantine status) have been worked out step by step in several countries. Currently most procedures that are implemented are derived from guidelines published in the USA (Arthropod Containment Guidelines 2003, Scott 2005) and in the UK by DEFRA (see Appendix IV with many specific measures listed). It should be noticed that these arthropod containment levels (ACL's) have no legal status and should not be confused with biosafety levels which are used in the European procedures for GM-organisms. They may, however, help to classify arthropods in groups that require additional attention.

Arthropod Containment level 1 is usually applied for non-GM species which are naturally occurring in area of where research is done, the non-GM species does not transmit any pathogen, and the non-GM species is not likely to spread into the environment because of lower fitness or low survival probability. Basic measures are taken to avoid undesirable escape, nuisance to people and negative effects on the environment.

This level is used for native and non-modified species but also for exotic species that are neither vector of any disease and unable to survive or settle in the natural environment. Standard measures to prevent escape such as window screens, sticky traps and well-sealed cages and containers are often used.

For genetically modified arthropods this basic containment level is insufficient in many cases since escape of the arthropod should be completely prevented.

Arthropod Containment level 2 are often applied when the species is genetically modified but not associated with any pathogen. This means that these ACL 2 measures should be applied within the (GMO) D-I area in order meet the GMO regulations. In case the species is a vector of a pathogen, the containment level criteria and measures of the pathogen should be conform legal regulations for that pathogen. In case the combinations of the insect and the pathogen cause additional risks even more strict measures should be considered.

In practice, facilities used at this containment level for arthropods are similar to biosafety level 2 for pathogens (including facility requirements, working protocols and instructions, working surfaces, handling disposals and disinfections).

First level containment measures are directed to prevent specimens escaping from tubes, cages, containers, Petri dishes, etc. into the contained area. For this many solutions have been proposed (see next chapter) but in particular during handling accidental escape could potentially occur.. Working protocols should be focused on optimizing first level containment.

In the contained area and in the ante-rooms appropriate traps can be placed to monitor and catch accidental escapes (in particular for mobile/ flying arthropods).

Second level containment should prevent that arthropods that are handled within the experimental area (laboratory, climate room, greenhouse) can escape into neighbouring rooms or corridors and other nearby spaces.

The contained area should preferably have a double door ante-room to experimental laboratories and rearing facilities with self-closing doors. Additional curtains and screens with appropriate mesh (tested on all stages) may form additional barriers to escape.

In many cases, there are good arguments to take measures additional to those prescribed above. This as a precaution for arthropods that are known to have a stronger tendency to escape (small but strong flying insects), to be easily transported without notice (thrips, mites), or for which the biology is not very well known and potential risk is expected.

Some options that are utilized by research groups:

- Reduced air-pressure within experimental area.
- Air curtains in ante-room.
- Species specific traps and monitoring inside and outside the contained area.
- Shoe covers and hair covers (that are not strictly required at level 2).
- All air conditioning and ventilation system can be equipped provide with Hepa filters.

It should be realized that many containment measures are not only effective to prevent escape from the experimental units but also to prevent contamination from outside.

Arthropod Containment level 3 This level may be required for GM vectors of human pathogens, high risk GM quarantine species and high risk modifications.

The requirements for this level are basically similar to those for microbes at biosafety level 3 as described by the World Health Organization (2004) . Also arthropod specific measures such as described for arthropod containment level 2 should be included. Often it is the associated pathogen that determines safety protocols. In such cases the specific behavioural characteristics of the arthropods need extra attention in order to consider special measures that are often most appropriate and effective. Avoiding direct contact with the arthropods by using high quality sleeve cages (see Figure 1), immobilization tools (Figure 2), and protocols to transfer material between different containers and experimental units, and applying very clean working protocols should have priority.

