Model-Like Implementation of Integrated Control Strategies Targeting *Aedes Albopictus* (Diptera: Culicidae) In Southwest Germany With Special Consideration of the Sterile Insect Technique

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Research Article

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Abstract

Background

In recent years the invasive species *Aedes albopictus*, known as the Asian tiger mosquito, has undergone an extreme expansion by steady introductions as blind passengers in vehicles from the Mediterranean to South-West Germany. Nowadays, more than 15 established populations are known in the State of Baden-Württemberg and Palatine (South-West Germany) which locally constitute a significant nuisance and public health threat. Therefore, the species deserves special attention as vector of several arboviruses like dengue, Chikungunya or Zika virus. As a consequence, immediate surveillance and control activities against *Aedes albopictus* have been implemented in the infested areas under the auspice of health departments and regulatory offices.

Methods

The control strategy comprises 3 columns: a) community participation (CP) based on detailed information for the elimination and sanitation of breeding sites as well as the use of fizzy Bti-tablets containing the pro-toxins of *Bacillus thuringiensis israelensis* (Bti); b) Door-to-Door (DtD) activities by trained staff including the application of Bti (Vectobac WG) at high dosages to achieve a sustained killing effect of the mosquito larvae; and c) the Sterile Insect Technique (SIT) to almost wipe out or eliminate the remaining *Aedes albopictus* population after intensive Bti-applications. In the laboratory and in semi-field tests, the different elements of the control strategy are evaluated and the efficacy of the integrated control strategy is assessed in routine treatments in the cities of Ludwigshafen (Palatine) and Freiburg (Baden-Württemberg) with special emphasis on the release of sterile *Aedes*/males.

Results

Following our information campaigns, more than 60% of the residents were practicing CP focusing on environmental sanitation and the use of fizzy Bti tablets. Although CP is an essential element of the integrated control strategy, it was shown that the strongest asset in our programme was the DtD activity and the application of a water suspension of Vectobac WG (2700 ITU/mg) to all potential breeding sites at dosages of 10g/rainwater container and 2.5g/smaller container which provided an effect for more than a month. The mean time for the inspection and treatment of a property was 27 minutes. As a result of the larval source management, the container index for *Aedes albopictus* achieved no more than 0.5% in Ludwigshafen. The mean number of *Aedes* eggs/ovitrap in Ludwigshafen was 4,3 and in Freiburg - Metzgergrün (SIT area) 18.23 and Freiburg-Gartenstadt-Freiburg (Control area) 22,4 eggs/trap. After the strong reduction of the *Aedes* population by Bti-application, the weekly release of 1013 (Ludwigshafen) and 2320 (Freiburg) sterile *Aedes albopictus* males/ha from May until October resulted in an overall sterility by egg counts 82.61% ((Ø60.52±42.88%) in Ludwigshafen and 62.68±28.21% in Freiburg compared to a natural sterility of 16.93±13.5% in the SIT untreated area. The field results are proven by the data achieved in cage tests in the laboratory. The mating of wild females with sterile males showed sterility rates of 87.53±9.15% whereas the sterility of eggs laid by females mated with unirradiated males

was only 3.3±2.8%. The most effective ratio of wild to sterile males is 1:5. The overall sterility of about 83% in Ludwigshafen indicates that our goal to almost eradicate the *Aedes albopictus* population could be achieved. The hydrogen-peroxide bleaching technique allowed a quick assessment of embryogenesis.

Conclusions

In this study, we clearly prove that an integrated control program based on a strict monitoring scheme is most effective when it comprises three columns, namely a) community participation, b) DtD activities including long-lasting Bti-larviciding of all possible breeding sites to strongly reduce the wild *Aedes albopictus* population as a basis for the successful application of SIT, and c) SIT to almost wipe out the *Aedes albopictus* population. The combination of Bti and SIT guarantees that two of the most selective, save and effective tools are employed against one of the most dangerous mosquito vector species *Aedes albopictus*, the Asian tiger mosquito.

