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(54) **Title:** MOSQUITO OVIPOSITION SUBSTRATE, METHOD AND KIT

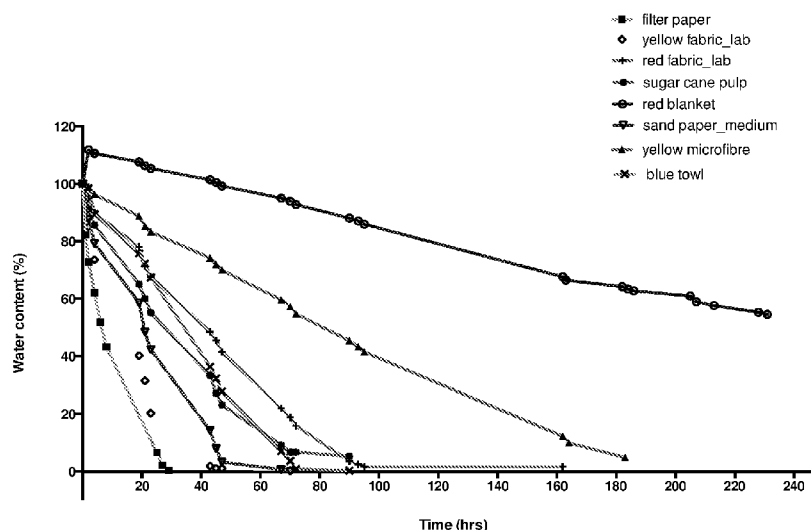


Figure 1

(57) **Abstract:** Mosquito egg oviposition substrates comprising a surface that facilitates a controlled arrangement or distribution of mosquito eggs substantially improves egg laying, incubation and hatching. A preferred oviposition substrate comprises embossed box board. This may be particularly efficacious for "in-field" kits to monitor local "wild-type" mosquito populations in a particular environment or to release mosquitoes into the environment as a biocontrol measure. A particular form of the invention relates to a method, substrate and kit for releasing mosquitoes infected with a mosquito-adapted *Wolbachia* bacterium into the environment to thereby modify mosquito populations and control mosquito-borne diseases such as dengue fever.



TITLE

MOSQUITO OVIPOSITION SUBSTRATE, METHOD AND KIT

FIELD OF THE INVENTION

THIS INVENTION relates to environmental control of mosquito-transmitted diseases. More particularly, this invention relates to an oviposition substrate for introducing mosquitoes into an environment to act as an agent for biocontrol of diseases transmitted by mosquitoes.

BACKGROUND OF THE INVENTION

Mosquitoes are a source of, or transmit, many diseases and conditions in humans and other animals. Mosquitos are responsible for the transmission of disease-causing pathogens such as arboviruses, flaviviruses and protozoa. These disease-causing pathogens are responsible for a variety of different diseases of humans and other animals including malaria, Dengue fever, Eastern Equine encephalitis, Western Equine encephalitis, Venezuelan equine encephalitis, Japanese encephalitis, Murray Valley encephalitis, West Nile fever, Yellow fever, LaCrosse encephalitis, Asian spotted fever, Q fever, Chikungunya fever, Zika fever and Ross river fever. More recently, in addition to Zika fever, Zika virus has been identified as capable of causing microencephaly and Guillain-Barre syndrome in humans.

Most pathogens that are transmitted by mosquitoes share a requirement to undergo a significant period of development in their insect vector before they can be transmitted to a new host. After a female mosquito ingests an infectious blood-meal, parasites or arboviruses, such as dengue, penetrate the mosquito's midgut and replicate in various tissues before infecting the salivary glands, where they are transmitted to a new host during subsequent blood-feeding. This time period from pathogen ingestion to potential infectivity is termed the extrinsic incubation period (EIP), and lasts approximately two weeks for both dengue (Siler *et al.*, 1926, Philipp. J. Sci. **29** 1; Watts *et al.*, 1987, Am. J. Trop. Med. Hyg. **36** 143.) and malaria (Gilles *et al.*, 2002, Essential Malariology (Arnold, London, 4th ed). A female mosquito must survive longer than its initial non-feeding period (usually less than 2 days) plus the EIP to successfully contribute to pathogen transmission.

Mosquito survival is therefore considered a critical component of a vector population's capacity for pathogen transmission (Dye, 1992, *Annu. Rev. Entomol.* **37** 1.). Interventions that aim to reduce the daily survivorship of adult mosquitoes, such as the spraying of residual insecticides in houses and insecticide-treated bednets for malaria control, yield large reductions in pathogen transmission rates (Schellenberg *et al.*, 2001, *Lancet* **357** 1241) because of the sensitive relationship between mosquito survival and vectorial capacity (Garrett-Jones, 1964, *Nature* **204** 1173; MacDonald, 1957, *The Epidemiology and Control of Malaria*. (Oxford University Press, London).

The control of diseases such as dengue primarily targets *Aedes aegypti*, a domesticated mosquito that prefers to live in and around human habitation (Gubler *et al.*, 1997, *Dengue and Dengue Hemorrhagic Fever*. D. J. Gubler, G. Kuno, Eds. (CAB International, New York, N.Y.) pp. 1-22.). With few exceptions, dengue management strategies have been complicated by the inability to completely eradicate *A. aegypti* from urban settings, and the ineffective application of long lasting vector control programs (Morrison *et al.*, 2008, *PLoS Med.* **5** e68.). This has led to a worldwide resurgence of dengue, and highlighted the urgent need for novel and sustainable disease control strategies.

A strain of the obligate intracellular bacterium *Wolbachia pipientis*, wMelPop, has been described that reduces adult lifespan of its natural fruit fly host *Drosophila melanogaster* (Min & Benzer, 1997, *Proc. Natl. Acad. Sci. USA* **94** 10792). *Wolbachia* are maternally-inherited bacteria that use mechanisms such as cytoplasmic incompatibility (CI), a type of embryonic lethality that results from crosses between infected males with uninfected females, to rapidly spread into insect populations (Hoffmann & Turelli, 1997, S. L. O'Neill, A. A. Hoffmann, J. H. Werren, Eds. (Oxford University Press, Oxford, UK), pp. 42-80).

However, life-shortening *Wolbachia* strains do not occur in mosquitoes naturally and experimental transfer of *Wolbachia* between host species (transinfection) has lacked success (Van Meer & Stouthamer, 1999, *Heredity* **82** 163). In some cases, transferred strains can be stable and maternally inherited, primarily when *Wolbachia* is transferred within or between closely related species

in a family or genus (Boyle *et al.*, 1993, Science **260** 1796 ; Xi *et al.*, 2005, Science **310** 326; Zabalou *et al.*, 2004, Proc. Natl. Acad. Sci. USA **101** 15042). In other cases, the new infection appears poorly adapted to its new host, showing fluctuating infection densities and variable degrees of transovarial transmission.

5 The result is often loss of infection within a few host generations. *Wolbachia* infections tend to be more susceptible to loss when they have been transferred between phylogenetically distant hosts (Kang *et al.*, 2003, Heredity **90** 71; Riegler *et al.*, 2004, Appl. Environ. Microbiol. **70** 273). Similarly, those species that do not naturally harbour *Wolbachia* have proven refractory to transinfection (Curtis

10 & Sinkins, 1998, Parasitology **116** Suppl: S111-5; Rigaud *et al.*, 2001, J. Invertebr. Pathol. **77** 251).

SUMMARY OF THE INVENTION

An object of the invention is to provide an improved substrate for oviposition of mosquito eggs. One particular object of the invention is to use the

15 substrate for improved surveillance of wild mosquito populations. Another particular object of the invention is to use the substrate to improve the introduction of mosquitoes into mosquito environments as a biocontrol system. A non-limiting object of the invention is to improve the introduction of *Wolbachia*-

20 infected mosquitoes into the natural environment as a vector control measure.

Broadly, the invention is directed to an oviposition substrate that optimizes mosquito egg laying in a regular, controlled arrangement.