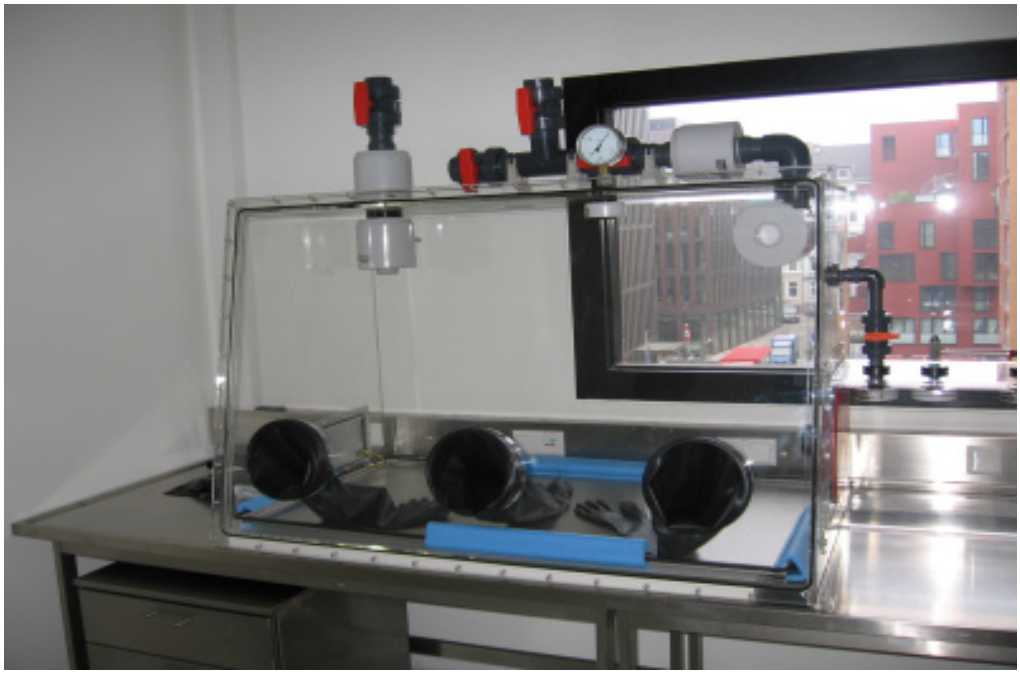

Figure 1. *High containment can be achieved by using high quality sleeve cages and experimental units to prevent escapes.*

5. Containment measures for GM arthropods

Spatial location and facility arrangements

In case of arthropod containment facilities (laboratories, climate rooms or greenhouses) at ACL 2 or higher, these should preferably be isolated, or away from other non-contained working areas where human activity is high in order to prevent accidental contact with experimental organisms involved. Access should be restricted and entrances be accessible for licensed personnel only.

Greenhouses used for GM research should preferably either be located in strict connection with an institutional building (with access from inside the building), or isolated from it surrounded by buffer zones free of public human traffic and dense vegetation or crops, and inaccessible for unauthorized people.

In case of arthropods that are disease vectors, care should be taken that infected and non-infected material are strictly separated (for example in the breeding units). This also counts for wild type and modified strains, and for uninfested and infested plants. There is a potential conflict between separation (GM/non-GM, infected/uninfected material) and keeping everything in one contained area to minimize transport of arthropods outside the contained area. Workflows and protocols to prevent contaminations should be optimised.

Facility entrance and notification

At the entrance door, there should be a biosafety sign indicating the containment level of the area and that work is done with genetically modified arthropods. The kind of arthropods (species) handled in the contained area should be indicated as well as specific risks related to that species. All requirements for people that are allowed to enter the area should be described as well. Personalized electronic door keys are a good option to prevent unauthorized people entering the area. Keep a log of what employees enter the facilities by date and time.

Construction quality.

Facilities should be tightly finished which means that any cracks and slits in walls, window / door frames that can serve as escape routes should be minimized as well as places that can serve as breeding spots (e.g. little holes where water can accumulate). Furniture should be smooth and easily cleaned. White colours are preferred for cleanliness and ease to spot accidentally escaped arthropods. Windows and glass walls should be fully sealed. Special attention should be given to hidden escape routes such as ceilings / machine room connections, electric cables plugs and so on.

Air treatment

Air conditioning should be realized by recirculation / air treatment in the laboratory area or by external outlets which are equipped with very small mesh filters or screens (in case of greenhouses) or preferably by HEPA filters which are necessary for many tiny arthropods such as thrips, mites and midges.

Air streams

In case of insects that fly active or passive it should be realized that air streams can cause unintentional displacement of insects and small spiders. Recognize also that many insects can easily fly upstream. This must be taken into account before using upstream or downstream flow cabinets or air-pressure gradients (under and over pressure). Sliding doors can be used preferred instead of inward opening doors when species tend to fly upstream. Air curtains with appropriate flow streams can also be used to prevent insects from flying out from a contained area. It should be known how the specific organism respond to air movements.

Ante-room

Entrance via an ante-room (interlock) is required from ACL 2 and higher. Both doors from the contained area to the ante-room and to the corridor should be sealed well and opening inward and automatically only one at a time. The ante-room is used to change cloths in such a way that working cloths are only present in the ante-room or in the contained area (jackets, overshoes, and hair covers). Plastic curtains, air-curtains or insect-proof gauze screens (small mesh) can be placed in order to attain an extra barrier between the experimental area and the ante-room.

Light

Light intensity differences can be used between the rooms when insects and other arthropods are attracted to either light or dark, in order to keep accidentally escaped animals in the contained area. This principle can also be used inside working cabinets in such a way that when animals escape during handling they always fly in the opposite direction from the exit side of the cabinet.

