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Biological Control of Dengue Vectors

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1. Introduction

Biological control is the deliberate use of natural enemies to reduce the number of pest organisms. It comprises methods that have gained acceptance for controlling nuisance arthropods partly due to the emergence of insecticide resistance and also because people have become more aware about the need to limit environmental pollution. In the case of arthropod-borne disease vectors, biological control is a potentially effective strategy for regulating and preventing transmission of diseases such as dengue, malaria and lymphatic filariasis, amongst others. Dengue is an arbovirus transmitted by species of *Aedes* mosquitoes. *Aedes aegypti* and *Aedes albopictus* are the primary and secondary worldwide vectors; they breed in peridomestic man-made water containers, and their control is the most effective way to reduce the viral transmission.

In this chapter we present an outline of the conceptual development of biological control since it was proposed by Harry S. Smith in 1919, to the current understanding of applied biological control involving basically autecology of insects that has led to Integrated Pest Management (IPM) principles. The potential of a natural enemy to regulate vector abundance will provide quantitative insight into Paul DeBach's principles, *ie.* an optimal biological agent according to its search capacity, host specificity, and tolerance to environmental factors. We will also explain Pavlovsky's theory to understand the origin of dengue as a human disease evolving from enzootic cycles. Likewise, we will introduce the reader to population regulation and transmission control describing the concepts of "functional response" and "numeric response" according to the classical Holling modelling.

We then explain the evolution of vector synanthropism and outline why dengue transmission can only be reduced by controlling the *Aedes* mosquito vector. In this regard we introduce the reader to the parasites, predators and pathogen complexes of the dengue vectors so that the he understands the present situation in terms of the biological control of the *Aedes* mosquito. We will use classic and recent papers and reviews to describe novel lines of research, and pros and cons of the use of natural enemies for dengue vector control. We hope that the chapter will work as a source of key literature references for students and researchers. Finally, the need for an integrated vector management (IVM) strategy aimed at controlling dengue will be put forward, and the potential for the deployment of biological control tools in future programmes will be made.

2. Biological control: Basic concepts

Biological control is part of a larger phenomenon called natural control, because the environment is always exerting selection pressure on populations. The selection is expressed in terms of mortality rates inflicted by all environmental factors and living beings as natural control. In this context, Harry S. Smith in 1919 proposed the term "natural control". In reality, this idea is closely linked to the Darwinian concept of "struggle for the existence or survival of the fittest" because a given species interacts within its ecosystem, and coexists surviving the attack of microorganisms, animals and plants that depend directly or indirectly on it. All these consumers who share resources (competitors) in the context of Smith, make up the complex known as "biotic" factors, which are constantly adapting and optimizing the manner of obtaining energy from the species in question. Conversely, all those factors from the environment such as temperature, humidity, pH, chemicals, substrates, etc., which also cause mortality on populations, constitute the complex of "abiotic" factors, the other component of natural control.

Four decades later in 1964, Paul DeBach, a student of Dr. Harry S. Smith, emphasized the term "regulation" as synonymous of "control" to specify the total mortality exerted by the biotic and abiotic factors on populations. He explained that since the mortality is dynamic, the population size will also be fluctuating in time and space. In theory, if a graph is depicted using the population changes against a reasonable period of time, such as a year, we might determine the average density (equilibrium) around which decreases and increases may occurred in the population. DeBach used this criterion to define the concept of biological control: If we may remove all biotic factors acting on a particular species, obviously its average density would be higher than normal. He pointed out that the difference between both average densities (with and without biotic factors) is the "effect" of biological control. In addition to the large number of studies published by DeBach on biocontrol, he also has the merit of having edited and published in 1964, together with Evert L. Schlinger, the book entitled "Biological Control of Insect Pests and Weeds", which is the classic in this discipline.

Now then, biotic factors include the "natural enemies", a term used by DeBach and by Huffaker and Messenger (1976) (another classic book as well). Natural enemies are the predators, parasites, parasitoids, and pathogens of each species. Predators kill rapidly, and require several preys, usually smaller in size, to complete their life cycle. Parasites are generally much smaller than the host, live on or inside it, and may or may not kill it. Pathogens are smaller still; they are the microorganisms that consume nutrients from a host which may be killed or not (Price, 1970). A parasitoid is an insect similar in size to its host, which is parasitized in immature stage and always dies. Whilst all the other types can be used against *Aedes* mosquitoes, parasitoids cannot. Biological control is divided into two types: natural and applied. The former is geared to the regulation of the populations by natural enemies without any human intervention, while the latter is obviously artificial. Applied biological control in turn can be divided into three types: 1) Classic biological control includes cases where there is an introduction of a foreign or exotic natural enemy to a region or country where it does not exist, 2) The increase of local natural enemies which can be performed by inoculative (if the natural enemy establishes itself in the habitat and in the target organism using a single release) and inundative releases (where the natural enemy regulates the target population temporarily, or only while he remains alive and

therefore many releases may be required), and 3) Applied biological control by conservation of natural enemies, which include all those agricultural practices (plowing, planting dates management, irrigation, etc...) or other activities (manipulation of weeds, with alternate preys and hosts or maintaining the nectar source for the natural enemies as adults) aimed at increasing the level of regulation of the pest population.

3. Quantitative expression of the regulation capacity of a natural enemy

Populations change in time and space. Changes can occur in the density of a species whether host or prey, affecting the biology of its parasites, parasitoids and predators. Scientists have been modelling functional responses since the 1920s although the term "functional response" was introduced in 1949 by Solomon. Holling (1959) explained the concept of functional response as the population rate of a host or prey consumed by a carnivore per time unit, and the concept of numerical response as those changes induced by the host or prey on survival rate, emigration, and mostly fecundity of the carnivore, which in turn depends on the food amount eaten. Therefore, functional response determines the numerical response. The former was considered as a response of the carnivores at individual level while the later as response at population level.

Since the beginning both concepts called the attention of ecologists. Functional response is crucial because taking the number of prey eaten per individual at the end of a period of time, the product of this estimator by the density of the predator will allow prediction of the host or prey population consumed after that time interval (Royama, 1971). In other words, if the predation rate increases, also the reproduction of the carnivores will increase and this will be reflected in an increment of its population; its density will follow the one of the host or prey resulting in a plot where oscillations of both will be very close. This is a top-down regulation system with negative feedbacks because when the host or prey is scarce it diminishes the carnivore population and this permits the host or prey to recover its original density (Holling, 1961). However these models rely on theoretical assumptions that must be accomplished, for instance, host searching has to be a random process, its populations should have a stable age structure, spatial distribution without clusters, no emigration, etc., and these requirements do not exist in nature. Despite these drawbacks the concept is applied to have an idea of how much a predator could diminish the prey population.

Holling (1966) proposed three basic types of functional response: Type I is a linear relationship where the predator consumes the same rate along the prey density until it reaches a "satiation state" which is a plateau in the graph. Type II describes a situation in which the number of prey consumed per predator initially rises quickly as the density of prey increases but then levels off with further increase in prey density. Finally, Type III resembles Type II in having an upper limit to prey consumption, but differs in that the response of predators to prey is depressed at low prey density (Figure 1). Explanation of the three models is not in the objectives of this chapter, but we will describe only the characteristics of the Holling Type II model also known as the "disc equation" because it has been the most widely used and accepted by researchers to describe numerous prey-carnivore systems (Tully, et al. 2005). We recommend to the reader the review of Jeschke et al. (2002) in which the authors carry out a detailed analysis of 47 models of functional response, 32 of them were for parasites and predators.

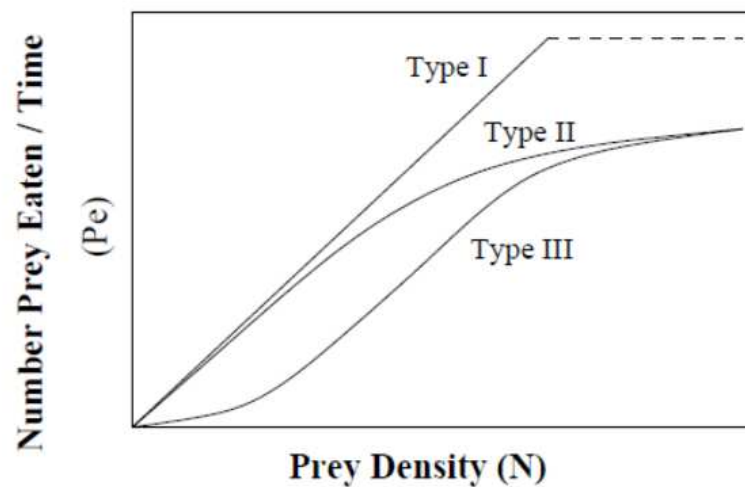


Fig. 1. Three types of functional response relating prey density (N) and the number of prey eaten by one predator (Pe).