In a first aspect, the invention provides a method of controlling oviposition of mosquito eggs including the step of providing a substrate to an egg-

25 laying mosquito so that a substantial proportion of the eggs are laid by the mosquito in a controlled arrangement on the surface of the substrate.

In a second aspect, the invention provides a mosquito egg hatchery comprising a plurality of mosquito eggs and a substrate wherein a substantial proportion of the plurality of mosquito eggs are located in a controlled

30 arrangement on a surface of the substrate.

Suitably, the mosquito egg hatchery further comprises a housing in which the substrate is located.

In a third aspect, the invention provide a mosquito egg hatchery kit comprising a housing and a substrate wherein a substantial proportion of the plurality of mosquito eggs may be located in a controlled arrangement on a surface of the substrate.

In a fourth aspect, the invention provides a method of producing a mosquito egg hatchery including the step of applying a plurality of mosquito eggs to a substrate so that a substantial proportion of the plurality of mosquito eggs are located in a controlled arrangement on a surface of the substrate.

In a fifth aspect, the invention provides a method of releasing mosquitoes into an environment, including the step of introducing a mosquito egg hatchery into said environment under conditions that facilitate hatching of mosquito eggs into mosquito larvae, said mosquito egg hatchery comprising a plurality of mosquito eggs, wherein a substantial proportion of the plurality of mosquito eggs are located in a controlled arrangement on a surface of the substrate.

In one embodiment of the aforementioned aspects, the mosquitoes are wild-type or otherwise naturally-occurring mosquitoes that exist in a local environment, region or habitat.

In another embodiment of the aforementioned aspects, the mosquito eggs are infected with or otherwise comprise a mosquito-adapted bacterium capable of modifying one or more biological properties of a mosquito, wherein said mosquito-adapted bacterium does not normally colonize, inhabit, reside in, or infect said mosquito host.

Accordingly, in a particular aspect the invention provides a method of modifying a mosquito population, including the step of introducing a mosquito egg hatchery into an environment under conditions that facilitate hatching of mosquito eggs into mosquito larvae, said mosquito egg hatchery comprising a plurality of bacterially-modified mosquito eggs located in a controlled arrangement on a surface of the substrate, wherein release of the mosquito larvae

into the environment modifies one or more biological properties of said mosquito population.

In a preferred embodiment, said mosquito-adapted bacterium is of the genus *Wolbachia*.

5 In another preferred embodiment, said isolated mosquito-adapted bacterium is of the species *Wolbachia pipientis*.

In one particularly preferred embodiment, said isolated mosquito-adapted bacterium is wMel.

10 A further aspect of the invention provides a method of obtaining eggs from a mosquito, said method including the step of providing an oviposition substrate whereby one or more mosquitoes can oviposit eggs on a surface of the substrate in a controlled arrangement.

The substrate may be provided in a kit or housing as hereinbefore described.

15 In some embodiments, the eggs obtained by the method may be used for maintaining and/or supplementing laboratory colonies of uninfected (e.g. *Wolbachia* free) mosquitoes.

In some embodiments, the eggs obtained by the method may be used to screen for *Wolbachia* infected mosquitoes to determine the spread of *Wolbachia*-
20 infected mosquitoes in release areas.

In other embodiments, this aspect of the invention may be applied to monitoring other mosquito control strategies, where mosquitoes are released to decrease local mosquito populations through sterile breeding.

25 In a preferred embodiment of the aforementioned aspects, the substrate is or comprises embossed box board.

Throughout this specification, unless otherwise indicated, “comprise”, “comprises” and “comprising” are used inclusively rather than exclusively, so that a stated integer or group of integers may include one or more other non-stated integers or groups of integers.

30 BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Analysis of the water content of potential oviposition substrates for

Aedes aegypti mosquitoes measured over time. The graph demonstrates the rate of drying for each substrate. Only substrates that reached a 45% moisture content within a period of 61 h were selected for further analysis.

Figure 2. Comparison of the distribution of mosquito eggs laid on different substrates: (a) Filter paper; (b) “Red duck” material; and (c) embossed sugarcane pulp.

Figure 3. Example of the indented or dimpled egg-laying surface (upper panel) and planar base (lower panel) of embossed sugarcane.

Figure 4. Testing of cloths, fabrics and textured papers as suitable oviposition substrates.

Figure 5. Papers and cardboards tested as suitable oviposition substrates. Embossed patterns and egg distribution on papers and boards are shown for each substrate tested.

Figure 6. (A) An embodiment of a mosquito egg hatchery kit with a closed housing; and (B) An embodiment of a mosquito egg hatchery kit with an open housing.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention is predicated on the discovery that oviposition substrates comprising a surface that facilitates a controlled arrangement or distribution of mosquito eggs substantially improves egg laying, incubation and hatching. A preferred oviposition substrate comprises embossed box board. This may be particularly efficacious for “in-field” kits to monitor local “wild-type” mosquito populations in a particular environment or to release mosquitoes into the environment as a biocontrol measure. Accordingly a particular form of the invention relates to a substrate and kit for releasing mosquitoes infected with a mosquito-adapted *Wolbachia* bacterium into the environment to thereby modify mosquito populations and control mosquito-borne diseases such as dengue fever.

For the purposes of this invention, by “isolated” is meant material that has been removed from its natural state or otherwise been subjected to human manipulation. Isolated material may be substantially or essentially free from

components that normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together with components that normally accompany it in its natural state.

As used herein “*mosquito*” and “*mosquitoes*” include insects of the family *Culicidae*. Preferably, mosquitoes are of the sub-families *Anophelinae* and *Culicinae*. Even more preferably, mosquitoes are capable of transmitting disease-causing pathogens, including viruses, protozoa, worms (*e.g.* nematodes) and bacteria to a host. Non-limiting examples include species of the genus *Anopheles* which transmit malaria pathogens, species of the genus *Culex*, and species of the genus *Aedes* (*e.g.* *Aedes aegypti*, *Aedes albopictus* and *Aedes polynesiensis*) which transmit nematode worm pathogens, arbovirus pathogens such as *Alphaviruses* (*e.g.* Eastern Equine encephalitis, Western Equine encephalitis, Venezuelan equine encephalitis and Chikungunya virus), *Flavivirus* pathogens that cause diseases such as Japanese encephalitis, Murray Valley Encephalitis, West Nile fever, Yellow fever, Dengue fever and Zika virus-associated conditions such as Zika fever, microencephaly and Guillain-Barre syndrome, and *Bunyavirus* pathogens that cause diseases such as LaCrosse encephalitis, Rift Valley Fever, and Colorado tick fever, although without limitation thereto. Non-limiting examples of worm pathogens include nematodes (*e.g.* filarial nematodes such as *Wuchereria bancrofti*, *Brugia malayi*, *Brugia pahangi* or *Brugia timori*), which may be transmitted by mosquitoes.

Disease-causing pathogens transmitted by mosquitoes also include bacteria (*e.g.* *Yersinia pestis*, *Borellia* spp, *Rickettsia* spp, and *Erwinia carotovora*).

Non-limiting examples of pathogens that may be transmitted by *Aedes aegypti* are dengue virus, Zika virus, Yellow fever virus, Chikungunya virus and heartworm (*Dirofilaria immitis*).

Examples of pathogens that may be transmitted by *Aedes albopictus* include West Nile Virus, Yellow fever virus, St. Louis Encephalitis, dengue virus, Zika virus and Chikungunya virus although without limitation thereto.

Pathogens frequently transmitted by the mosquito vector *Anopheles*

gambiae include malaria parasites of the genus *Plasmodium* such as, but not limited to, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium berghei*, *Plasmodium gallinaceum*, and *Plasmodium knowlesi*.

5 In one embodiment, said mosquito is of a genus selected from the group consisting of *Culex*, *Aedes* and *Anopheles*.

 In a preferred embodiment, said mosquito of a species selected from the group consisting of *Aedes aegypti*, *Aedes albopictus*, and *Anopheles gambiae*.