Prevent transport between areas

When animals need to be moved frequently from different subunits (breeding, experiment, handling and so on), it is preferred that these subunits are within one containment area. However care should be taken to avoid contamination of different cohorts, such as wild type and GM strains, and infected and uninfected vector species. There are specific procedures for transport (bijlage 9 van de Regeling ggo, (http://wetten.overheid.nl/BWBR0009653), that are used for various organisms and essentially the same for insects.

Handling insects and other arthropods

For experimental purposes it is often necessary to take individual arthropods out of rearing cages and tubes, and to manipulate them in free space. In that case it is important to deactivate flying or otherwise actively moving specimens by either CO_2 or cooling them down to below 10 degrees or lower. Note that activity / mobility in arthropods strongly depends on temperature. Clean and tidy working places limit? the occurrence of places where escaped arthropods may hide or even reproduce.

Keeping animals

Containers, tubes, bottles, jars for keeping animals should be preferably easy to handle (in such a way that animals can easily be taken out and put in), sufficiently closed to fully prevent escape. For employees it is important to be experienced in handling the arthropods and the experimental species in particular. Specific devices and methods are available for easy and safe handling with or without immobilization (special tubes, aspirators) (Figure 2). Workers new in the field should be instructed by experienced entomologists.

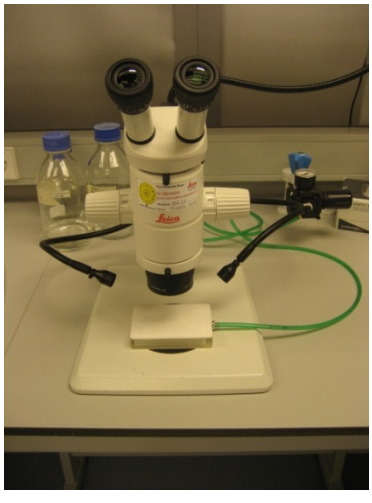

Figure 2. Using a CO_2 emitting work plate to keep insects immobilized when handling under the microscope.

Numbers of arthropods

If possible records should be kept on the number of individuals to be used and maintained before and after handling. In that way it is possible to check if any individuals are missing. In some species where numbers tend to be high (aphids, thrips and mites in colonies), however, this is virtually impossible and only indicative figures may be monitored.

6. Protocols and working procedures for arthropods

General working procedures for arthropods are similar for those applied to micro-organisms which are extensively described for the different biosafety levels for example by the World Health Organization (http://whqlibdoc.who.int/publications/2004/9241546506_partI.pdf). These include the way people should be instructed, the way material is handled, the way finished materials are disposed of, the way lab journals are handled and so on.

Training of people

Scientist and technicians have to be instructed and trained how to work safely with arthropods in general and genetically modified arthropods in particular. A basic understanding of how arthropods behave, reproduce and develop under both laboratory and natural outdoor conditions is necessary to understand why and how they should be handled in order to prevent unwanted escapes and spread. The basic response of arthropods to light, temperature, handling and killing agents should be known for the species to work with. It is advisable to train working protocols with non-GM material.

Testing and monitoring effectiveness of containment measures

General measures for containment (anti-escape) as well as specific trapping and killing protocols should be tested on their effectiveness with wild type material before being implemented in the experimental setting with GM arthropods.

Protective wearing / clothing

Persons entering the containment should always wear white laboratory coats, which should be taken from the ante-room on entrance and are left there on exit. In addition to lab coats, it is advisable to wear disposable overshoes and hair nets. The latter is essential when working with smaller flying insects such as thrips, aphids and midges, but also for mites and ticks that easily adhere to skin parts and hide between hairs without being noticed. It is advised when different containment levels are present, each level should have differently coloured clothing or colour marked collars.

Killing of material from finished experiments

Most arthropods can be effectively killed by freezing at minus 20oC for one day (however, killing rates should be tested for each separate species!) or by putting them into ethanol (70%).

To be absolutely sure for frozen material to be dead the material can be autoclaved or put in designated bins for GMO waste, after freezing. This might also be necessary when it concerns a vector level 2 pathogen or when eggs may have been laid in available substrate, since eggs are often more cold tolerant.

Minimizing escape of arthropods during handling

To minimize this risk of escape during handling it helps a lot when proper cages, jars and containers are used that are well closed and easy to work with. Working with sleeve cages, for example, is effective in most cases. Working in separate working units within the contained area can also be helpful. This makes it easier more easy to recapture any escaped individual. Where possible sleeve-cages can be appropriate. Keeping track of the number of arthropods per tube, box or experimental unit is very helpful to check for lost individuals but this not always feasible (e.g. when working with colonies of thrips, aphids or mites).