Type II response incorporates predator handling time, which refers to the act of subduing, killing, and eating a prey, including cleaning and resting before moving on to search for more prey (Juliano and Williams, 1985). The number of prey attacked increases at a constant initial rate under this model, but then increases at an ever decreasing rate as satiation is approached. The result is the hyperbolic curve of the “disc equation” which is mathematically equivalent to the Michaelis-Menten model of enzyme kinetics and the Monod formula for bacterial growth (Abrams, 2000). This name derives from the way Holling conducted his experiment; he distributed randomly paper discs on a table (available preys), and a blindfolded person (the predator) with his fingertips “searched” around on the table the discs. Each disc was removed but replaced immediately after each encounter, and using the number of “consumed” preys from the exposed ones per time unit, he found his deterministic disc equation:

$$Na = \frac{aTNo}{(1 + aThNo)}$$

Where Na is the number of prey killed, No is the initial density of prey, T is the time available for searching during the experiment, a is the instantaneous rate of discovery or also known as attack rate varying with No , and Th is the total amount of time (constant) the predator handles each prey killed. So, the first step is to plot the numbers of eaten prey per density per unit time. Once detected the tendency of the hyperbolic curve, the computation of a and Th proceeds in the second step, which is the simplification of the equation by reciprocal linear transformation (Livdahl and Stiven, 1983) which is as follows:

$$\frac{1}{Na} = \frac{1}{aTNo} + \frac{Th}{T}$$

This equation is the linear regression model $Y = \alpha + \beta X$, where the intersection value α is the reciprocal of the attack rate $1/a$ and the slope β is the factor Th/T , however T is constant

and the factor stays just as Th and is equal to $-\frac{\beta}{a}$; then both values are used in the disc equation as follows:

$$Na = \frac{\alpha * No}{\left(1 + \alpha * \left(-\frac{\beta}{a}\right) * No\right)}$$

Therefore, after conducting an experiment, coefficients α and β are computed regressing Na/No as the response variable on No . The maximum predation rate is $1/Th$ and is the maximum value that Na/No can have. Attack rate, a determines how steeply the curve rises with increasing prey density (No). Once the expected values are plotted together with the observed ones, the degree by which the model explains the functional response is computed by the determination coefficient R^2 and a χ^2 goodness of fit test.

Another similar option to analyzing data of the same experiment is to calculate the reciprocals $1/Na$ and $1/N$ and conducting a regression of the former as the response variable on the later (Williams and Juliano, 1985); this method also produces the next linear equation:

$$\frac{1}{Na} = \frac{1}{aN} + Th$$

Here, the attack rate a is $1/\beta$, and Th is the intercept α , both coefficients are used in the disc equation to have the hyperbolic curve. To know which method is better the determination coefficient R^2 for each regression is calculated; the higher value will indicate the better fitting. Nevertheless, regardless of the method, linearization is polemic because it may produce bias in parameter estimates. To directly fit the disc equation with data generated from experimental protocols, or to use nonlinear procedures (logistic regression), the reader could examine the excellent review of Juliano (2001).

4. Origin and ecology of dengue

4.1 How diseases adapt from zoonoses to anthroozoonoses

Pavlovsky in 1962 published his book in Russian about the theory of natural nidality of transmissible diseases and the book was available in English in 1964. The *nidus* is a nest or focus of infection, *i.e.*, a place where a disease occurs in the wild and is then transmitted to humans by arthropod vectors when they invade the *nidus*. These diseases are zoonoses. It is the triangle host-pathogen-vector interactions that functions on a permanent and tridimensional space within a "pathobiocenosis" which is the community where the three species converge. The conceptual framework includes what Pavlovsky pointed out as "polivectorial focus" where several vectors and pathogens interact in the same three-dimensional space. For example, a polivectorial focus is the nest of the cactus rat *Neotoma spp.* in North America where the *Lutzomyia spp.* sand flies with the protozoan *Leishmania mexicana*, and the *Triatoma infestans* kissing bug with the flagellate *Trypanosoma cruzii* coexist infecting the rodent. Humans that inhabit areas near to the rat nests could potentially be

bitten by those vectors seeking a blood meal and so the parasites that produce the leishmaniasis or Chagas disease will be spread to the humans. If the parasite transmission cycle occurs among the rats only it is known as enzootic cycle or zoonose. The rat is the primary host, but if the human is infected by the vector's bite, then it becomes a secondary host and the disease shifts from zoonose to anthroponose. Given that the human invaded the *nidus*, he is said to be infected by tangential transmission, and the rodent is the reservoir or amplifier host. The reservoir is the host where the pathogen survives in the wild as the mosquito *A. aegypti* for dengue viruses. Usually, the primary hosts or reservoirs have co-evolved with the pathogens and developed immune defenses that will cause them to become asymptomatic. On the other hand, the secondary hosts have evolved recently, so there is still some susceptibility to the infection with the pathogen that produces symptoms ranging from minor to quite severe ones which may even cause death.

4.2 The evolution of dengue (Pavlovsky's principle)

According to Pavlovsky a dengue *nidus* is an enzootic cycle in Asia with *Aedes (Finlaya) niveus* mosquitoes as vector and several primates of the genus *Macaca* as hosts. Or in Africa with *Erythrocebus patas*, *Cercopithecus aethiopica*, or *Papio anubis* monkeys as reservoir hosts (Roche 1983) and as vectors, the mosquitoes *Aedes (Stegomyia) africanus*, *Aedes (S.) luteocephalus*, *Aedes (S.) opok*, *Aedes (Diceromyia) taylori*, *Aedes (D.) furcifer* or *Aedes polynesiensis* in the South Pacific islands (Moncayo, et al. 2004) (Figure 2).

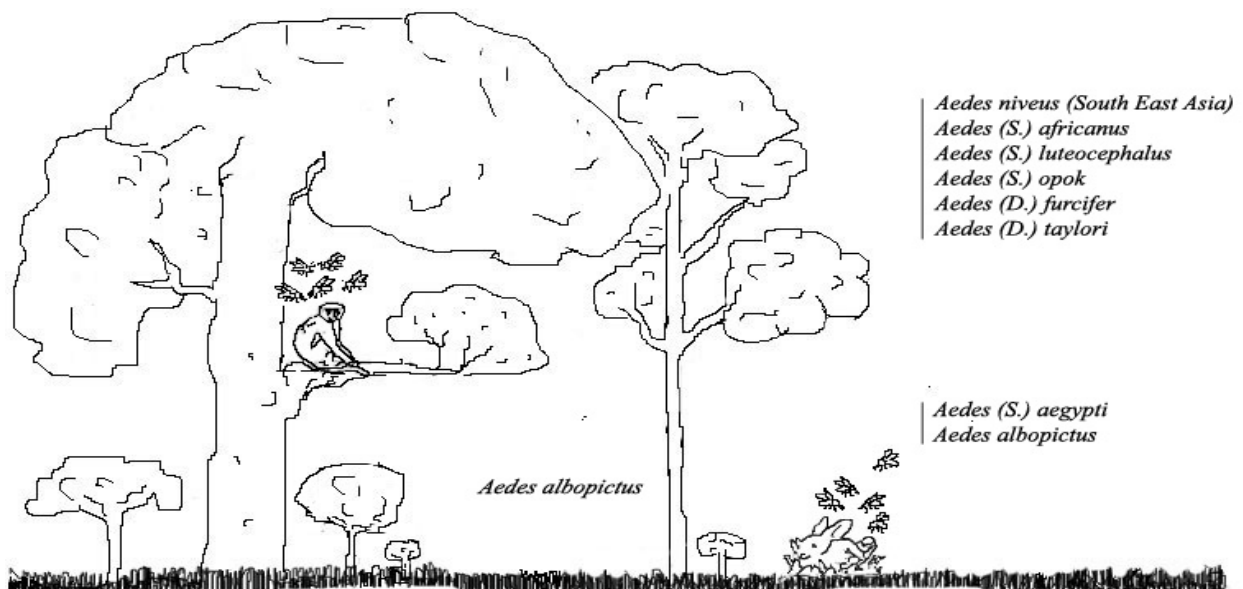


Fig. 2. Zoonotic cycle of dengue viruses circulating between *Aedes* mosquitoes and monkeys in the jungle. *A. aegypti* is adapted to live near to the ground, while *A. albopictus* tends to be catholic, and the other vectors are canopy residents.