 In a particularly preferred embodiment, said mosquito of the species *Aedes*
10 *aegypti*.

 A “host” may be any animal upon which a mosquito feeds and/or to which a mosquito is capable of transmitting a disease-causing pathogen. Non-limiting examples of hosts are mammals such as humans, domesticated pets (*e.g.* dogs and cats), wild animals (*e.g.* monkeys, rodents and wild cats) livestock animals (*e.g.*
15 sheep, pigs, cattle, and horses) and avians such as poultry (*e.g.* chickens, turkeys and ducks), although without limitation thereto.

 An aspect of the invention broadly relates to a substrate that is suitable for oviposition, laying or placement of mosquito eggs. Suitably, the substrate facilitates oviposition of the mosquito eggs such that the arrangement of the laid
20 eggs can be controlled. In this regard, by “arrangement” is meant the distribution, location, frequency and/or density of the eggs on the substrate. It is a preferred objective of the invention to achieve a patterned array of eggs that minimizes the laying of eggs on top of each other, thereby resulting in an egg monolayer.

 The substrate preferably comprises an indented or dimpled surface
25 comprising alternating portions of peaks and troughs, whether the alternating portions are regularly spaced (*e.g.* patterned or arrayed) or irregularly spaced. In a preferred form, the indented or dimpled surface comprises alternating portions that are regularly spaced (*e.g.* patterned or arrayed), such as comprising alternating peaks and troughs that are regularly spaced (*e.g.* patterned or arrayed). The
30 indented or dimpled surface may be produced by any means known in the art including stamping, embossing, pressing, engraving, moulding or etching,

although without limitation thereto. An advantage of this indented or dimpled surface is that gravid female mosquitoes lay eggs in a patterned array that minimizes the laying of eggs on top of each other, thereby resulting in an egg monolayer. Typically, a substantial proportion of the eggs are laid in the troughs.

5 Suitably, the substrate further comprises a substantially planar (*i.e.* non-dimpled or indented) opposite surface, base or underside that facilitates placing the substrate in egg cups. As will be understood by persons skilled in the art, egg cups are cups of any suitable size, filled with water, in which the egg laying substrates are placed in such a way so that the substrates are partially submerged.

10 The substrate may be of any material that supports the laying, survival and hatching of mosquito eggs.

Non-limiting examples of materials include paper, cardboard and other cellulose-based substrates such as wood and sugarcane pulp, sandpaper and sponges, microfibre and cloth-based substrates such as “red duck” material.

15 In a preferred form, the material has a moisture content that is advantageous for mosquito egg survival and hatching. More particularly, a suitable moisture content is that which prevents the mosquito eggs hatching prematurely.

20 Suitably, the material has a moisture content of no more than 55%, no more than 50%, or preferably no more than 45% or about 15-25%. Typically, this is measured after a period of drying. Suitably, the period of drying is up to 72 hrs, up to 64 hrs, up to 56 hrs or typically about 61 hrs.

25 A preferred material is embossed box board. Suitably, the embossed box board is about 350 gsm embossed box board. An even and regular pattern may be embossed on the box board. The mosquitoes lay their eggs in these patterns, resulting in an even distribution of eggs while also minimizing oviposition of eggs on top of each other, particularly when this substrate is used in conjunction with controlled densities of gravid females. Additionally, the box board has a tendency to sink or otherwise be submersible in water, which allows the box
30 board material to retain an optimal amount of moisture to allow for controlled drying of eggs.

The substrate may be positioned or located within a housing that facilitates egg survival and hatching to produce larvae, pupae and subsequent development and release of adult mosquitoes into the environment. The housing is suitably a partially enclosed container or vessel that allows the exit of mosquitoes that have hatched and developed therein. The housing is suitably formed of a material that is at least partly water-repellent to prevent rain and moisture from compromising the structural integrity of the housing. The housing or “Mosquito Release Container” (MRC) can be made from any suitable water resistant material. Typically this material is plastic or water resistant card board.

Preferably, the material is biodegradable.

Accordingly, a kit may be provided which comprises the substrate and the housing, which can be assembled so that the substrate is positionable within the housing.

Another particular aspect of the invention provides a method of obtaining eggs from a mosquito, said method including the step of providing an oviposition substrate whereby one or more mosquitoes can oviposit eggs on a surface of the substrate in a controlled arrangement.

The substrate may be provided in a kit or housing as hereinbefore described.

Suitably eggs are initially laid on the substrate by gravid female mosquitoes and the substrate then placed in the housing for hatching of the eggs.

The housings or MRCs are designed specifically to minimise the opportunity for gravid females to enter and lay eggs. This could potentially result in the laying of eggs by unwanted mosquitoes, such as non-*Wolbachia* infected dengue vectors. Typically, the housing or MRC will include:

- a. a suitable amount of water to sustain mosquito development;
- b. a suitable amount of food to sustain mosquito development; and
- c. a suitable amount of eggs to maintain an optimal larvae to food ratio.

In some embodiments, the invention may facilitate monitoring the size of a wild mosquito population. By way of example, mosquito eggs laid in a controlled way are more easily counted and thereby used to obtain estimates of

mosquito population size. It will be appreciated that the population size may be monitored in the absence of a control strategy to thereby estimate abundance and hence the risk of disease transmission potential for a given human community.

By way of example, the substrate can be fixed in a bucket or surface near
5 water that may be located in an area to determine the level of mosquitoes breeding in an area. The substrate may be collected, photographed and subjected to image analysis.

In one particular example, due to the uniformity of the egg laying on the substrate it will be possible to count the number of eggs and potential future
10 mosquitoes in a given area.

In a particular example, due to the uniformity of the egg laying on the substrate it will be possible to discriminate the substrate egg laying mosquito species in a given area by the size and shape of the mosquito eggs.

These embodiments of the invention, the determination of the number of
15 mosquitoes breeding in an area may be facilitated by image analysis, which may include software that assists counting wild mosquito eggs on the substrate. Once the software that counts eggs in an area identifies a threshold number has been reached in that area, programs to reduce or eradicate wild mosquitoes such as spraying can be implemented. The determination of the number of wild
20 mosquitoes in a given area is therefore useful for pest control and also the control of mosquitoes harbouring known pathogens. Pathogens include flaviruses such as dengue and other arboviruses that cause disease in man, as hereinbefore described. The determination of the number of mosquitoes breeding area, such as facilitated by software as described herein, may be generally useful for pathogen
25 control in a given area and for the implementation of disease prevention measures that may include mosquito eradication.

In typical embodiments, such software-based imaging methods may include one or more of: capturing of an image of the eggs laid on the substrate, such as by a digital camera, lens, scanner or other image-capturing device;
30 computer-implemented analysis of the image to determine a mosquito egg count or number and, optionally, a statistical determination as to any error (*e.g.* standard

deviation, variance or the like) associated with the count or number; optionally, remote communication of the mosquito egg count or number to a database that records the mosquito egg count or number; and determination of the number of wild mosquitoes in a given area.

5 A non-limiting example of a computer-implemented, imaging system for counting mosquito eggs may be found in International Publication WO/2007/073591.

In another particular form of the invention, the mosquito eggs are infected with or otherwise comprise a mosquito-adapted bacterium.

10 By “*mosquito-adapted*” bacterium is meant a bacterium (*e.g.* of the genus *Wolbachia*) that has been taken out of its native host environment and adapted to a mosquito host, in which environment said bacterium does not naturally reside. Accordingly, a non-limiting example of a mosquito-adapted bacterium is a *Wolbachia* bacterium that has been isolated from its native host (*e.g. Drosophila*
15 *melanogaster*) and adapted to infect, colonize or reside in, a mosquito.

It will be appreciated that the term mosquito-adapted bacterium encompasses any bacterium that is capable of colonizing, infecting, or residing in a mosquito host within which it does not normally reside.