Trapping and monitoring accidentally escaped arthropods

Due to the mobility of most arthropods accidental escape from cages, tubes or boxes during handling could occur. Even though other measures (such as curtains) will hinder further escape from the contained area, additional measures can be taken to recapture, kill and monitor such escaped individuals.

For this purpose often general or species specific traps are placed in the contained area (experimental part and ante-room). Often sticky traps (with different colours), UV-traps, light traps, and pheromone-baited traps are used and in many cases quite effective for this purpose. These traps are also helpful to detect other arthropods species that are present in the area unintentionally, entering or brought in from outside or emerging from soil or plant material. It is important to identify and register all the species caught frequently. In some cases (e.g. at ACL 3) it might be useful for extra safety options to place also traps outside the contained area (corridor or open space) to detect any leakage from the contained area.

Removal of non-adult life stages

Arthropods have life stages that are not only morphologically different but also behave differently and hence generate additional risks. Eggs, pupae, and larvae may adhere to materials substrates or even working cloths that seem free of adult arthropods. Therefore it is crucial that all material leaving the containment should be cleaned properly and preferably be autoclaved for a fixed appropriate period. Care should be taken with waste water where eggs can be deposited (such as those of mosquitoes and certain other Diptera). Such waste water should be treated as GMO contaminated water.

Transfer of genetically modified arthropods

Transport of life GM arthropods between locations should be avoided as much as possible. There are strict guidelines for packaging (double packed, unbreakable), transport and registration procedures that have to be followed. In the case of arthropods, transport containers should be absolutely escape safe and unbreakable. Clear instructions should be included for delivery.

Independent auditing

It is necessary that experiments with GM arthropods are licensed by the responsible authorities and follow the official regulations and guidelines. The guidelines and or specific containment measures of the license often need to be translated into working protocols by the scientist. These protocols are developed and proposed by the scientist based on the biological characteristics of the species to work with, in particular when additional measures are required, such as for quarantine organisms. It is essential, however that professional independent biosafety officers approve these measures and protocols.

7. Containment measures for specific arthropod groups

Historically, arthropods containment practices were primarily focused on insects that transmitted human diseases such as mosquitoes that transmit malaria parasites. When human health is involved safety measures are quite restrictive to protect researchers. Similar containments have gradually been elaborated for quarantine organisms and genetically modified arthropods. Potentially, all arthropods that are affecting human or animal health (disease vectors) or threaten ecological resources (quarantine organisms and crop pests) are subject to genetic and ecological studies where genetic modification can be useful for study or manipulation. In addition also beneficial insects can be interesting for genetic modification. Although the escape-behaviour and risk varies enormously among species even within taxonomically related groups, some categorisation can be helpful to develop group-specific guidelines.

Arthropod groups that are likely to be used for genetic studies or genetic modification include those having specific economic or scientific and human impact:

- Plant feeding arthropods that directly affect crop production or natural resources such as forests or ornamentals.
- Beneficial insects and arthropods such as predators, parasitoids and pollinators.
- Vector species that may transmit plant, animal of human pathogens such as viruses, bacteria, phytoplasms and fungi.
- Blood sucking arthropods that may or may not transmit human and animal diseases.
- Research model insects such as Drosophila that are often used for genetic studies.
- Genetically modified flies and mosquitos, like Sterile Insects Technology or SIT, and Release of Insects carrying a Dominant Lethal trait, or RIDL.
- GM arthropods for biomass production.

It should be noticed that most containment guidelines are developed for mosquitoes, drosophila fruit flies, moths and ticks. Virus transmitting aphids, leafhoppers, white flies and midges have recently got more attention and other groups can become relevant in the future.

Group specific risks and containment measures

Although the number of GM arthropod species used for basic and applied research is growing, still most work is focused in the taxonomic groups discussed in this chapter. For a detailed review of environmental risks of GM arthropods to be placed in the market the reader is referred to Benedict *et al.*, (2010). This chapter summarizes containment information for the most relevant taxonomic groups. It is not fully extensive and no other comparable information source for containment per taxonomic group could be found.

Fruit flies (Diptera: Drosphilidae and Tephritidae)

Most relevant traits for containment are the high capacity to fly (though many wingless strains are available for research), rapid reproduction on various sources of decaying matter (not only fruit and growing media), several species are of economic importance and designated as quarantine species.

Drosophila is still regarded as a genetic 'model' species and there is a well-developed history of how to handle them in experimental conditions. Most genetic modification is directed to elucidate basic molecular mechanisms.