In Malaysia, blood samples were taken from over 2,300 domestic (in urban areas) and wild (in the forest) animals belonging to 55 species and 28 genera to detect anti-dengue antibodies. In addition, 25,000 adult mosquitoes were tested for dengue viruses but all were negative despite high levels of antibodies in the majority of wild monkeys leading to the first evidence of dengue zoonoses (Rudnick, 1965). However, these zoonoses could

become epidemics if the viruses are spread by vectors at high rates in monkey populations as in Sri Lanka where the exposure rate of dengue epizootic, based on antibody detection in asymptomatic monkeys, had been reported to be up to 94% in an area of 3 km² (Peiris, et al. 1993; de Silva, et al. 1999). Humans could become tangentially infected when invading this dengue *nidus*, permitting the transfer of arboviruses from zoonoses to anthroozoonoses.

In theory, dengue hemorrhagic fever (DHF) occurred after the dissemination and co-adaptation of the dengue viruses in the vector (reservoir) and in the human (host). The first DHF cases were reported in Manila, Asia, in 1954 (Quinlos, et al. 1954), and in 1981 in Cuba, America (Guzman, et al. 1995). Thus, according to Pavlovsky, the benign form of dengue fever is associated with those from the original *nidus*. The circulating serotypes in the urban areas are thought to be the same as those in enzootic cycles, though they differ genetically due to independent evolution (Cordellier, et al. 1983; Roche, et al. 1983). Five, six, four, and two genotypes of the serotypes 1, 2, 3, and 4, respectively have been defined, hence, dengue serotypes contains a total of 17 dengue genotypes; two (belonging to serotype 2 and 4) out of which are circulating in the *nidus* amongst monkey populations in the forest (Holmes and Twiddy, 2003).

According to Pavlovsky principle, the older a serotype is that is circulating in a particular area the less pathogenic it will tend to be to humans. It has been documented that *A. aegypti* and *A. albopictus* are less susceptible to a wild genotype strain than to an epidemics genotype of the serotype 2 (Moncayo, et al. 2004). Similarly, the American genotype (AM) of serotype 2, the oldest strain thought to cause the first dengue fever epidemics in the Americas, has been, in appearance, supplanted by the Southeast Asia (SA) genotype, a more pathogenic strain to humans that is efficiently transmitted by *A. aegypti* mosquitoes (Armstrong and Rico-Hesse, 2003). In addition, an emerging genotype of the serotype 3 had evolved in Sri Lanka which is more pathogenic to humans and transmissible by *A. aegypti* mosquitoes. This apparently has displaced the native genotype which is less pathogenic to humans, and there is some evidence that *A. aegypti* is less competent at acquiring and transmitting the native strain (Lambrechts, et al. 2009). Thus, the incidence of DHF cases seems to be normal during dengue fever outbreaks because of the serotype and genotype diversity circulating which leads to multiple infections in the human populations.

4.3 Co-evolution between viruses and *Aedes* mosquitoes

Although the terms vector and transmitter are used interchangeably in the literature, the term vector involves a more intimate evolutionary co-existence among blood-sucking arthropods and the pathogens they transmit; this has led to the concept of biological transmission, which is a basic concept in medical entomology. Extended co-existence allows adjustment in the arthropod bionomics to acquire more tolerance (vector competence) towards a virulent pathogen. This gradual adaptation of the viruses to the vectors could lead to a successful evasion of their immune defenses resulting in minimal damage. Consequently, a pathogen that has achieved such adaptation to survive optimally in the vector populations could be successfully transmitted to healthy individuals from an exposed host population. This phenomenon may be observed in the case of vectors-viruses interactions.

The dengue virus was isolated by first time at the end of World War II by Dr. Susumu Hotta's group (Kimura and Hotta, 1944). They found the viruses in blood samples taken from Japanese soldiers. Two decades later, in Singapore the presence of dengue viruses in field-collected *A. aegypti* and *A. albopictus* mosquitoes was shown (Rudnick and Chan, 1965). *A. aegypti* females are easily infected with the serotype 2 which is transmitted successfully; conversely, this is not the case when using the other three serotypes. If mosquitoes were fed with blood contaminated with similar viral titres, the amount of viruses which could reach salivary glands of those infected females would be higher with serotype 2, than that of those mosquitoes fed with other serotypes. In addition, the viruses will infect the salivary glands of mosquitoes in a shorter period of time (a shorter extrinsic incubation period) than that when using the other dengue serotypes. This phenomenon has been documented for the SA genotype of the serotype 2 which showed a higher replication rate in both the vector and definitive hosts than that of the AM genotype in America (Cologna, et al. 2005).

4.4 Evolution of vector synanthropism

Vectors for dengue fever including the primary *A. aegypti* and secondary *A. albopictus* mosquitoes are species whose origin was in a forest habitat; this could be also alike for other vector species associated with dengue zoonoses in Africa and Asia (Figure 1). The wild vectors in the forest prefer tree heights and foliage-canopy, depositing eggs in breeding sites of rainwater accumulated in hollow trees and in the axils of epiphytic plants such as the family Bromeliaceae. The forest habitat is a permanent shaded site; hence, these mosquitoes exhibit negative phototaxis during visual flight orientation and show specific preferences for resting or moving towards dark sites. This innate behavior facilitated their adaptation to survive in shacks or huts of the primitive man. Today it is common for people in underdeveloped tropical areas around the world to store drinking water in containers of clay or other material that remain in or close to their houses. These containers are dark and relatively cold, and serve as the perfect replacement of the typical larval breeding site, as mentioned, that was in a hollow tree in the forest. These artificial containers would represent the transition between the forest and urban habitats.

A. albopictus feeds less on humans than *A. aegypti* which is highly anthropophilic. *A. aegypti* shows evident endophilic and endophagic behaviour. It appears that females tended to stay indoors because there was availability of blood from people and oviposition sites in a form of artificial containers, which allows the survival of successive generations within the same household. When more large human settlements appeared, the vector adapted to standing water in flowerpots, buckets, old tires, etc. which are abundant in the exterior and interior of houses in villages and modern urban areas. A feature of the *Aedes* lifecycle that lent itself to the utilization of these small and artificial larval habitats is that the eggs can survive desiccation. This allowed them to utilize small habitats that potentially dry out.

Aedes mosquitoes are active during the day, and as such they cannot be controlled using insecticide-treated bednets in the same way the malaria vectors can be controlled. And yet it is becoming increasingly important that these mosquitoes are effectively controlled. Dengue is reported to be the most rapidly spreading mosquito-borne disease in the world (World Health Organisation, 2009). Recent estimates are that 50 million dengue infections occur each year, with 2.5 billion people at risk of infection in dengue endemic countries. Dengue distribution is spread across the tropics but also reaches sub-tropical areas too. Given the

high mutation rate of dengue viruses it is difficult to develop a dengue vaccine. Nevertheless, vaccine development is ongoing but dengue is rapidly becoming a public health problem in the Americas, Africa and Asia, and at present the only feasible way to control it is by controlling the *Aedes* mosquito vector (World Health Organisation, 2009).

There are many ways in which *Aedes* mosquitoes can be controlled. But as with other mosquito vectors, *Aedes* mosquitoes are becoming resistant to the insecticides used, and in any case, people are becoming more sensitive to environmental pollution. This is especially true because a major source of mosquito larvae are people's drinking water storage jars. Thus in the rest of this chapter we outline the different types of biological control available to use against *Aedes* mosquitoes, and we will explain how these biological tools are playing a role in IVM programmes.

5. Biological control tools

5.1 Single celled organisms

Aedes aegypti is host of entomopathogenic microorganisms but historically just a few species have been reported and isolated from the dengue vector as natural host (Hembree, 1979). There is a lot of information about entomopathogens evaluated against *A. aegypti* but most are at experimental level. We think that the spore-forming bacteria *Bacillus sphaericus* (*Bs*) and *Bacillus thuringiensis israelensis* (*Bti*) are regarded as the most promising microbial control agent against the dengue vector.