In a preferred embodiment, said isolated mosquito-adapted bacterium is of
20 the genus *Wolbachia*.

Wolbachia includes strains such as *wMel*, *wMelPop*, *wMelPop-CLA*, *wAlbB*, *wAu*, *wNo*, *wHa*, *wMau* and *wCer2*, although without limitation thereto.

In one particular embodiment, said isolated mosquito-adapted bacterium is
25 *Wolbachia pipientis*.

Suitably, the mosquito eggs are infected with the mosquito-adapted bacterium as described in United States Patent 9090911 and in McMeniman *et al.*, 2009, Science **323** 141. As originally described in United States Patent 9090911 and in McMeniman *et al.*, 2009, *supra*, *Wolbachia pipientis*-infected mosquitoes
30 have a shorter life-span, reduced fecundity, altered feeding behaviour, reduced pathogen transmission and/or a lower susceptibility to flavivirus pathogens such

as Dengue virus. As more recently described, *Wolbachia pipientis*-infected *Aedes aegypti* mosquitoes displayed lower Chikungunya virus and Zika virus prevalence and intensity, decreased disseminated infection and blocked viral transmission (Aliota *et al.* 2016 PLoS Negl Trop Dis **10** e0004677; Dutra *et al.*, 2016, Cell Host & Microbe **19** 771).

A mosquito comprising the isolated mosquito-adapted bacterium of the aforementioned aspects may be referred to as a “*modified mosquito*”.

In one embodiment, said mosquito-adapted bacterium shortens a life-span of a mosquito.

In another embodiment, said mosquito-adapted bacterium reduces a susceptibility of a mosquito to a pathogen.

As used herein, a mosquito that has a “*reduced susceptibility*” to a pathogen is less likely to become infected by, carry and/or transmit a pathogen than a wild-type counterpart.

As referred to herein, a pathogen may be a virus, a fungus, a protozoan, a worm or a bacterium, as hereinbefore described.

In yet another embodiment, said isolated mosquito-adapted bacterium introduces a reproductive abnormality in a mosquito host such as, but not limited to, parthenogenesis, feminization, male killing, and cytoplasmic incompatibility (CI).

Typically, according to this embodiment, said reproductive abnormality reduces a fecundity within a mosquito population.

As used herein, the term “*fecundity*” refers to the ability of a mosquito, or a population thereof, to reproduce. In one particular embodiment, said reduced fecundity may result in a loss of progeny following a cross between a modified male mosquito and a wild-type female mosquito.

In yet another embodiment, the mosquito-adapted bacterium reduces the ability of a mosquito to feed from a host.

Typically, according to this embodiment, the mosquito may have a reduced ability to obtain, ingest, or otherwise acquire blood from a host (*e.g.* a human) compared to a corresponding wild-type mosquito.

In another embodiment, the modified mosquito has a reduced life-span.

Typically, according to this embodiment, said “*reduced life-span*” is shorter than an average life-span of a wild-type of said modified mosquito. Accordingly, said reduced life-span may be 10%, 20%, 30%, 40%, 50%, or up to
5 80 % shorter than the average life-span of a wild-type of said mosquito.

It will be appreciated that the modified mosquito may be less likely to transmit a pathogen than its wild-type counterpart, since most pathogens have to undergo a relatively long incubation period in a mosquito vector before they can be transmitted to a new host.

10 In yet another particular embodiment, the modified mosquito may produce eggs from a modified female mosquito have a reduced tolerance to desiccation and a shorter life-span compared to eggs from a wild-type mosquito.

As used herein, “*reduced tolerance to desiccation*” refers to a reduced, diminished or decreased ability of eggs from mosquito to withstand or endure
15 extreme dryness or drought-like conditions.

According to this particular embodiment, the life-span of eggs from a modified mosquito may be at least 4 weeks, at least 8 weeks, at least 12 weeks, and up to at least 18 weeks shorter than eggs from said wild-type mosquito.

Wolbachia can also be used to reduce the mosquito population through a
20 phenomenon called cytoplasmic incompatibility (CI). CI is one of the reproductive modifications *Wolbachia* introduce into their hosts to drive the spread of infection. It means that if an infected male mates with an uninfected female, the offspring will be non-viable. This means that if instead of infected females, infected males are released, the resulting population of *Wolbachia*-
25 uninfected mosquitoes will decrease.

Accordingly, in a particular aspect the invention provides a method of modifying a mosquito population, including the step of introducing a mosquito egg hatchery into an environment under conditions that facilitate hatching of mosquito eggs into mosquito larvae, said mosquito egg hatchery comprising a
30 plurality of bacterially-modified mosquito eggs located at a controlled density on a surface of the substrate, wherein release of the mosquito larvae into the

environment modifies one or more biological properties of said mosquito population.

As will be appreciated from the foregoing, the one or more biological properties may include fecundity, tolerance of eggs to dessication, pathogen susceptibility, viability of offspring, average life-span of a mosquito population and/or the ability to transmit vector-borne diseases such as, but not limited to, malaria, dengue fever, and lymphatic filariasis, although without limitation thereto.

Thus according to this particular aspect of the invention, the oviposition substrate and/or kit may be used to facilitate the release of modified mosquitoes into the natural environment to thereby modify the local mosquito population as a means of reducing or controlling the transmission of mosquito-borne diseases such as dengue fever.

In some other embodiments, the eggs laid on the substrate may be used for maintaining and/or supplementing laboratory colonies of uninfected (e.g. *Wolbachia* free) mosquitoes.

In some embodiments, the eggs obtained by the method may be used to screen for *Wolbachia* infected mosquitoes to determine the spread of *Wolbachia*-infected mosquitoes in release areas.

In other embodiments, this aspect of the invention may be applied to monitoring other mosquito control strategies, where mosquitoes are released to decrease local mosquito populations through sterile breeding.

In one embodiment, mosquito control is achieved through an RNAi-based approach where an essential gene for male reproductivity is knocked out or otherwise inhibited using a small interfering RNA molecule to create “sterile males”. The sterile males may be released and compete with healthy males to mate. Matings with modified males doesn't result in offspring and the mosquito population is reduced or "crashed". This may be referred to as a “Sterile Insect Technique” (SIT).

Another embodiment relates to a mosquito control system known as Release of Insects with Dominant Lethality (RIDL), which is- a modified version

of SIT. In this embodiment, male mosquitoes are genetically modified to contain a homozygous pair of lethal genes. One copy of these genes is passed on to offspring, so that without an antidote (*e.g.* tetracycline), these offspring do not survive. Thus, genetically modified males compete with unmodified males to mate, resulting in non-viable offspring and the mosquito population is reduced. By way of example, reference may be made to Massonet-Bruneel *et al.*, 2013, PLOSOne DOI: 10.1371/journal.pone.0062711 for an example of RIDL control of dengue fever.

So that the invention may be fully understood and put into practical effect, the skilled reader is directed to the following non-limiting detailed Examples.

EXAMPLES

EXAMPLE 1

The water content of several candidate materials for use as an oviposition substrate was measured and compared after drying. As shown in FIG. 1, there was a wide variety of moisture contents across the different materials tested. Drying was performed by placing the egg substrates on a suitable absorbent material for a prescribed amount of time at 26 °C and 65% humidity. Water content was measured by comparing the weight of completely dry substrates to substrates that have been exposed to water.

A cutoff of no more than a 45% moisture content after 61 hrs of drying was considered to distinguish preferred materials from less preferred materials. This cutoff was determined empirically. If the egg substrates contained a higher than 45% moisture content after 61 hrs, the eggs will start hatching prematurely.

A comparison of the distribution of mosquito eggs laid on different substrates, namely filter paper, “Red duck” material and embossed sugarcane pulp was performed. The eggs were laid by gravid female *Aedes aegyptii* mosquitoes. The results showed that the most regular and even distribution of eggs was on embossed sugarcane pulp. Generally, the eggs were not laid on top of each other and tended to be laid in the indentations or troughs on the embossed surface.