Ceratitis capitata, the Mediterranean fruit fly, has quarantine status and is widely studied also under contained conditions. Sterile male techniques are applied and further developed with genetic modification. At present the

sterile male technique using GM approaches is widespread and applied to control many harmful species (Benedict 2010).

As long as no species from the quarantine status are used, the basic guidelines as described in the Dutch Guidelines for Animal Housing (with additional measures as formulated in Appendix I) will be sufficient to contain most fruit flies.

Mosquitoes (Diptera: Culicidae)

The most relevant traits for containment of mosquitoes are their ability to fly, being attracted to animals, having a strong light-dark response, being vector of human and animal pathogens, and having eggs and larvae in moist substrates and water.

Methodology, protocols and facilities have been fairly well developed in the e.g. USA, Europe and Canada. Special handling cabinets, the use of CO2 for immobilization, freezing as killing method, registration of each individual mosquito, light traps in case of escape, and a double door system seem to work quite well. No escapes from the containment have been recorded and no persons were reported to be bitten during the work. D-I and Additional measures such protective clothing as or even containment level 3 measures may be required if higher risk organisms would be studied (such and the combination of Asian tiger mosquito and Western Nile Fever.

Midges (Diptera: Ceratopogonidae)

Midges are more and more relevant for genetic studies in Western Europe because they are vectors of animal pathogenic viruses such as Bluetongue and Schmallenberg virus that recently were epidemic.

The most relevant characteristics for containment in this group is their status as disease vector, being very tiny and hard to detect, easily flying and transported by air currents, being attracted to humans and animals for blood feeding, laying eggs in different moist organic substrates.

To study transmission routes, infection cycles and so on genetic studies have been started. For handling different strains of both midges and the viruses they transmit ACL 2 is the minimum requirement though this is extended with additional measures that come close to ACL 3. These include working with shoe covers, hair nets, all air conditioning and ventilation equipped with Hepa filters, and including very strict working protocols. It is advisable to be advised by an experienced laboratory that have a permit to work with GM-midges such as Pirbright in the UK.
Aphids and Leafhoppers (Hemiptera: Aphididae and Cicadellidae)

The characteristics involved with high risk of this arthropod group are that they are often maintained in colonies, and the younger stages often escape attention. As they can fly and adhere to clothing and hairs, there is a potential difficulty for effective containment. As they can easily spread, reproduce rapidly and asexually, escape prevention should be strict. However, for many species risk after escape is reduced as they cannot survive without the proper host plant. These tiny insects, in particular the younger stages, are easily overlooked. The adults, which are small and fly quite easily, are easily overlooked, and are not easy to recapture because sticky traps are not working 100% effectively.

Aphids and leafhoppers are widely studied also because there are many pest species of virus vectors among them. Combinations of quarantine insect species and quarantine plant pathogens such as viruses are usually handled at arthropod containment level ACL 2 or higher and appropriate measures should be taken. These include working with overshoes, hair nets, all air conditioning and ventilation with Hepa filters, and including very strict working protocols. It is recommended to be advised by an experienced laboratory that has a permit to work with virus transmitting insects, such as the John Innes Centre in the UK or INRA in Montpellier in France.

Moths (Lepidoptera)

Up till now mostly larger species are studied that can be quite safely handled and easily recaptured (e.g. by light of pheromone-baited traps). They often reproduce slowly. If necessary, both adults and larvae can be easily killed

chemically or by freezing. For the larger species most risk is probably in the egg stage as they can adhere to substrates and survive adverse conditions. Most containment experience is obtained from the silk moth Bombyx mori with the aim of producing improved silk and proteins (Prudhomme & Couble *et al.*, 2002). No information could be found on GM research in Microlepidoptera, which might be more difficult to handle.

In general basic D-I requirements with ACL2 measures should be sufficient for the species currently under study.

Mites (Acari)

Only one record was found for genetically modified mites up to now, but as they are of economic importance in both plant and animal health it seems likely that GM studies will be started in the near future. Even though most species are not fast moving they tend to adhere to substrates and often are tough survivors (frost and drought tolerant). In laboratories they can be a nuisance for all microbial cultures when established and for example blood mites in chickens can survive for months without food. As they are very tiny (in particular the young stage), they can easily escape through tiny openings and are often unnoticed.

Fleas and ticks (Pulicidae and Siphonaptera)

Both groups are carrier of human and animal diseases so in most cases research is carried out at arthropod containment level ACL 3. Although escape may at first sight be less likely than in flying insects these animals, the risk for escape is high because they are small and easily attach to cloths, hair and the skin. They are often occurring in high numbers in the breeding facilities. The combination with small mammals as a host may pose an additional risk. In that case the working protocols for GM animal housing would be sufficient and should be strictly followed.