As soon as *Bs* and *Bti* appeared, they demonstrated their usefulness as control tools particularly when the dengue vector began to show signs of resistance to chemical insecticides (Sun, et al. 1980). *Bs* was discovered in 1964 (Kellen and Meyers, 1964) while *Bti* was isolated in 1977 (Goldberg and Margalit, 1977). Both are highly effective not only at killing larvae of Culicidae and Simuliidae (Federici, 1995) but also at killing adults of *Aedes*, *Culex* and *Anopheles* mosquitoes (Klowden and Bulla Jr., 1984). However *Bs* is more selective than *Bti* because it is specially toxic to *Culex* and *Anopheles* larvae, and tolerant to high levels of organic pollution (Regis, et al. 2000); however some mosquitoes already are resistant to *Bs* (Rodcharoen and Mulla, 1994). *Bti* acts when its spore-crystal containing toxic proteins (protoxins) is ingested by larvae. Then the pro-toxins are solubilized in the alkaline pH of the gut and activated into toxins which cause a detergent-like rearrangement of lipids in the epithelial membrane, leading to its disruption and cytolysis (Gill, et al. 1992). The mode of action of *Bs* is similar but less known. Since their isolation, both bacteria have been intensively investigated and virtually thousands of papers have been published. Most papers have been focused on enhancing the toxicity of the proteins associated to the crystals, and currently hundreds of bio-formulates have been produced biotechnologically. We will mention as an example the Programme for Eradication of *Aedes aegypti*, launched in 1997 in Brazil to fight dengue fever transmission. Although today still in Brazil the use of biological agents to control mosquitoes has been restricted to experimental and operational research, they have discovered new technologies to improve the efficacy of these bacteria. For example, the *Bti* tablet experimental formulation C4P1-T, shows good persistence, killing more than 70% of *A. aegypti* larvae within 40 days after treatment of tanks in shade, and 25 days in tanks exposed to sunlight. In addition, the *Bs* formulations showed up to 100 days persistence against *Culex quinquefasciatus* larvae after the third application in shaded tanks,

as did a *Bti* formulation, Inpalbac, after the 4th treatment. Tested under identical conditions, some of the experimental formulations revealed performances almost or equally as good as the best commercial products tested, VectoBac and VectoLex (Regis, et al. 2000).

For the rest of entomopathogens there are only evaluations at laboratory or semi-field conditions, for instance, some iridoviruses have been explored in relation to their sublethal effects on *A. aegypti* (Marina, et al. 2003), the protozoan *Ascogregarina culicis* has been explored against larvae in cemeteries (Vezzani and Albicocco, 2009), and the microsporidian *Edhazardia aedis* examined at semi-natural and laboratory conditions (Becnel and Johnson, 2000; Barnard, et al. 2007). In conclusion, the only promising entomopathogen in this group is *Bti* especially those new formulates with better efficacy than the traditional formulates. However, *Bti* does not always persist for a long time under field conditions.

5.2 Fungi (Ascomycetes: Hypocreales)

Entomopathogenic Ascomycetes could be a promising biological control tool. The conidia of these fungi, once germinated, directly penetrate the adult mosquito cuticle then produce a blend of organic compounds, causing internal mechanical damage, nutrient depletion and death (Gillespie and Clayton, 1989). These fungi have been successfully used under field conditions to kill malaria vectors (Scholte, et al. 2005), and to modify wild mosquito blood feeding behaviour (Howard, et al. 2010). While a wide range of these fungi have been used in experiments with dengue vectors (Scholte, et al. 2004), there are two main species that are currently being used by many laboratories worldwide: *Metarhizium anisopliae* and *Beauveria bassiana*.

Most work has been carried out against adult mosquitoes. Scholte, et al. (2007) found that *M. anisopliae* caused significant mortality to *A. albopictus*, and found high levels of infection. Studies have showed that *A. aegypti* had significantly increased mortality after exposure to *M. anisopliae* (Scholte, et al. 2007; de Paula, et al. 2008, Reyes-Villanueva, et al. 2011) and *B. bassiana* (de Paula, et al. 2008, Garcia-Munguia, et al. 2011). Worryingly, susceptibility to fungal infection is significantly reduced following a blood meal, but after digestion fungal susceptibility returned to pre-feeding levels (de Paula, et al. 2011a). Also wild strains may be less susceptible to fungal infection than some colony strains used (de Paula, et al. 2011b). Nevertheless fungal virulence can be increased by the co-exposure to an insecticide (de Paula et al. 2011a).

In addition to direct effects on mortality, an interesting pre-lethal effect has been seen. Recently our group has shown that fungal infections can affect fecundity of female *A. aegypti*. When infected with *M. anisopliae*, fecundity was reduced by up to 99% (Reyes-Villanueva, et al. 2011) and after infection with *B. bassiana*, fecundity was reduced by 95% (Garcia-Munguia, et al. 2011). Whilst no field trial data has been published using entomopathogenic fungi against dengue vectors, field trials are reported to be underway (de Paula, et al. 2011a) and hopefully the results will soon be known.

Many different methods have been put forward for the deployment of entomopathogenic fungi, these include black cotton sheets attached the ceilings (Scholte, et al. 2005), direct application onto mud walls (Mnyone, et al. 2010) and treated window covers (Howard, et al. 2010). Our team in Mexico have shown that both *M. anisopliae* (Reyes-Villanueva, et al. 2011)

and *B. bassiana* (Garcia-Munguia, et al. 2011) can be transferred from infected *A. aegypti* males to females during mating, and this could be utilised in field applications in the future.

Although much of the recent work has focussed on the adults, fungi can be used to kill *A. aegypti* larvae as well. For mosquito larvae the fungal conidia are either ingested through the mouth or enter the siphon. Here they can cause a physical blockage by vegetative growth of the fungi, and the release of midgut toxins causes death. *M. anisopliae* is effective at killing *A. aegypti* larvae (Ramoska, et al. 1981) but there are conflicting reports with one study stating that *B. bassiana* is pathogenic (Miranpuri and Khachatourians, 1990), while other studies found that it is not (Clark, et al. 1968; Geetha and Balaraman, 1999). Work targeting *A. aegypti* eggs has also been carried out. Both *M. anisopliae* and *B. bassiana* have proved to be ovicidal (Luz, et al. 2007), but high levels of humidity were required (Luz, et al. 2008). Further work using *M. anisopliae* found that oil-based formulations can enhance the ovicidal effect (Albernaz, et al. 2009).

Questions still remain about fungal longevity and viability under tropical conditions. In Tanzania, *M. anisopliae* in suspension did not lose viability whereas when the fungus was impregnated onto black cotton cloths and exposed to the ambient heat and humidity, the viability had reduced to 63% three weeks after application (Scholte, et al. 2005). Similarly, in Benin, *B. bassiana* conidia in suspension did not lose viability, but after 20 days exposure to field conditions viability of *B. bassiana* on polyester netting was reduced to 30% (Howard, et al. 2011b). This inability of entomopathogenic fungi to withstand tropical temperatures has also been found in several laboratory studies (Rangel, et al. 2005, Lekimme, et al. 2008, Darbro and Thomas, 2009) and could pose an obstacle for the deployment of these entomopathogenic fungi for dengue vector control. Work focussing on the formulation needs to be carried out to ensure that this promising control tool can withstand the tropical climates in which it is likely to be used.

5.3 Invertebrates

5.3.1 *Toxorhynchites*

Toxorhynchites is the largest genus (52 species out of 90) of four in the subfamily Culicinae of Culicidae (Diptera) (Harbach, 2011). *Toxorhynchites* mosquitoes are diurnal and carnivorous in its larval stages but not haematophagous as adults (Steffan and Evenhuis, 1981). Larvae are generalist predators and their range of prey is so wide that they exhibit a strong cannibalism with the biggest larvae easily consuming the small ones of their own species. All these species have a precocious vitelogenesis since the pupal stage (Watts and Smith, 1978), which allows them to oviposit a short time after mating. This is because they are inhabitants of tropical and subtropical forests where the availability of temporary breeding sites for oviposition is unpredictable. *Toxorhynchites* usually lay eggs in rock depressions and tree holes, axils of bromeliads, cut bamboo canes, and so on, where they find live mosquito larvae as prey (Clark-Gil and Darsie, 1983). For species living in suburban or urban habitats egg-laying occurs in man-made water containers, such as discarded tires, buckets, cans, and graveyard flowerpots, where there are larvae of domestic mosquitoes such as *Culex spp.* and *A. aegypti* and *A. albopictus* (Rubio and Ayesta, 1984).