Figure 3 shows a comparison of the embossed surface of the sugarcane

pulp substrate and the substantially planar base or underside of the substrate.

EXAMPLE 2

Initial testing involved a range of commercial products such as cloths, fabrics and textured papers for *Aedes aegypti* oviposition, as shown in FIG. 4.

Initially, it appeared that the embossed sugarcane pulp or biocane substrate may have been best suited as an oviposition substrate. The tight, 1 mm x 2mm “criss-cross” pattern promoted egg-laying uniformity. However, subsequent analysis found that the hydrophobicity and buoyancy of the biocane substrate in water made it unsuitable as an oviposition substrate. Therefore, further experiments were undertaken to select a less hydrophobic and buoyant substrate that would at least partially sink or submerge in water.

As shown in FIG. 5, a series of paper and cardboard products were tested. As it was difficult to find a commercial product that had the specified tight “criss-cross” pattern, we attempted to recreate this pattern on sinkable papers and cardboards using a sheet press and wire mesh as embossing template. The following products were first tested for sinkage:

1. Blotting paper GB003
2. Box board 350gsm
3. Box board 450gsm
4. Box board 600gsm
5. Box board 900gsm
6. Filter paper standard grade
7. Manila folder
8. Notepad insert
9. Printing paper 80gsm
10. Printing paper 250gsm
11. Quill blank flash card 300gsm
12. Square card 1
13. Sugar cane board
14. Quill board 210gsm

Papers and card boards that were found to sink in water included blotting

paper, box board (350gsm-900gsm), filter paper standard grade, square card 1 and quill board. We next tested these papers with different types of mesh with various patterns:

1. SF A series splatter screen
- 5 2. Metaltex splatter screen 29cm
3. 30 mesh, 30 gauge, 0.53mm aperture, 0.31mm wire ST/304
4. 40 mesh
5. 600 micron screen, 0.45mm wire

These studies showed that box board 350gsm box board was the most optimal
10 material to use as an egg substrate as it can be easily embossed with the desired pattern, which in turn better facilitates egg laying uniformity across the strip. In addition, this material also resulted in high fecundity and fertility.

Referring to FIG. 6A and 6B, there is shown an embodiment of mosquito egg hatchery kit 1 comprising a housing 10 that comprises a lid 11, body 12 and
15 internal cavity 15 into which can be placed an oviposited embossed box board substrate 13 and food source 14. Typically, up to about 300 mL of water (not shown) can be placed in internal cavity 15. Housing 10 may further comprise flaps 16A, 16B respectively comprising apertures 17A, 17B that allow suspension of the housing 10 above ground, such by way of a cable or thread. Food source 14
20 may be a dried pelleted material such as fish food sold commercially as Tetramin or Veggie Flakes.

Throughout this specification, the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. Various changes and modifications
25 may be made to the embodiments described and illustrated herein without departing from the broad spirit and scope of the invention.

All computer programs, algorithms, patent and scientific literature referred to herein is incorporated herein by reference in their entirety.

CLAIMS

1. A method of controlling oviposition of mosquito eggs including the step of providing a substrate to an egg-laying mosquito so that a substantial proportion of the eggs are laid by the mosquito in a controlled arrangement on the surface of the substrate.
5
2. A mosquito egg hatchery comprising a plurality of mosquito eggs and a substrate wherein a substantial proportion of the plurality of mosquito eggs are located in a controlled arrangement on a surface of the substrate.
3. The mosquito egg hatchery of Claim 2 which further comprises a housing in which the substrate is located.
10
4. A mosquito egg hatchery kit comprising a housing and a substrate wherein a substantial proportion of the plurality of mosquito eggs may be located in a controlled arrangement on a surface of the substrate.
5. The mosquito egg hatchery of Claim 2 or Claim 3 or the kit of Claim 4, further comprising a food source.
15
6. A method of producing a mosquito egg hatchery including the step of applying a plurality of mosquito eggs to a substrate so that a substantial proportion of the plurality of mosquito eggs are located in a controlled arrangement on a surface of the substrate.
7. A method of obtaining eggs from a mosquito, said method including the step of providing an oviposition substrate whereby one or more mosquitoes can oviposit eggs on a surface of the substrate in a controlled arrangement.
20
8. A method of releasing mosquitoes into an environment, including the step of introducing a mosquito egg hatchery into said environment under conditions that facilitate hatching of mosquito eggs into mosquito larvae, said mosquito egg hatchery comprising a plurality of mosquito eggs, wherein a substantial proportion of the plurality of mosquito eggs are located in a controlled arrangement on a surface of the substrate.
25

9. The method of Claim 1, Claim 6 or Claim 7 or Claim 8, the mosquito egg hatchery of Claim 2 or Claim 3 or Claim 5 or the kit of Claim 4 or Claim 5, wherein the substrate is, or comprises, embossed box board.

10. The method of any one of Claims 1 or Claims 6-9, the mosquito egg hatchery of Claim 2, Claim 3, Claim 4 or Claim 9 or the kit of Claim 4, Claim 5 or Claim 9, wherein the mosquitoes are wild-type or otherwise naturally-occurring mosquitoes that exist in a local environment, region or habitat.

11. The method of any one of Claims 1 or Claims 6-10, the mosquito egg hatchery of any one of Claims 2, 3, 4, 9 or the kit of Claim 4, Claim 5, Claim 9 or Claim 10, wherein the mosquito eggs are infected with or otherwise comprise a mosquito-adapted bacterium capable of modifying one or more biological properties of a mosquito, wherein said mosquito-adapted bacterium does not normally colonize, inhabit, reside in, or infect said mosquito host.

12. A method of modifying a mosquito population, including the step of introducing a mosquito egg hatchery into an environment under conditions that facilitate hatching of mosquito eggs into mosquito larvae, said mosquito egg hatchery comprising a plurality of bacterially-modified mosquito eggs located in a controlled arrangement on a surface of the substrate, wherein release of the mosquito larvae into the environment modifies one or more biological properties of said mosquito population.

13. The method of Claim 11 or Claim 12, the mosquito egg hatchery of Claim 11 or the kit of Claim 11, wherein said mosquito-adapted bacterium is of the genus *Wolbachia*.

14. The method, mosquito egg hatchery or the kit of Claim 13, said isolated mosquito-adapted bacterium is of the species *Wolbachia pipientis*.