1. Background

Out of more than 3500 mosquito species worldwide, only about 30 have begun to spread far beyond their original geographical borders [1,2]. The most successful invasive species are *Aedes aegypti*, *Ae. albopictus*, *Ae. japonicus, Ae. koreicus*, *Ae. atropalpus* and *Ae. triseriatus.* Of these, *Aedes albopictus* deserves special attention considering that it is a vector of at least 22 arboviruses, including dengue, Chikungunya, Zika and Yellow fever viruses, and its worldwide spread, including the temperate climate zone [3-8].

In the early 1990s, *Ae. albopictus* was passively introduced into Italy, via the international trade of used tires followed by a rapid spread into other areas in Italy [9,10]. Having become established in Italy, *Ae. albopictus* was spread by vehicles and boats along the Mediterranean coast, including France, Spain, Croatia, and other Balkan countries, as well as Greece and Turkey. Today, the species is present in the whole northern and some parts of the southern Mediterranean basin, with an increasing tendency of spreading northwards across the Alps into central European countries. Considering that Italy is a tourist destination for Germans, the risk of the introduction of *Ae. albopictus* into Germany via returning tourists in vehicles from Italy and therefore an increasing public health risk is evident [11-14]. This risk must be considered as serious as the Asian tiger mosquito is already involved in the autochthonous transmission of chikungunya, dengue and Zika viruses in Europe [6,7,8,15-18].

Whereas the intercontinental spread is mainly facilitated by the global transport of goods, especially used tires, the spread of females of *Aedes albopictus* within and between neighbouring countries takes place with them as “blind passengers” in vehicles [2,14].

As a consequence, the German Mosquito Control Association (KABS) started a monitoring program in 2005 from Basel to Heidelberg along the A5 (E35) motorway coming from Italy as a suspected port of entry for *Ae. albopictus* adults. In the frame of the first monitoring program in the period from 2005 to 2009, *Ae. albopictus* eggs were found the first time in an ovitrap at a resting station north of the city Weil am Rhein [13]. In order to assess the risk of the introduction of *Ae. Albopictus*, a collaboration of scientific, traffic and governmental institutions was initiated in 2011 [2,19]. Furthermore, public awareness has been increased by spreading detailed information to the public to enable them to recognise Asian tiger mosquitoes and report them to the KABS in Southwest-Germany or nationwide to the "Mückenatlas" [20]. The highway monitoring revealed that about 40% of all service and resting stations and some campgrounds along the highway A5 between Basel and Hesse were infested by *Ae. Albopictus*, indicating the regular introduction of the Asian tiger mosquito into Germany 2 [2].

The increased public awareness resulted in numerous records of adult *Ae. albopictus* females by alert people who sent emails or pictures of tiger mosquitoes - mainly taken by mobile phones - to the KABS, IfD or the Mückenatlas. The first record of a larger population in the area of the Upper Rhine was reported by a gardener in an allotment garden in Freiburg in September 2014. In the following years, increasing numbers of established populations (established is a population which has overwintered and produced at least three consecutive wild populations) were recorded in the cities of Heidelberg (2016), Sinsheim (2017), Lörrach (2018), Karlsruhe (2018), Frankfurt (2018), Östrich-Winkel (2019), Weinheim (2019), Ludwigshafen am Rhein (2019), Germersheim (2019), Weil am Rhein (2019), Graben-Neudorf (2019), Stuttgart (2019), Offenburg (2019), Bad Bellingen (2019), Bad Neuenburg (2019), Korntal-Münchingen (2020), Grenzach-Wylen (2020), Bad Krotzingen (2020) and Meckesheim (2020) (Fig. 1). Additional campgrounds along highway A5, including in Lörrach, Bad Bellingen or Achern, were infested with *Ae. albopictus* populations [55].