Both sexes of the Neotropical *Toxorhynchites theobaldi* (currently *T. moctezuma*) stay close to the breeding sites from where they emerged waiting for females to mate. Most females mate

during the first ten days in the laboratory, kept in a cage without a cup with water, but after introducing the cup into the cage they oviposit within the following four days. Eggs are white or yellowish and hydrophobic floating individually on the water surface (Rodriguez and Reyes-Villanueva, 1992). The pattern of oviposition of all species have a bimodal activity with two peaks; the lower in the morning and the higher one in the afternoon (Arredondo-Bernal and Reyes-Villanueva, 1989; Bonnet and Hu, 1951). The preferences for oviposition in flowerpots in a Mexican cemetery were described by Reyes-Villanueva, et al. (1987) for *T. theobaldi* (today known as *T. moctezuma*). They examined 584 containers and found 1,009 eggs in 204 flowerpots (35%). Most eggs (66%) were found in shady flower containers, which indicate the preference of females to stay and oviposit in the shady microhabitats.

There are few field studies evaluating the impact of *Toxorhynchites* adults released in areas with man-made containers harboring *A. aegypti* larvae. One 4th instar of *T. brevipalpis* at 22-25°C living in tires consumes around 12 *A. aegypti* larvae during 24 hours, while in the laboratory they eat on average 16 prey larvae at 26°C (Trpis, 1972). In a survey in Africa nine tires and nine tins were sampled weekly between April 1969 and March 1970 registering the number of *T. brevipalpis* and *A. aegypti* larvae per container. So, to estimate the larval population of both predator and prey per hectare which was the surface of the tire dump, an extrapolation was done based on the mean number of larvae per container and the percent of containers with water in the dumping (Trpis, 1973). By this way, he was able to obtain the numerical response of *T. brevipalpis* to *A. aegypti*, existing a lag time of a month between both, and with the predator population always following the prey one.

The same author estimated the absolute population of *T. brevipalpis*. Two hundred wild specimens of both sexes of *T. brevipalpis* were collected by hand-nets from a 1-ha tire dump at Dar es Salaam, Tanzania; each specimen was marked after anaesthetization by applying a spot of enamel paint to the front of the mesonotum. However, only 195 marked mosquitoes (140 males and 55 females) were released in the middle of the habitat. Then, 24 h after the release a new capture was carried out. From the number of mosquitoes marked and released (M) and the number marked (m) in the total recapture sample (T), the size of the population (N) was estimated according to the formula $N=MT/m$, which is the Lincoln index. Of the 337 mosquitoes in the second collection, 19 (15 male and 4 female) had been marked. The size of the *T. brevipalpis* population of the 1-ha habitat was therefore estimated as 3,459 mosquitoes. The author also calculated the *A. aegypti* population in the area and this was of 570 females, which was around 33% compared to the predator population (Trpis, 1973).

In New Orleans, USA, *T. amboinensis* was examined by Focks, et al. (1985). They did 29 weekly releases of 6-8 day old females; releases comprised 100, 200 and 300 females per block of a neighborhood formed by 16 blocks (4x4 area) during March-July 1982. The *A. aegypti* population was monitored by using two ovitraps per block. The response variables measured weekly were three: the average number of *A. aegypti* eggs per ovitrap, number of exuviae of *Aedes spp.* and *Culex spp.* per container in treated and control blocks, and the proportion of containers in treated area positive for predators. There was a reduction of 45% in the *A. aegypti* population compared to the one of control blocks after the release of 100 *T. amboinensis* females, while no significant increase in control was achieved at 200 and 300 females. Likewise, the *C. quinquefasciatus* population also was diminished by around 40% with 100 females; while ovitraps placed around the experimental areas demonstrated that the females released had little dispersion between blocks. This study showed the potential of

T. amboinensis as biocontrol agent used in inundative releases at urban habitats infested with the dengue vector.

T. moctezuma oviposition rate was examined at Northeast Mexico by Alvarado-Castro and Reyes-Villanueva (1995). They performed six releases of 20, 40 and 80 inseminated, 10-day old females in the center of a pecan orchard *Carya illinoensis*, with a discarded tire filled with 3 liters of water, and fastened at the trunk of each tree. Ten trees with tires comprised the experimental area arranged in two lines of five trees each, predators were released at the center, and the egg number per tire of the predator were counted daily for 17 days after each release. Daily means were 48.23 and 35.88 eggs for both 20-female releases, 95.65 and 65.12 eggs, and 242.94 and 108.12 eggs for both 40- and both 80-female releases, respectively. There was a linear trend well defined only for the releases of 20 and 40 females with a decrease rate of oviposition of 0.25 per day, and most eggs (56-66%) were laid during the first four days after release.

Although in the above experiment there were no larvae of *A. aegypti* in tires, the high numbers of eggs deposited daily by the released females of *T. moctezuma*, suggest this predator is promising as biocontrol agent against the dengue vector larval populations. Females are able to locate larval breeding sites of *A. aegypti* and oviposit on them, but a strong limiting factor is the expensive production of adults of this predator. At least for the experiment of Alvarado-Castro and Reyes-Villanueva (1995) to develop and produce 100 pupae of the predator required the use of around 10,000 larvae of *A. aegypti*.

5.3.2 Copepods

The most successful type of invertebrate used for mosquito larva control is the cyclopoid copepods, most notably *Mesocyclops*. These are 1-2 mm long crustaceans that are one of the most numerous multicellular organisms on earth that can be found in many geographical locations, and therefore the use of copepods for mosquito control does not require exotic introductions. Because of their size, copepods mainly kill the first instar larvae, and they prefer *Aedes* larvae over *Anopheles* and *Culex* larvae. Copepods can live for 1-2 months, are quite hardy and they self-replicate readily. Because they eat a variety of aquatic prey, they can maintain populations in water storage containers even if mosquitoes are not found (Marten and Reid, 2007). They can also be easily moved from one container to other container habitats. Therefore they offer the potential of sustainable mosquito control. Furthermore, copepods can be easily and cheaply mass produced and transported, even under field conditions where they are required. Nam, et al. (2000) used a method using plastic garbage bins in which thousands of copepods could be produced in just 3 weeks. They then transported these copepods to the various field locations using hollowed out polystyrene blocks that they were able to send using the Vietnam postal service. No seriously adverse environmental effects have been reported from the use of copepods.

The major success story for the use of *Mesocyclops* against dengue vectors comes from Vietnam. A study carried out in northern Vietnam using *Mesocyclops* as the primary control measure was able to reduce *A. aegypti* levels to 0-0.3% of baseline estimates and *A. albopictus* to 0-14.1% of baseline levels (Kay, et al. 2002). This project was then expanded into 3 provinces in central Vietnam, with similar findings. The authors report that *Aedes* mosquitoes were eliminated from several study communes and several years into the

programme no dengue was detectable in the three treated rural communes (covering a population of 27,167), but dengue transmission was still evident in the control areas (Nam, et al. 2005). Following country-wide programme expansion it was reported that *A. aegypti* had been eradicated from 32 of 37 communes, covering a human population of 309,730. Dengue has not been reported in the treated areas for years, where the authors estimate that 386,544 people have been protected, but dengue transmission remained in the untreated areas, (Kay and Nam, 2005). *Mesocyclops* use is also proving to be sustainable; 7 years after official involvement ceased, *Mesocyclops* are still being used by community members to keep *Aedes* populations at bay and local transmission of dengue has been eliminated in areas where they are being used (Kay, et al. 2010).

It is not just in Asia that copepods have been successful in field trials. A field trial in Mexico used copepods in water tanks, tires and vases to control *A. aegypti*. It was found that the most effective control was in the cemetery vases, with 67.5% reduction over the 3 month study period (Gorrochotegui-Escalante, et al. 1998). Copepods have also been used to successfully control *A. aegypti* populations in Argentina (Marti, et al. 2004) and *A. albopictus* populations in Japan were effectively controlled by *Mesocyclops* and *Macrocyclus* copepods (Dieng, et al. 2002).

A disadvantage of *Mesocyclops* is that they are the intermediate hosts for the Guinea worm *Dracunculus medinensis*. This is a helminth human parasite that infects people when they ingest infected *Mesocyclops* in drinking water. Therefore, *Mesocyclops* cannot be used to control dengue vectors in areas where Guinea worm transmission still takes place. There is a global Guinea worm eradication programme that has made great progress, however, Sudan, Ethiopia, Ghana and Mali all remain endemic for Guinea worm. Chad is the only other country that reported cases in 2010. Another disadvantage is that as with some other biological control options, *Mesocyclops* are susceptible to insecticides like Temephos (Kaul, et al. 1990), but they are unaffected by *Bti*. In addition they are sensitive to chlorine in the water (Marten and Reid, 2007). Nevertheless, despite these disadvantages *Mesocyclops* have been sustainably used to almost eradicate dengue from areas of Vietnam (Kay, et al. 2010), and along with fish (see below) are probably the best biological control tool of dengue vector mosquitoes that is currently available for operational use.