15. The method, mosquito egg hatchery or the kit of Claim 14, wherein said isolated mosquito-adapted bacterium is wMel.

16. The method of Claim 12, wherein the mosquito egg hatchery is according to any one of Claims 2, 3, 4, 9-11 or 13-15.

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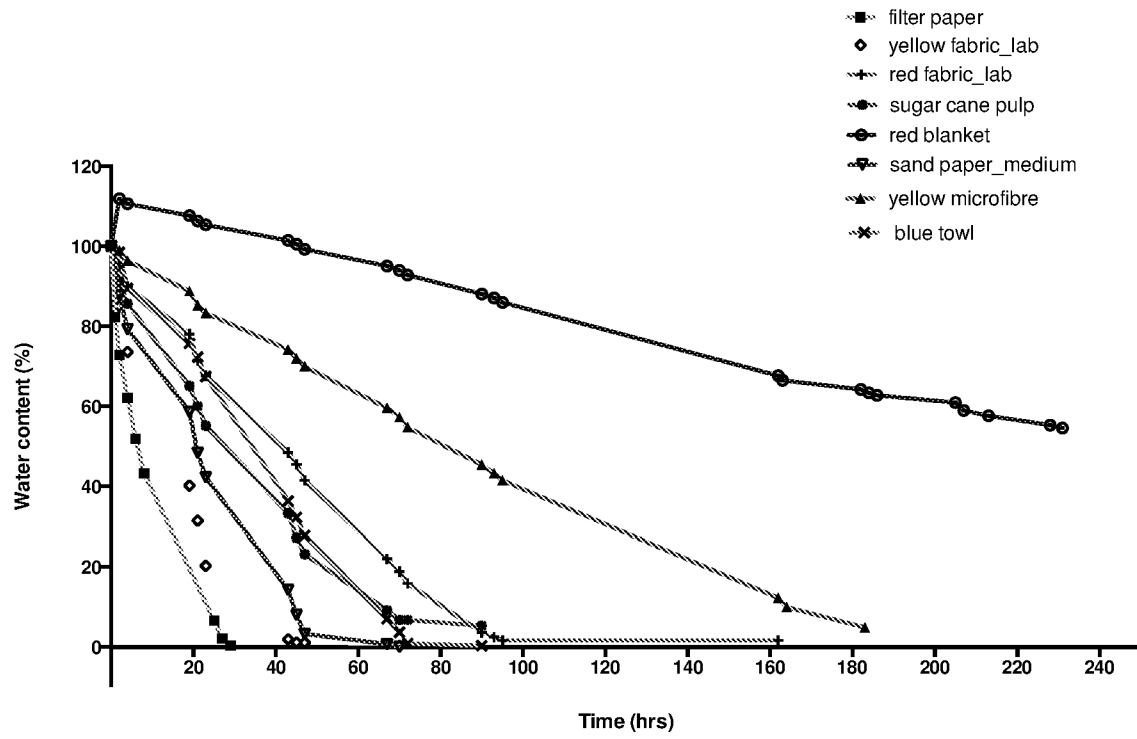


Figure 1

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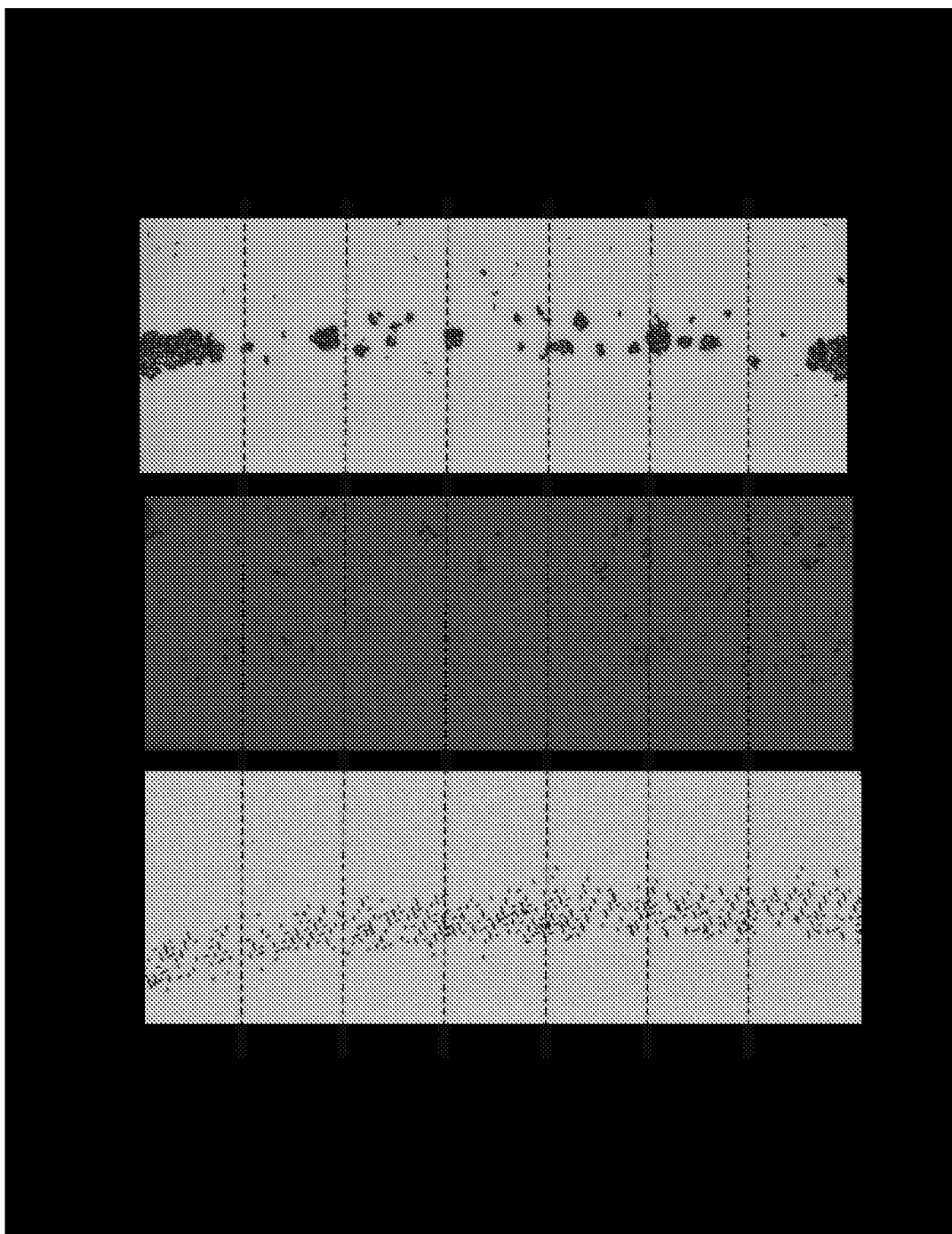


Figure 2

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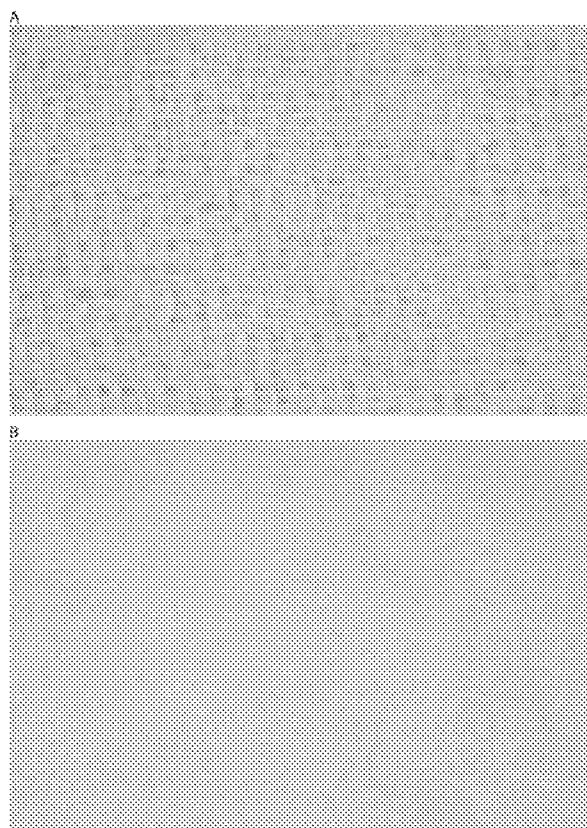
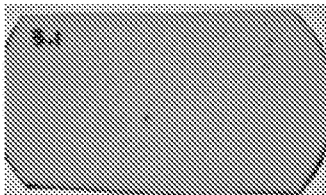
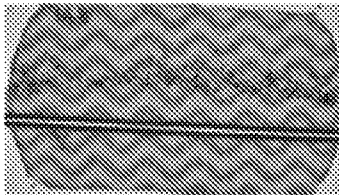
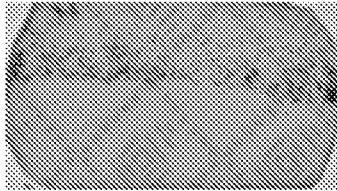


Figure 3

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Cloths

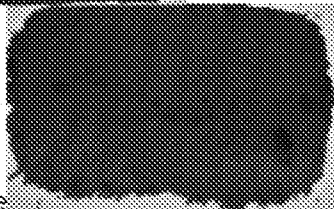
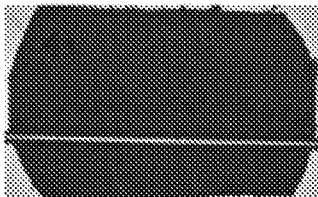
1. All purpose cleaning cloths
2. Chux