The rapid increase in the recorded Asian tiger mosquito populations can be explained by the effect of climate change with the highest summer temperatures ever recorded in South-West Germany of 42°C in 2019 and 38°C in 2020 (DWD 2019,2020) and the increased awareness of people. Furthermore, in Northern Italy, the Asian tiger mosquito population peaked in July/August (personal communication Alessandro Albieri, CAA), which is the time at which many tourists return from holidays in Mediterranean areas to their home countries.

In all colonised areas, immediate surveillance programs have been initiated to assess the size of the infested area and the abundance of *Ae. albopictus*. Our ultimate goal is to identify newly developing *Ae. albopictus* populations as early as possible and to initiate immediate control activities conducted by KABS/IfD/ICYBAC experts under the auspice and financial support of the community authorities (guided by the health departments and regulatory authorities). Immediate actions are necessary to either avoid the permanent establishment of the Asian tiger mosquito or prevent the nuisance potential and the risk of disease transmission. This can only be achieved when an integrated control program is immediately initiated and the vast majority (aiming 100%) of all breeding sites are treated in the infested areas, or when the remaining *Ae. albopictus* populations are knocked down by applying SIT.

In the Upper Rhine Valley, the KABS established the modern biological control of mosquitoes mainly based on *Bacillus thuringiensis israelensis* (Bti) about four decades ago, targeting floodwater mosquitoes (e.g. *Ae.vexans* and *Ae. sticticus*) and the so-called house mosquito *Culex pipiens* s.l. [23]. Since 2015, control activities in the Upper Rhine Valley have also focused on the control of *Aedes albopictus* populations. Principally, some of the control tools against container-breeding house mosquitoes can also be utilised against *Ae. albopictus*. However, the strategy has had to be modified due to the specific biology and relevance of *Ae. albopictus* for the public. In the frame of a research program financed by the State of Baden-Württemberg, the KABS/IfD evaluated all possible control tools and designed the most appropriate strategy with which to tackle the Asian tiger mosquito populations under local circumstances [24]. Based on these evaluations, the effect of different control approaches was tested in the cities of Ludwigshafen (Palatine) and Freiburg (Baden-Württemberg).

After careful evaluation, the actual control strategy comprises the following three columns: 1) community participation including the distribution of Bti-fizzy tablets, 2) Door to Door (DtD) activities including the treatment of all breeding sites by people trained with Bti in about three weeks intervals, and 3) the integration of the Sterile Insect Technique (SIT) to cover *Ae. albopictus* populations from cryptic and/or non-accessible breeding sites to possibly wipe out the population. The strategy also comprises an intensive surveillance program to identify any hotspots of *Aedes albopictus* populations.

1.1. Community participation (CP)

Community participation (CP) focuses on the increase in public awareness of the need to avoid mosquito breeding and to record the occurrence of *Ae. albopictus* as an “early warning system” conducted as a citizen science approach (so-called passive monitoring). The CP is achieved by detailed information given to the public via press releases, TV air time, flyers, web pages and information events, e.g. at schools, in city halls or meetings of gardener associations. The information events serve as platforms to provide details on morphological characteristics, distribution, and the biology of the Asian tiger mosquito, as well as measures to avoid the further proliferation of the mosquito. This includes traditional tools like the elimination of breeding sites and environmental sanitation, e.g. by the use of totally fitting lids and the treatment with Bti-fizzy-tablets (Culinex Tab plus). Bti has specific advantages for CP because it is safe for humans and the environment [25-28]. The tablets are distributed free of charge in city halls or areas that are heavily infested by DtD activities including the distribution of flyers with detailed information about the biology of *Ae. albopictus* and the use of Bti-tablets. With the distribution of Bti-fizzy-tablets “help for self-help” is provided to the public in situations where the active control of breeding sites by DtD activities conducted by special expert teams cannot be afforded. Additionally, in mass-infested areas, mosquito nets are distributed free of charge to thoroughly cover rainwater containers and prevent female mosquitoes from entering the container via uncovered slits or holes. The involvement of the public should also lead to people recording suspected mosquitoes to achieve area-wide passive monitoring.