5.4 Fish

Another biological control method that has been used to control mosquitoes is the deployment of fish that will eat the mosquito larvae and pupae. Many different types of fish are used, but to avoid damaging ecosystems the World Health Organisation (WHO) advocates the use of native larvivorous fish (World Health Organisation, 2002).

Fish can be incredibly effective at reducing *Aedes* mosquito numbers under field conditions. In Mexico, the mean container index (CI) (percentage of water-holding containers infested with *Aedes* larvae or pupae) in cement tanks was around 87% before indigenous fish species were introduced, and mosquito numbers were recorded for a year. The results show that each of the 5 fish species eliminated mosquito breeding in the tanks, while the CI in the control remained at 86% (Martinez-Ibarra, et al. 2002). Similarly, the Chinese cat fish *Clarias fuscus* reduced the Breteau Index (BI) (the number of positive containers per 100 houses) from 50 (before fish introduction) to 0 just 15 days after fish introduction (Neng, et al. 1987).

In Northeastern Brazil, before the deployment of *Betta splendens* fish, 70.4% of the tanks were infested with *A. aegypti*, one year later the infestation rate was just 7.4%, dropping to 0.2% 11 months later (Pamplona, et al. 2004). Furthermore, a study in Thailand found that in rural areas 43.7% of containers without fish had *A. aegypti* larvae, compared to just 7.0% of containers that had fish; this effect was also seen in an urban area (40.6% vs 8.3%) (Phuanukoonnon, et al. 2005). This study in Thailand compared a range of control methods and found that keeping fish was the most effective (Phuanukoonnon, et al. 2005).

Larvivoracious fish have further advantages. Unlike some of the invertebrate predators, people feel familiar with fish (Martinez-Ibarra, et al. 2002), and this means that they are able to apply this control tool themselves. This happened in Brazil where the successful use of *B. splendens* fish was broadcast in the media, resulting in the people placing these fish in their water storage containers of their own accord (Lima, et al. 2010). In addition, the success of a trial in Mexico was attributed in part to the adoption of the larvivoracious fish as pets by the local children (Martinez-Ibarra, et al. 2002). As well as being pets, some fish can be farmed and eaten by local communities (Howard, et al. 2007). Several indigenous Mexican species used to control *A. aegypti* can be eaten (Martinez-Ibarra, et al. 2002) and the Chinese cat fish *C. fuscus* is not only edible but also highly larvivoracious and tolerant of harsh environmental conditions (Neng, et al. 1987). Keeping fish can also be more cost-effective than other control methods like insecticide spraying (Neng, et al. 1987) and larvicide application (Seng, et al. 2008). Furthermore, fish have not only been found to be more cost-effective and long lasting than *Bti*, but they were also found to be much more effective as a control method (Lima, et al. 2010). Further advantages of larvivoracious fish are that they are self-sustaining, so in general water bodies only have to be treated once, or at least less frequently than for other control tools. This can lead to sustainable mosquito control. In addition, fish survival does not depend on the presence of mosquito larvae whereas other biological control agents often depend on the mosquito population not being entirely eliminated (Wright, et al. 1972). Fish are effective at controlling the older larval stages of *Aedes*, something that is not readily achieved by the copepod predators (Russell, et al. 2001). Also, unlike for chemical larvicides, mosquito larvae cannot build up a physiological resistance to fish.

As with all mosquito control tools, there are some disadvantages of using fish. Larvivoracious fish can only be used in certain water bodies conducive to their survival (Lima, et al. 2010), and they will only thrive and reproduce under certain conditions that can be specific to the different fish species. In addition, not all containers that allow *Aedes* breeding are suitable for fish. Fish obviously cannot be used in habitats that are prone to drying out. They are also not well suited to the smaller containers where the water may become too hot during the day, and where oxygen levels may not be high enough. The ability to withstand chlorine can be an important characteristic because in many countries chlorine is added to the drinking water, and that is then stored in large tanks by householders. A study comparing the chlorine tolerance of two larvivoracious fish, *B. splendens* and *Poecilia reticulata*, found that *P. reticulata* was unable to withstand chlorine concentrations within the limits for human consumption in Brazil (Cavalcanti, et al. 2009). Not always but, somehow there are reluctance of certain individuals to use fish in tanks because their presence stinks drinking water.

Under laboratory conditions, *B. splendens* repelled *A. aegypti* females from laying eggs in the water where the fish were, but *P. reticulata* (Pamplona, et al. 2009) and *Gambusia affinis* (Van Dam and Walton, 2008) did not. This repellency can be a problem because these fish cannot be

very effective at controlling successive generations, especially when untreated oviposition sites are available (although in an integrated approach, those sites could be removed or treated with another control tool). Fish can also have an effect on non-target organisms. A study comparing *P. reticulata* with a native Australian fish found that the Australian fish outperformed in terms of the larvivorous potential, but this fish species also ate native tadpoles, and as such should only be utilised in water containers where the tadpoles would not be found (Russell, et al. 2001). Some fish can also reduce their larval intake in the presence of commercial fish food (Ekanayake, et al. 2007). Whilst the use of fish has proved popular in certain trials, and shows great promise for sustainable control of dengue vectors, the implementation of larvivorous fish should be accompanied by adequate participatory education to make it more acceptable for communities, and therefore potentially more sustainable.

5.5 Plants

As entomopathogenic fungi seem promising for adult control, plants could be a promising biological control tool for aquatic stage mosquitoes. Plants produce compounds to protect themselves from insects, and these compounds can effect insect development in many ways. Hundreds of plant species have been tested for their effects against mosquitoes (Shaalan, et al. 2005) with a recent review published by Fallatah and Khater (2010). Much of the research against *A. aegypti* mosquitoes has focussed on by-products of plants already utilised for economic gain, or on already recognised medicinal plants. In the former bracket, avocado seed extracts were found to be able to kill *A. aegypti* larvae (Leite, et al. 2009). Similarly, unripe black pepper extracts were found to be effective at killing pyrethroid-resistant *A. aegypti* (Simas, et al. 2007). Ethanolic extracts also fall into this category, since ethanol is a by-product from sugar cane refinement (Wandscheer, et al. 2004). In the latter bracket, 14 Mexican medicinal plants were tested and a range of toxicity was found, with some being highly toxic and others showing very little larvicidal effect (Reyes-Villanueva, et al. 2008). The neem tree (*Azadirachta indica*) is a well known medicinal plant that has been widely tested against mosquitoes (Howard, et al. 2009; Fallatah and Khater, 2010). When tested against dengue vectors neem was found to be effective at relatively low doses (Wandscheer, et al. 2004) but oviposition was inhibited (Coria, et al. 2008). It is important that oviposition is not affected, because if mosquitoes do not expose their progeny to the neem then control cannot be sustainable (Howard, et al. 2011a).

Plants have not yet been used to control dengue vectors in field trials, and are not currently under consideration for inclusion into IVM trials, but many laboratory trials have been conducted with a view to identifying promising candidates. However, as well as testing whether plant extracts can kill mosquitoes, it is important that the effect on non-target organisms is evaluated. These could be native aquatic fauna, other biological control tools, or mammals that have access to the water into which the botanical larvicides are to be placed. A recent laboratory study tested the bioefficacy of two plants against *A. aegypti* mosquitoes and the larvivorous fish *P. reticulata* (Patil, et al. 2011). Both plants were found to be highly effective as larvicide but *Plumbago zeylanica* was found to have a slight toxic effect against the fish, although the authors concluded that these plant species could be used alongside this larvivorous fish in IVM programmes (Patil, et al. 2011). Sodium anacardate from cashew nut shell liquid was evaluated against *A. aegypti* eggs, larvae and pupae and found to be highly toxic to all life stages, although the dose required to kill the pupae was

much higher than that needed to kill larvae (Farias, et al. 2009). This is not uncommon for botanical products (Howard, et al. 2009). The authors also tested the effect against mice. They used a dose much higher than the dose required to kill the mosquitoes, and found that even at 0.3 g/kg there was no apparent damage to the mice. They concluded that this botanical mosquitocidal compound was safe for mammals (Farias, et al. 2009).