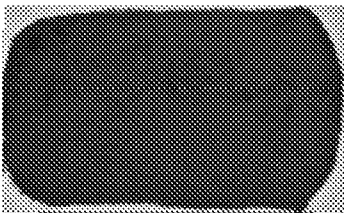
3. Microfibre

Fabrics

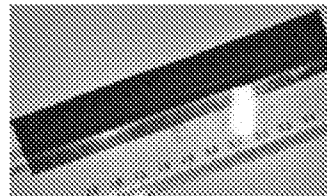
1. Assort red fabrics



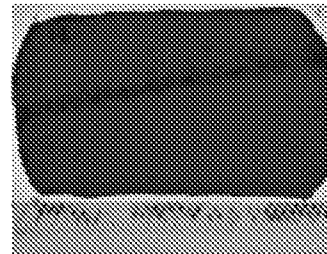
2. Coral fleece
3. Cotton French terry
4. Duck
5. Hessian
6. Nylon mesh
7. Polypop



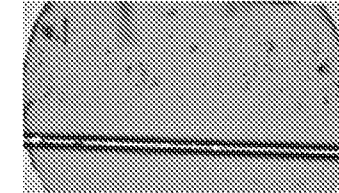
8. Premium polar fleece
9. PP spunbond non woven 655gsm
10. Reusable shopping bag



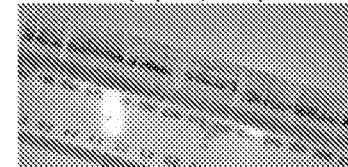
11. Ribbing



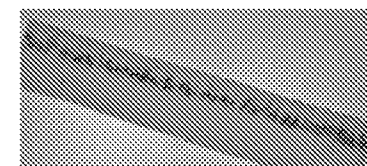
12. Yellow fabric

Textured Papers

1. Basket paper (front)



2. Basket paper (back)



3. Biocane plate

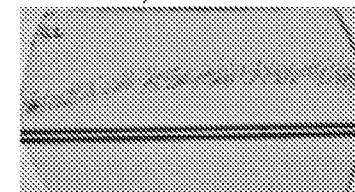
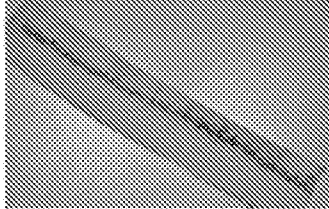


Figure 4

4. Copricated card



5. Kimsoft paper towel



6. Newspaper recycle pulp



7. Oak



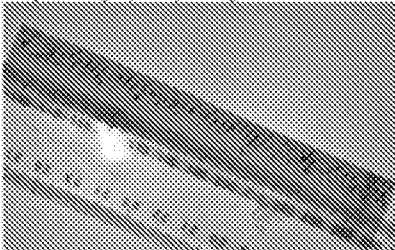
8. Pinstripe paper



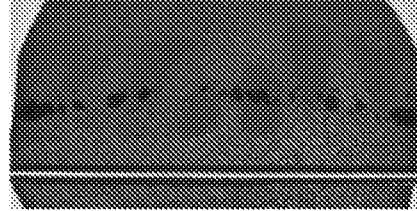
9. Popcorn paper (front)



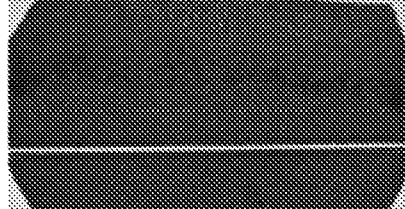
10. Popcorn paper (back)



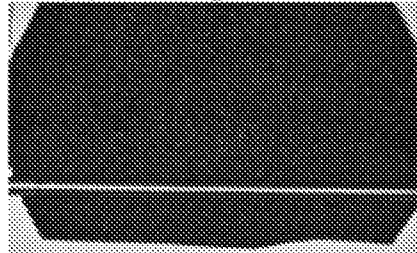
11. Sandpaper fine grains



12. Sandpaper medium grains



13. Sandpaper coarse grains



14. Square card 1



15. Square card 2



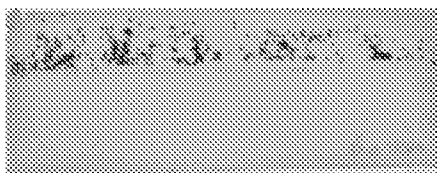
16. Zsa Zsa



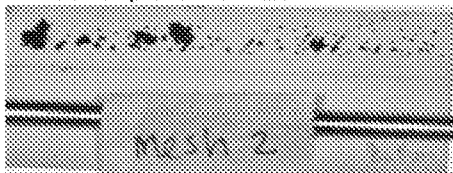
Figure 4 cont'd

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- 1 Filter paper embossed with SF A series splatter screen



- 2 Manila folder embossed with Metaltex splatter screen



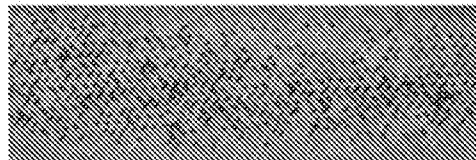
- 3 Square card 1 embossed with SF A series splatter screen



- 4 Box board 350gsm embossed with 30 mesh



- 5 Box board 350gsm embossed with 40 mesh



- 6 Box board 350gsm embossed with 600 micron mesh

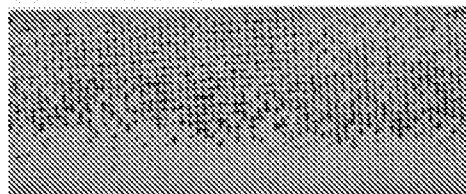


Figure 5

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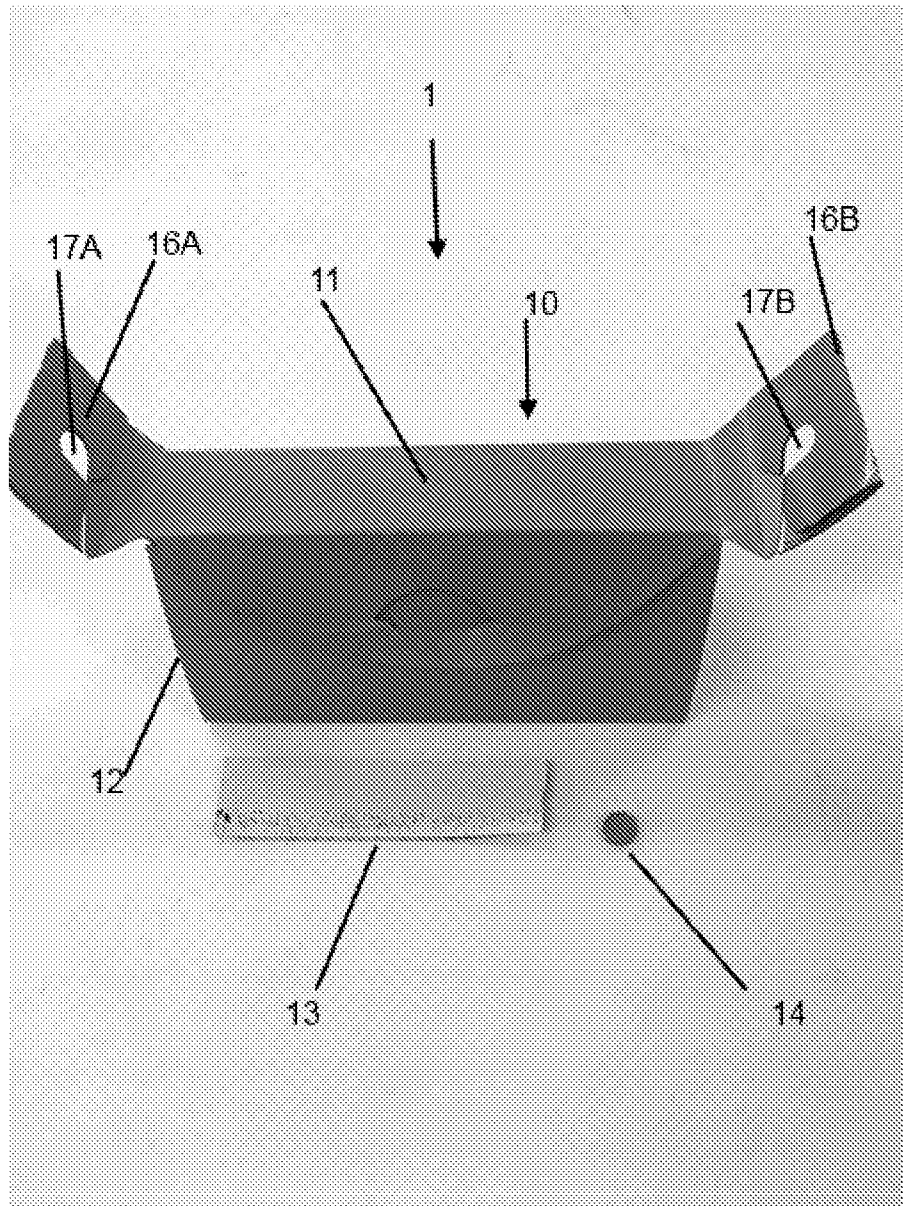