8. Conclusions and Recommendations

Most containment guidelines for GM arthropods are derived from microbiological experiences. Additional measures for arthropods are often taken from research with either medical entomology (mosquitoes) or quarantine arthropods.

Because of strict containment requirements for GM arthropods additional measures are often needed. In practice, this means that the animal facility D-I, as prescribed by the order on GMOs (Appendix I), is not sufficient. Instead, work is done at D-I (or sometimes ML-I) that is upgraded to arthropod containment level ACL 2, often in combination with additional measures. We think that this should be recommended in general for flying insects and other mobile arthropods as the tendency to escape or to be unintentionally be transported by human activities is significant.

Knowledge on the biological characteristics of the GM arthropod of interest and training to understand the routes for loss and escape of animals from experimental situations and containment is crucial to ensure that the right species-specific containment measures and to safeguard that good working procedures are taken. Before getting started, we recommend to use a containment checklist for working with GM arthropods (Appendix II). Arthropod-specific cages, tools, devices, laboratory materials are available and described. Before starting a study on GM arthropods it seems obvious to test the effectiveness of those tools on wild type strains. Setting up a (European) database with laboratory tools that can be used for biosafety and containment measures for specific arthropods could be helpful as well as developing better trapping / recapture tools and genetic traits to prevent any escape. We also recommend to get advise from experienced laboratories that work with arthropod containment and GM arthropods in particular (Appendices III and IV).

In various countries, such as the UK and Canada, preliminary lists of measures for different containment levels have been described and are still being developed. With the fast development and implementation of new genetic tools in entomological research it seems worthwhile to make that kind of information and the information given in this report more accessible to researchers and biosafety officers in the Netherlands.

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Appendix I.

Formal regulations concerning GM animal housing in the Netherlands

In 2013 a new ministerial order on GMOs ("Regeling genetisch gemodificeerde organismen") will be released. Animal housing rules, i.e., room design and operation procedures will be described in Annex 4 of that order. Below the part relevant to GM animals is depicted. The final text however can be different.

D-I Animal Housing

Closed animal housing (small animals)

Construction:

a. The access door:
    1. Has a sign stating that this is a D-I animal facility;
    2. Shows the names and telephone numbers of at least one of the individuals responsible for the activities, and of the 'biologischeveiligheidsfunctionaris' (BSO);
    3. Has a sign, which warns that a facility may not be entered while activities are in progress;
b. The facility can be closed and locked and is constructed in such a way that genetically modified animals cannot leave the facility except due to the deliberate actions of people and/or the result of a calamity.
c. (Not applicable to insects).

Working instructions:

a. The facility is closed and locked when no personnel is present;
b. Eating, drinking, smoking, the presence of food- or drinking ware, applying cosmetics and storage of human foods and drinks is prohibited;
c. Vermin must not be present;
d. Access must be restricted to authorized persons;
e. The housing of GM animals must be labelled clearly in order to distinguish them from non-genetically modified animals;
f. The animals must be kept in the facility in such a way that no unintended mating can take place;
g. Small genetically modified laboratory mammals are kept in a cage inside the facility. When staff open the cage, the access door to the facility is kept closed and there is an indication on the outside that no one may enter during this process;
h. Other animals are kept in appropriate housing. The way they are housed is clearly written in the notification of activities.
i. Not applicable for the housing in this report;
j. During activities, the door of the animal facility must be kept closed.

Additional new guidelines for *Drosophila melanogaster* have been proposed. The preliminary text is given below (in Dutch).

Voor activiteiten met genetisch gemodificeerde *Drosophila melanogaster* gelden, bovenop de inrichtings- en werkvoorschriften voor een D-I dierverblijf als geformuleerd onder 4.1.4.1 van bijlage 4 van de Regeling ggo, de volgende aanvullende voorschriften:

1. Het verblijf heeft een sluis waarvan de opening aan de buitenkant is voorzien van een deur en de opening naar de binnenkant is afgesloten met een gordijn van insectengaas. De deur is aan de onderzijde voorzien van veegborstels en aan de zij- en bovenkant zijn tochtstrips in de sponning aangebracht;
2. Op de toegangsdeur dient de tekst 'Genetisch gemodificeerde insecten, geen toegang voor onbevoegden' aangebracht te worden en een signalering die waarschuwt dat het verblijf niet betreden mag worden gedurende de uitvoering van de werkzaamheden;
3. De ramen van het verblijf zijn afgekit;
4. Alle kieren zijn afgekit;
5. Alle ventilatieopeningen zijn voorzien van insectengaas;
6. Een voor de betreffende insecten aangepaste elektrische insectenval, vangplaten en, indien bruikbaar, een voedselval zijn in het verblijf en de sluis aangebracht;
7. Kapstokken voor dagelijkse kleding zijn buiten het verblijf aangebracht; kapstokken voor de werkkleding zijn in de sluis aangebracht;
8. Een diepvriezer (- 20°C) is in het verblijf aanwezig;
9. Werkkleding wordt gedragen. Alvorens de werkkleding het verblijf verlaat wordt deze, gedurende 10 uur, in een in het verblijf aanwezige diepvriezer (-20 °C) geplaatst om eventueel aanwezige insecten te doden;
10. In het verblijf mogen uitsluitend niet-genetisch gemodificeerde insecten aanwezig zijn indien zij deel uitmaken van het experiment;
11. Voorafgaand aan het openen van de insectenkooien worden de insecten geïmmobiliseerd met een gevalideerde methode;
12. Besmet materiaal en afval dienen te worden ontsmet door minimaal 10 uur bevriezing bij -20 °C. Hetzelfde gebeurt aan het eind van het experiment met de insecten.

In de huidige vergunningen zijn meer aanvullende voorschriften opgenomen, maar die zijn in de (concept) nieuwe Regeling weggelaten om de volgende redenen:

a. Voorschriften met betrekking op het voeren van een juiste administratie (logboeken, etikettering): hiervoor geldt het Artikel in de Regeling ggo betreffende 'Administratie'.
b. De methode van immobilisatie, onder 11, is veralgemeniseerd ten opzichte van reeds afgegeven vergunningen (voorafgaand aan het openen van de insectenkooien worden de insecten geïmmobiliseerd door gedurende minimaal 60 seconden pure CO_2 in een container (van maximaal 75 ml) met maximaal 300 vliegjes te blazen. Om de vliegjes immobiel te houden worden ze in een op ijs gekoelde petrischaal geschud of op een matje waar CO2 uitkomt gehouden. Na afloop van de handelingen worden de vliegjes overgezet in een container die op ijs is geplaatst. De immobilisatie wordt opgeheven door de container met vliegjes vervolgens bij kamertemperatuur te plaatsen).
c. Het voorschrift met voorwaarden waaronder insecten buiten het verblijf mogen worden bestudeerd is weggelaten omdat hiervoor Bijlage 9 van de Regeling geldt (vervoer van ggo's) of bovengenoemd voorschrift, onder 12, met betrekking tot besmet materiaal.

Nota bene: de genoemde inrichtings- en werkvoorschriften gelden uitsluitend voor activiteiten met genetisch gemodificeerde ***Drosophila melanogaster***. Voor andere (genetisch gemodificeerde) insecten geldt dat te allen tijde gegevens moeten worden voorgelegd met betrekking tot de wijze van inperking, immobilisatie en afdoding (inclusief validatie).

Appendix II.

Containment checklist for working with GM arthropods

Check the following questions when planning to work with genetically modified species and for primary containment level and additional measures!

Apply a risk analysis and identify and apply appropriate containment measures, by further checking this report and consulting containment experts when:

- The species is not native (exotic) or on a quarantine list.
- The species is potentially harmful to any crops or commodity.
- The species is a (potential) vector of any plant-, animal- or human pathogen.
- The (intended) modification may enhance fitness of the organism.
- The species/strain is able to survive and reproduce under outdoor conditions or in human settlements and buildings.
- The species is hard to contain at a small (cage/ container) or larger scale (contained area) because of moving, flying or attaching to clothes, hair or skin.
- Any of the life stages of the species (adults, larvae, or eggs) are tiny and hard to detect.
- Any stage of the animal has a tendency to hide or to adhere to cloths or hair.
- It is not easy to recapture escaping individuals efficiently by hand or any appropriate device or trap.
- The species responds to light, temperature, air currents or moisture regimes that may help them to leave the contained facilities.
- Any stages are resistant or tolerant to methods that are commonly used to kill finished material (e.g. freezing).
- The killing methods have not been validated (e.g. with wild type specimens).

Larvae of eggs of the species can survive in waste or water.

Before starting any project it should be warranted that employ working with arthropods instructed, trained to work with GM organisms, and aware of the risks in relation to the features of the species.

All working protocols should be approved by an independent biosafety officer.

Appendix III.
Information sources

Advices concerning containment of GM insects are available for the public on the COGEM website www.cogem.net (mostly in Dutch), including many details for working procedures and containment measures.

Further information about working procedures was obtained from personal communication and permits from the laboratories below. Information is public available for fruit flies, parasitoids and mosquitoes.