There are several advantages that plants offer. Plants could be used in water sources that are too small to house larvivorous fish or that have a tendency to dry up completely for long periods of time. These small habitats are more prone to fluctuating temperatures, and evidence has shown that some plants can be effective at a range of temperatures, with increasing toxicity at the higher temperatures (Wandscheer, et al. 2004; Patil, et al. 2011). In addition, many plants are widely available where they are required, and they can be grown by rural communities which could provide sustainable and relatively cheap mosquito control. Plants are biodegradable, relatively safe for the environment and communities are familiar with many of the plants that have proven insecticidal. As with the other biological control tools discussed, they can be used to manage insecticide-resistant mosquito populations.

Despite these advantages, there are several reasons why plants are not being used in IVM programmes. Most plants toxic to larvae of *A. aegypti* are wild species, and therefore not cultivated. In addition to the fact that they are not available in practical amounts, phytochemicals can display heat and UV instability which can reduce the applied dose to levels that are no longer effective. Some plant parts are more effective than others; for example root infusions of *Solanum nigrescens* were toxic but leaf infusions were not (Reyes-Villanueva, et al. 2008), and variation can occur between the same plant products produced in different geographical areas (Schmutterer, 1995). Further disadvantages of using botanical products to control *A. aegypti* include the pronounced taste of some of the plants. For example the use of black pepper may not be acceptable in drinking water (Simas, et al. 2007). Whilst aqueous extracts are normally less effective than organic chemical extracts (Simas, et al. 2007), they could be more applicable for use by rural communities. Thus effectiveness in the laboratory may not immediately translate to field success, especially when community-based control tools are required. Work should be carried out looking at the most ubiquitous and larvicidal plants with a view to community deployment in future IVM trials. In addition, work should continue towards the commercialisation of botanical products for dengue vector control.

6. Integrated vector management

Integrated vector management (IVM) is a comprehensive strategy which aims to achieve a maximum impact on vector borne diseases like dengue. IVM was adopted by the WHO in 2004 (World Health Organisation, 2004) as a strategy to improve the cost-effectiveness, efficacy, ecological soundness and sustainability of vector control. The emphasis of IVM is on examining and analyzing the local situation, making decisions at decentralized levels, and utilising the appropriate mosquito control tools (World Health Organisation, 2009). One of the features of IVM is the use of a range of interventions, often in combination and simultaneously, that work together to reduce dengue transmission.

For dengue control, there are three main categories of intervention. These are biological, as described in detail above, the use of chemicals to kill the adult and immature mosquito stages, and the physical removal, periodic cleaning or covering of container habitats. These

categories can be used to target all life stages of the *Aedes* mosquito, as shown in (Table 1). The use of education is also an important component, because communities need to know how and why to control dengue vectors. Community-participation in these methods is not only crucial for sustainability (Wang, et al. 2000), but also leads to more effective *Aedes* control, as shown in an IVM trial in Guantanamo, Cuba (Valerberghe, et al. 2009).

There is no silver bullet for dengue vector control, and each of the intervention categories has their disadvantages. Biological tools are not always feasible in certain small container habitats. Chemicals can pollute the environment, be expensive, and insecticide resistance has developed (World Health Organisation, 2009), and not all water storage containers can be removed/cleaned/covered. One of the benefits of IVM is that it overcomes the disadvantages of using individual methods, and a combination of mosquito control tools can be more effective than any tool used in isolation. Authors of a study in Taiwan concluded that integrated pest control was the best and most effective method for dengue control (Chen, et al. 1994). A study in Thailand that looked at the effectiveness of individual methods also concluded that a combination of the control methods increased effectiveness (Phuanukoonnon, et al. 2005). Furthermore, a systematic review and meta analysis of 56 publications detailing the results from field studies found IVM to be the most effective method of reducing entomological indices like the BI and CI (Erlanger, et al. 2008).

Life stage	Intervention	Measure	Sample reference
Egg	Chemical	Insecticide-impregnated ovitraps	(Perich, et al. 2003)
	Physical	Autocidal ovitraps Removing, cleaning of containers	(Cheng, et al. 1982) (Chen, et al. 1994)
Larvae and pupae	Biological	Fish	(Martinez-Ibarra, et al. 2002)
		<i>Mesocyclops</i>	(Nam, et al. 2005)
		<i>Bacillus thuringiensis israelensis</i> (Bti)	(Lima, et al. 2010)
	Chemical	Spinosad	(Darriet, et al. 2010)
		Temephos	(Phuanukoonnon, et al. 2005)
		Pyriproxyfen	(Darriet, et al. 2010)
Physical	Removing, cleaning of containers	(Chen, et al. 1994)	
Adults	Biological	Entomopathogenic fungi	(Reyes-Villanueva, et al. 2011)
	Chemical	ULV fogging	(Osaka, et al. 1999)
		Aerosol cans	(Osaka, et al. 1999)
		Repellents	(Jahn, et al. 2010)
		Lethal ovitraps	(Kittayapong, et al. 2008)
	Physical	House modification	(Vanlerberghe, et al. 2011)
		Sticky ovitraps	(Ordonez-Gonzalez, et al. 2001)

Table 1. Some methods that can be used in IVM programmes to control dengue vector mosquitoes, for a full list of possible control tools see World Health Organisation (2009).

Operational large-scale IVM programmes are already being carried out in a range of countries. IVM has been carried out in Singapore since the mid 1970s, in China since the

early 1980s (Neng, et al. 1987), and Taiwan since the late 1980s (Chen, et al. 1994, Wang, et al. 2000). A successful regional IVM campaign focussed on the use of predacious copepods was expanded to a national campaign in Vietnam in the mid 1990s (Nam, et al. 2000). Dengue control programmes in Brazil (Lima, et al. 2010) and Thailand (Phuanukoonnon, et al. 2005) are centred around community participation, health education, larval control (including biological control), chemical control of adult mosquitoes and physically removing/covering containers. In Cuba routine *Aedes* control comprises physically removing container habitats and chemical control of adult and larval mosquitoes, backed up by health education (Vanlerberghe, et al. 2009). Not only have these IVM control programmes been carried out for many years in some countries, but the notion of enforcement has been adopted in a few countries. A study from China describes how fines were handed out for non-compliance, with incentives given to those households adequately maintaining the dengue control methods (Neng, et al. 1987). Specific laws aimed at ensuring that householders carry out dengue control measures have been in effect since 1968 in Singapore, and 1988 in Taiwan (Chen, et al. 1994). As in these other countries, mosquito control legislation is enforced by handing out fines in Cuba (Vanlerberghe, et al. 2009).

Not only do IVM programmes show that *Aedes* mosquitoes can be successfully controlled, but more importantly, IVM can be effective at reducing dengue disease burden. An IVM trial that was targeted at high-transmission areas in Thailand used a combination of biological larval control, chemical adult control and physically preventing oviposition. Not only did they report a dramatic reduction in the number of *Aedes* positive containers, but there was also a significant reduction in adult *Aedes* mosquitoes. Crucially, there were no dengue cases reported in the treated area, whilst in the control there were 322.2 cases per 100,000 people; baseline data was similar for the two areas at around 230 cases per 100,000 (Kittayapong, et al. 2008). Similarly, a programme utilising all the major categories of intervention was carried out in Taiwan between 1987 and 1993 (Chen, et al. 1994). The authors reported that in 1988, there were 10,420 dengue cases however, between 1990 and 1993, no dengue cases were reported (Chen, et al. 1994). In a later report from Liu-Chiu island (off the coast of Taiwan) IVM was able to nearly eradicate *A. aegypti* mosquitoes and there were no dengue cases reported by the end of the study, even though mosquito habitats were still present (Wang, et al. 2000).

7. Potential of biological control methods in the future

At present, there is no vaccine for dengue, and vector control remains the cornerstone of any dengue control effort. The future of dengue vector control must involve IVM programmes, ideally with a combination of governmental top-down and community-based bottom-up approaches. Attention must be paid to the WHO guidelines on dengue control (World Health Organisation, 2009), as well as to new research that may also be effective. Ultimately, sustainable mosquito control requires behavioural change at both individual and community levels so that the number of larval habitats is reduced and remains low. Because the main dengue vector has a preference for breeding in domestic water containers, the potential of the community to sustainably control mosquito populations is probably higher than for malaria programmes, where the malaria vector breeds in natural habitats that are not always easy to find.