Figure 6A

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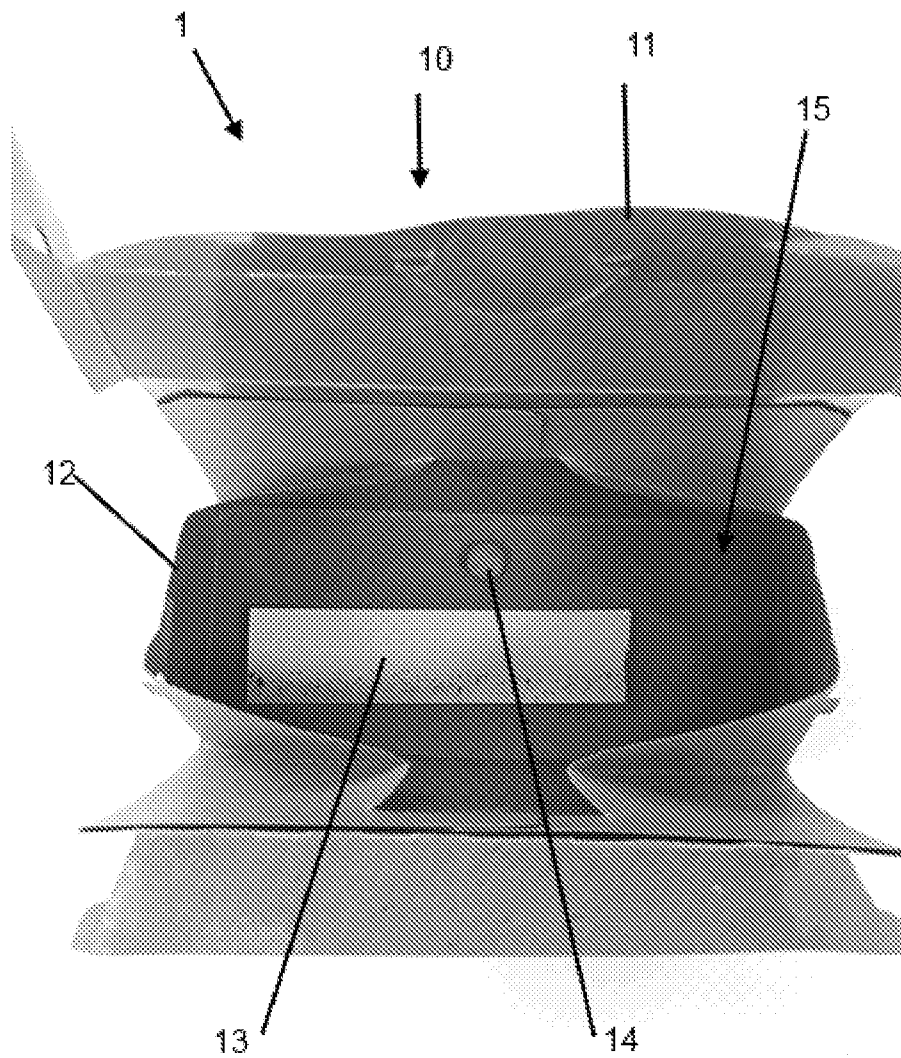


Figure 6B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2016/051124

A. CLASSIFICATION OF SUBJECT MATTER

A01M 1/02 (2006.01) A01M 1/10 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

INTERNET SEARCH

WPIAP, Epoque, TXTE: CPC/IPC: A01K67/0339, A01M1/02, A01M1/10, A01M1/026, A01M1/106 with keywords (Mosquito, Hatchery, oviposition, egg-laying) and like

Google Search, google Patents, Google Scholar: Keywords (Mosquito hatchery, oviposition, substrate)

Espacenet:

A01K67/0339, A01M1/02, A01M1/10, A01M1/026 with keywords (Mosquito, oviposition) and like

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 14 February 2017		Date of mailing of the international search report 14 February 2017	
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaaustralia.gov.au		Authorised officer Stephanie Pearce AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262832647	

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-10

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/AU2016/051124
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DESCHAMPS, A preliminary study of the attractiveness of ovitrap cups in collecting container species in Massachusetts,[Online], [Retrieved from the internet on 10th of January] <URL: http://www.cmmcp.org/2005ovitrap.htm > published on 7 Aug 2013 as per Wayback Machine Whole document	1-10
X	US 5896697 A (KANG) 27 April 1999 Columns 1-2, Figures 1-2 & 4	1-10
X	US 3997999 A (EVANS) 21 December 1976 Column 3 & Lines 1-9, Figure 1	1-10
A	WO 2015 A1 (WESTHAM LTD) 06 August 2015 Page 6, Lines 11-20	5, 9
A	WO 2012/112785 A2 (BARRERA) 23 August 2012 Whole document	1-10

Form PCT/ISA/210 (fifth sheet) (July 2009)

Supplemental Box**Continuation of: Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

This Authority has found that there are different inventions based on the following features that separate the claims into distinct groups:

- Claims 1-11 are directed to controlling mosquito oviposition by providing a substrate. The feature of obtaining mosquito eggs in a controlled arrangement on a surface of a substrate is specific to this group of claims.
- Claims 12-16 are directed to the use and method of producing bacterially modifying mosquitos. The feature of modifying a mosquito population via bacterially modified mosquito egg hatchery and releasing said mosquitos into the environment through the use of a hatchery is specific to this group of claims.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

The only feature common to all of the claimed inventions **is providing a substrate to an egg-laying mosquito such that a substantial proportion of the eggs are laid by the mosquito in a controlled arrangement on the surface of the substrate.** However it is considered that this feature is generic in this particular art.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a priori*.

Furthermore, claim 11 appended to claim 1, includes features related to the second invention, bacterially modified mosquito egg hatchery. Therefore search and examination of claim 11 will be reserved.

INTERNATIONAL SEARCH REPORT Information on patent family members		International application No. PCT/AU2016/051124	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
US 5896697 A	27 April 1999	US 5896697 A	27 Apr 1999
US 3997999 A	21 December 1976	US 3997999 A	21 Dec 1976
WO 2015 A1	06 August 2015	None	
WO 2012/112785 A2	23 August 2012	WO 2012112785 A2	23 Aug 2012
		AU 2012217569 A1	04 Apr 2013
		AU 2012217569 B2	28 Jan 2016
		MX 2013003508 A	16 Dec 2013
		US 2014223802 A1	14 Aug 2014
		US 9237741 B2	19 Jan 2016
		US 2016128313 A1	12 May 2016
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2009)			