1.2. Door-to-Door (DtD) activities and Bti-treatments

The most powerful tool with which to eradicate or strongly reduce *Ae. albopictus* populations in a certain area are DtD activities, including long-lasting Bti-treatments by trained staff. It has been shown that CP alone is not enough to reach the goal of strongly reducing or even eliminating the Asian tiger mosquito due to the ignorance of some people or insufficient treatments of almost all breeding sites. Bti offers special advantages next to its safety for humans and the environment; formulations such as water dispersible granules (WGs) can be easily mixed with water and applied with knapsack sprayers or pressurised hand sprayers. They cause a quick knock-down effect and can produce a long-term effect when an appropriate dosage is used.

1.3. The Sterile Insect Technique (SIT)

Our final goal is the elimination or at least significant reduction of *Ae. albopictus* populations by an integrated control strategy which should also include traditional genetic control using ▯-radiated sterile males. The biological features of *Ae. albopictus* are ideal for employing SIT, as the species is easy to mass rear, has a limited flight range, does not reproduce in enormous masses within a very short period like floodwater mosquitoes, and because breeding sites are usually defined, as a container-breeding species, and can more or less easily be controlled by larvicidal use to reduce the wild *Aedes* population [29].

Therefore, the SIT method is an excellent tool with which to overcome a general problem occurring by DtD activities, namely that not all properties can be inspected or that cryptic breeding sites are difficult to find.

A prerequisite for the successful application of SIT is a strong reduction of the natural *Ae. albopictus* population by all traditional tools, e.g. the mass application of Bti to a level with as few “wild” individuals left as possible before SIT is applied. After a significant reduction of the wild population, a sufficient number of sterile males has to be released over an appropriate time period so that they outcompete the wild males in terms of mating. As a consequence, the wild females inseminated by the sterile males will lay sterile eggs [30].

The goal of our pilot program is to assess the effect of various control techniques (the three column approach) in our integrated control strategy against *Ae. albopictus* in the laboratory, semi-field tests and routine field applications. A comprehensive surveillance program employing ovitraps serves as a tool with which to monitor the various effects. A cost-analysis should serve as a guide for the further planning of community-based activities.

2. Methods

2.1 Study areas

The study was conducted in two *Ae. albopictus* infested areas in Southern Germany: a) The “Melm” district in the city of Ludwigshafen (Palatine) and b) the “Metzgergrün” district in the City of Freiburg (Baden-Württemberg). The “Gartenstadt” district in Freiburg served as a control area to the SIT area “Metzgergrün”.

a) **Melm** is a new district of Ludwigshafen which was built at the end of 1990 and in the early 21st The total size is about 65 hectares and it is embedded in a green area with lakes for recreation. It consists of about 950 estates with single family and double houses, as well as some apartment buildings. Almost every house possesses an adjacent garden area which can provide potential breeding sites for *Ae. albopictus*. The area is well defined and well suited as a test area due to the complaints of the residents about nuisances caused by Asian tiger mosquitoes.

The study area of about 65 ha was divided in three sectors of almost equal size, each with specific mosquito control actions: Sector A: 23 ha (CP+DtD); Sector B (CP+DtD+SIT): 17 ha; and Sector C: 25 ha (CP+DtD) (Fig. 2).

b) **Metzgergrün** in Freiburg. The district has a size of 4.5 ha and is mainly characterised by apartment buildings with social housing along 4 streets (Fig. 3). This district was chosen to employ the SIT technique in addition to CP and DtD Bti treatments because this residential area includes a large portion of gardens adjacent to apartments/residential houses which include a vast number of breeding sites like used tires and garbage. The garden plots were usually locked and the owners were unknown. Frequently, the inhabitants were not cooperative, and it was difficult to enter the gardens to treat the breeding sites on a three-weekly interval from July until October 2020 as was done in the rest of the *albopictus* infested area in Freiburg.

c) **Gartenstadt** (Freiburg): This district of Freiburg is about 1 kilometre away from the SIT-area (Metzgergrün) and similarly infested by *albopictus* as Metzgergrün, therefore, this area was chosen as a control area where CP and DtD were deployed as well as ovitraps to assess the sterility of an SIT untreated area in comparison to Metzgergrün.