Laboratories in the Netherlands working with strict arthropod containment

- Leiden University Medical Centre (*Drosophila* and *Anopheles*).
- University of Groningen Faculty of Medical Sciences, (*Drosophila*).
- Wageningen University & Research Centre/ Plant Research International (*Spodoptera*).
- Radboud University Nijmegen (*Anopheles*).
- Nederlands Kanker Instituut, Amsterdam (*Drosophila*).
- NVWA, Wageningen, Containment facilities for quarantine insects (non-GM).
- Central Veterinary Institute, Wageningen UR, Lelystad (non-GM midges as vector for animal viruses).

Laboratories in the United Kingdom working with strong expertise in arthropod containment

- John Innes Centre, Dept. Disease and Stress Biology, Norwich, UK (aphids and leafhoppers).
- Institute for Animal Health Pirbright, UK (midges).
- Oxitec Ltd. Abington, UK (mosquitoes and flies, sterile male technique).

Appendix IV.
Defra containment reqirements 2008

http://archive.defra.gov.uk/foodfarm/farmanimal/diseases/pathogens/handlinga.htm

Containment Requirements for laboratories to be Licensed to Handle Arthropods Under the Specified Animal Pathogens Order 2008.

Arthropod accommodation (vectors or parasites).

If the arthropod is vectoring a specified animal pathogen then the containment condition appropriate for that pathogen will be required in addition to those for the arthropod.

A. Structure

(a) Rearing unit

1. Rearing rooms should be physically separate from other arthropod rearing rooms, from animals which may be infected and from cultures of pathogens.
2. Rearing rooms should have an ante-room arranged so that 2 solid or screened doors, opening into the room and closing automatically, are provided. The ante-room must be large enough to allow one door to be closed before the other is opened. It should also contain an insect-killing device.
3. Internal wall surfaces should be readily washable (e.g. tiles) and light coloured to facilitate detection and destruction of escaped arthropods. Cracks and crevices should be avoided.
4. Air ducts, lights and plumbing fittings and any other openings into the room should be suitably screened or sealed to prevent escape of stray arthropods.
5. For easy cleaning to prevent build-up of residues where arthropods or pathogens may persist, light removable shelving rather than fitted units should be provided.
6. Recapture devices, such as u.v. light electrocution traps, or sticky traps for flying insects, should be provided to prevent the survival of escaped arthropods.
7. Waste disposal outlets must be provided with a fine-mesh sieve to ensure the retention of the smallest larvae or other stages of arthropods in waste water or washings, and permit safe disposal of all solid waste.
8. No crevices or structure (e.g. humidifier) should be able to contain unmonitored sources of open water in which insects such as mosquitoes may oviposit.
9. As an extra precaution, windows and other outlets of rooms leading off an insectary containing insects which may carry or cause disease, should also be screened against flying insects.

(b) Experimental rooms and yards

1. Rooms and yards for experimental work with arthropod vectors or parasites should be separate from arthropod-reading accommodation, but should comply with the same basic structural requirements. If large animal hosts, e.g. cattle or sheep, are to be used, these may have to be modified, but this must be done in such a way as to ensure continued security.
2. Within the room, where arthropods carrying pathogens are concerned, fail-safe cages should be employed (e.g. use of safety cabinets for flying insects, or cages over trays of oil or glycerine for crawling arthropods).
3. Protective clothing should be provided as necessary to ensure against infection of operators by accidental arthropod bites, or abrasions, with face mask where necessary to avoid inhalation of pathogenic organisms in dust.
4. Special arrangements may be necessary for the sterilisation or disinfection of solid and liquid waste possibly contaminated by pathogens or infected arthropods.

5. Experimental yards for infested large animals (cattle and sheep) should be arthropod-proof and precautions should be taken against the spread of ectoparasites by birds and rodents. A moat containing disinfectant or acaricide may be necessary where crawling ectoparasites are involved.

B. Procedures

1. All stages of any arthropod should be killed before disposal in a sealed container or bag, using a suitable fumigant.
2. All larvae should be reared in a manner which will prevent the escape of emerging adults. Culturing procedure should be carefully timed where possible, so that the expected emergence date can be marked on cultures.
3. All arthropod cultures should be killed and disposed of as soon as their purpose has been completed.
4. If progeny are reared from virus-infected arthropods, these should be treated at all stages as infected individuals and kept in appropriate accommodation.
5. Small animal vertebrate hosts exposed to infected arthropods within the experimental rooms should be retained in screened accommodation and transferred to it in secure non-breakable containers, which must be sterilised after use.
6. Large animal hosts used for transmission experiments should be kept in screened accommodation, the arthropods involved being transported to and from the host in secure non-breakable containers, which must be sterilised after use.
7. When entering experimental yards containing livestock infested with specified arthropods (e.g. psoroptes mites, some ticks), experimental staff should wear special protective clothing which is left in a store at the entrance to minimise the risk of escape of non-enzootic pests.