The WHO says that IVM should be composed of an integration of non-chemical (biological) and chemical vector control methods. Furthermore, they say “productive larval habitats

should be treated with chemicals only if environmental management [physical] methods and other non-chemical [biological] methods cannot be easily applied or are too costly” (World Health Organisation, 2009). Thus, biological larval control tools appear to be given more emphasis than chemical tools. Perhaps because of this, there has been a shift towards using more biological control methods, with chemical control trials becoming less frequent (Erlanger, et al. 2008). A review of 21 studies comparing biological, chemical and educational dengue prevention programs found that biological interventions were the most effective; nearly all the biological interventions eliminated mosquito larval populations, whereas the chemical interventions were judged to be the least effective, and were not thought to offer a long-term solution (Ballenger-Browning and Elder, 2009). A separate review of 56 field studies found that the relative effectiveness of biological control was better than chemical or environmental/physical control measures, but that an integrated approach was best (Erlanger, et al. 2008).

This switch from chemical to biological control tools is in part due to raising insecticide resistance in mosquito populations. Another reason is that chemical control tools are usually associated with top-down campaigns, where the government was solely in charge of implementing mosquito control, like insecticide spraying. In these cases the insecticide and equipment used was rarely available to the communities themselves. Top-down campaigns usually relied on the mass-production of one product that was easy to store. Recently there has been a shift to more bottom-up campaigns because it has been recognised that these are more likely to be cost-effective and sustainable. The same characteristic that makes biological control unattractive from a commercial point of view (namely the difficulty in making money from organisms that cannot be mass produced, stored and shipped from cost-effective industrial plants) is especially appealing to the resource-poor community members affected by dengue, because many of the biological tools can be produced on a small-scale without the need for expensive and complicated infrastructure.

Utilising indigenous biological control tools is appropriate in under-resourced countries because biological control tools are *in situ* in many areas where they will be required. In addition, they can be easy to reproduce under field conditions. An important point is whether the control tool can be produced in large enough quantities to be used in control programmes. For example a simple and effective way of increasing and transporting copepod populations has been devised in Vietnam using polystyrene blocks (Nam, et al. 2000). Fish can be farmed where needed and locally-produced *Bti* was used in a trial in Vietnam where dengue transmission was successfully suppressed (Kittayapong et al. 2008). However, there are some tools whose biological characteristics do not lend themselves to intentional deployment such as the corixid bug *Micronecta quadristrigata*. Attempts to culture this invertebrate predator in the laboratory were unsuccessful because it readily flew from one container to the other (Nam, et al. 2000). In addition, some fish are not easily transported (Russell, et al. 2001). By their nature biological control tools are natural, living organisms and as such there are certain considerations to be made before deciding which should and can be used in certain settings. Their ability to survive in the intended control area is of course important, and for this reason some water bodies are more suited to invertebrate predators, and some to the vertebrate ones.

Biological control has an advantage over physical control due to the “egg trap effect”. In essence, if you remove containers then the reduction in mosquitoes is generally proportional, because there will still be some that *Aedes* mosquitoes can lay eggs in, and

from which they can emerge. But with biological control, mosquitoes that emerge from untreated containers waste most of their eggs on containers treated with the biological agent and this can cause a population collapse (Marten and Reid, 2007). This can be of particular importance in terms of dengue control because unlike for malaria, dengue can be transmitted vertically from infected adult mosquitoes to their eggs, and adult mosquitoes can emerge from water bodies already infected with and infectious for dengue.

The main risks with biological control are the safety of the biological control tool to non-target organisms, and the consequence of permanent establishment of the tool into areas where it may not naturally be found (Various, 1995). For this reason, WHO says that only native organisms should be used (World Health Organisation, 2002), and many native *Mesocyclops* species and fish types exist that can be used. Formulated and registered biopesticides such as *Bs* and *Bti* are being produced that could overcome the risks of classical biological control. These biopesticides are usually mass-produced and could complement the use of classical biological control tools in IVM programmes.

Being able to produce control tools where they are needed can lead to more cost-effective and sustainable control. Crucially, local production and trading of biological control tools could lead to an increase in the socio-economic status of communities. Control programmes incorporating biological control tools that lead to successful mosquito suppression, along with an increase in the socioeconomic status of the community, not only have the potential to be more sustainable than some top-down insecticide-based control programmes but they can also lead to an increased sense of understanding, ownership and empowerment among the community. This is important because eventually communities will be charged with monitoring and implementing mosquito control. This process will be made easier if the control tools used are already familiar to the communities and are readily available, like some of the biological control tools discussed above.

The successful future of dengue control lies in engaging, empowering and entrusting affected communities with mosquito control in their environment using many methods in an IVM approach. For this to occur, cheap, readily accessible and effective mosquito control tools need to be researched and developed. Biological control tools certainly have the potential to fulfil these criteria.

8. Conclusions

Traditionally, IVM control programmes have been based on two components: chemical control (temephos as larvicide and organophosphates and pyrethroids as adulticides applied by ultra-low volume space spraying), and the community contribution to remove the water in artificial containers. However, dengue is associated to the lowest socio-economical strata of the endemic (developing) countries worldwide where the community lacks a culture of participation. Therefore, although there are reports of resistance of *A. aegypti* to chemicals, nowadays their application is still a major tool that health agencies have against the vector, whose populations are invading new habitats due to the global warming. Nevertheless, the persistent use of chemicals conveys a high risk for a serious and real trouble of resistance; if their application continues it is not far from the day in which no chemical reduces sufficiently the vector densities to below their transmission threshold.

Despite the vast number of technical reports and scientific papers published yearly about *A. aegypti* biocontrol, most of the natural enemies of the mosquitoes *Aedes* incriminated in

suburban and urban transmission are at experimental level. Based on our review, we think that the efforts of Brazil will produce at short term, good formulates of *Bacillus sphaericus* and *B. thuringiensis* subsp. *israelensis* to control larval populations at accessible costs in developing countries and with no risk of pollution as threat to human and his environment.

So far, there no low-cost production of viruses with practical potential to be used against *A. aegypti* in developing countries; neither are there artificial cultures to produce the protozoans and microsporidians evaluated as parasites of the dengue vector. The *Toxorhynchites* mosquitoes are good larval predators but high numbers of *Aedes* larvae are required to produce sufficient adults that need to be used in inundative releases. Their production although easy is impractical; likewise the strong cannibalism tendency is an obstacle for their mass production. A similar case is the huge complex of plants reported as toxic to larvae or adults. It is difficult to cultivate them to use their crude extracts as bioinsecticides; what proceeds is to carry on research to identify the chemical structure of the active compounds to produce them synthetically and use them as bioinsecticides; but this needs of a lot of time and great investments in biotechnology, which is prohibitive for the economy of endemic countries. Also somehow impractical is the use of larvivorous fishes; most *A. aegypti* populations are produced in small man-made containers as tires, buckets, cans, bottles and so on, located at the backyards of houses. Nevertheless in the tropical Central and South American countries, it is common to have large cement-built deposits to store water in houses; the use of fishes in those structures is effective.

Copepods are the group with the most potential; they are very cheap to yield them as biolarvicides as today they are being produced and used in Vietnam. Actually copepods show a great potential to be used in the IVM control programmes against *A. aegypti* worldwide. Finally another promising group is formed by the fungus Ascomycetes *Metarhizium anisopliae* and *Beauveria bassiana*. They are effective to control immatures and adults, although we think they are more effective as adulticides by indirect exposure of mosquitoes to surfaces impregnated with conidia at doses superior to 10^8 spores ml⁻¹. Fungal dissemination among female populations by releasing conidia-contaminated virgin males of *A. aegypti* deserves further research. These fungi are cheaply produced by using natural substrates like rice, sorghum, etc. in plastic bags in laboratory, to have a low cost production in an IVM control programme for dengue in any developing country.

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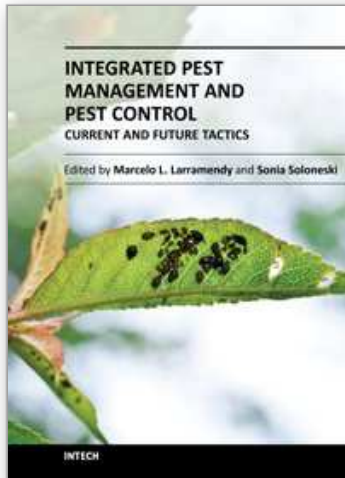
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