2.2 Assessment of the efficacy of the "three column strategy" including Community participation, DtD activities with Bti treatments and SIT

2.2.1 Community Participation

In the first week of May 2020, before the program started, citizens in Melm were informed via local media about the planned control activities against Tiger mosquitoes. The goal was to turn the residents from spectators to actors in the fight against *Ae. albopictus*. Between the 9th and 12th of May, three people distributed flyers to 1820 households with detailed information for the help of residents to control the Asian tiger mosquito on their properties. Each household received a flyer to which a paper box containing 10 Bti-tablets in a blister pack (Culinex® Tab plus, Lot: 0604783, activity: 1000 ITU/mg, Culinex GmbH, Ludwigshafen) including a package insert with instructions for application was attached by organic glue. The Bti-tablets were sterilised and contained no spores or living bacteria [44]. They had a weight of 550 mg/tablet and one tablet was enough to treat 50 l of water [14]. It was recommended that the treatment be repeated on a fortnightly interval.

Additionally, people were informed about the characteristics of *Ae. albopictus* and asked to record suspicious biting mosquitoes by killing them or making them immobile with a slight touch during biting. People were asked to send the mosquitoes to our laboratory or take a picture with a mobile phone showing the hind legs, head, or thorax for species determination. During the DtD activities, the number of accessed and non-accessed properties as well as the presence of container-breeding mosquitoes were recorded to determine the scope of CP. Furthermore, the residents of the estates were interviewed about their knowledge of control activities. During the last control round (no. 5) in September, the residents were asked if they had implemented the proposed control activities on their property by applying the Bti-tablets or environmental sanitation.

2.2.2. Assessment of the effect of Bti-treatments

Whereas the control of floodwater mosquitoes requires only a minimum effective dosage of Vectobac WG (strain AM5265, Valent Biosciences, Libertyville, USA), to achieve a knock-down effect of the floodwater mosquito larvae, which hatch during a flood [56], the control of container-breeding mosquitoes needs a long-term effect due to the usually constant follow-up of generations. Thus, the sequence of retreatments and the costs for manpower could be strongly reduced when a long-term effect is achieved.

In our routine control program against the third and early fourth instars of floodwater mosquitoes, we applied 500 g Vectobac WG (potency when sterilized 2,700 ITU/mg) which resulted in a dosage of 0.5 mg/l (1,350 ITUs/l) calculated to a depth of 10 cm of the breeding site. For the control of container-breeding mosquitoes, alongside the 0.5 mg/l concentration, we tested 10x (5 mg/l) and 100x (50mg/l) higher dosages to achieve the desired long-term effect.

2.2.2.1. Efficacy of Bti-treatment in large water containers (rainwater containers)

In a first series of tests, the effect of Bti-treatments in rainwater containers was simulated. The Bti mixture was prepared with sterilised Vectobac WG (Valent BioSciences, Libertyville, USA, lot: 294458PG00, manufactured Oct. 2018). Here, 250 grams of Vectobac WG was thoroughly mixed with 1.5 l of tap water (pH: 7.8; conductivity: 680 µS). Aliquots of the suspension were taken with an Eppendorf pipette by constant stirring of the suspension and applied to plastic buckets filled with 20 litres of tap water (1/10 of the usual water volume of a regular rainwater container) to achieve the desired dosage of 1 gr (6 ml of Vectobac WG solution), 0.1 gr (0.6 ml Vectobac WG solution), and 0.01gr (0.06 ml of Vectobac WG solution) per 20 l of water. Each dosage was tested in four replicates at a constant temperature of 24°C±1°C, with 4 buckets serving as a control. At the beginning of the test series, 20 third instar larvae from a laboratory-reared *Ae. albopictus* colony were introduced in each bucket. The mortality reading was taken at 24 and 48h after the release of the larvae. On a weekly basis, 5 l of water was taken from each container and replaced again with fresh tap water to achieve a total water volume of 20 l and simulate natural conditions when water is removed for watering the garden. Then, 20 third instar larvae were again released in each container and the mortality reading was performed after 24 and 48 h. At each 48h reading, the larval cadavers and living larvae were counted and removed. The test was conducted until the mortality rate was less than 60%.

2.2.2.2. Efficacy of Bti-treatment in small water containers

In a second series of tests, the efficacy of Vectobac WG in four different small water containers typically found in garden areas was assessed: a) terracotta flowerpots with rough surfaces (volume: 1400 ml); b) terracotta pots – with smooth walls (volume: 1400 ml); c) plastic flowerpots (volume: 950 ml); d) zinc pots (volume: 800ml); and e) terracotta flowerpot saucers (volume: 200 ml). Before application, the containers were scrubbed with flower soil (Compo Sana potting soil) and cleaned with water and dried for 24 h to simulate natural conditions before the application of Vectobac WG (lot: 294458PG00, manufactured Oct. 2018). Four of each type of container were homogenously sprayed by means of a Mesto pressurised sprayer (Mesto BUGSI 1.5L), with 15 ml of the Vectobac WG stock solution (250 g Vectobac WG mixed with 1.5 L of tap water) resulting in 2.5 g Vectobac WG/small container. The containers were dried for 48 hours, filled with tap water, and then 20 *Ae. albopictus* third instar larvae were added to each container. The mortality reading was performed 48 h after the release of the larvae. The containers were emptied after the mortality reading and dried again for 48 hours to fill the containers again with water and release new batches of larvae. The procedure was repeated until the mortality rate was less than 60%. Four containers of each type served as an untreated control. The test was conducted at 24°C±1.5°C and 80% RH.

2.2.2.3. Routine Bti-Treatments of the infested areas

Melm in Ludwigshafen: The first records of biting *Ae. albopictus* females were obtained in early August 2019. After species identification was confirmed, the health department of the county and the city authorities were informed. Immediately afterwards, the occurrence of a reproducing *Aedes* population was proven on the following days via the inspection of breeding sites along 4 streets equally distributed in the Melm district. Scattered across the district, several breeding sites (unused flowerpots, saucers, rainwater barrels) were proven to be positive for the developmental stages of *Ae. albopictus*. In total, 55 breeding sites were inspected with six positives for *Ae. albopictus* (CI: 10.9%). Thus, in addition to the complaints of residents in 2019, the existence of a reproducing widespread population was documented. In the middle of August 2019, representatives of the health department, city authorities and specialists from KABS/IfD met to discuss further actions. In the first step, the residents were informed via media, and a website was created as a platform to record more specimens reported by the residents. In the last week of August 2019, all households received flyers and Bti tablets for self-help. After the distribution of flyers, several citizens sent emails and reported a severe nuisance caused by *Ae. albopictus* in their garden area. Therefore, an action plan was designed in cooperation with authorities for 2020, which should serve as a proof of concept for a successful integrated control strategy. The plan includes:

1. Press releases and information of the public by local media in close cooperation with the local authorities (first half of May, 2020).
2. Training of field staff (6 people) in early May. The training course includes detailed information on: a) the biology and taxonomy of mosquitoes to be able to answer the questions of residents and to distinguish between *albopictus* and other container-breeding mosquitoes such as *Culex pipiens*

s.l./*Cx. torrentium*, *Culiseta annulata*, *Cs. longiareolata* or *Ae. japonicus*; b) the breeding habitats of *Ae. albopictus* and how to identify and record the breeding sites in a database; c) the characteristics and appropriate management of the biological larvicide Bti; d) the handling of mosquito traps; and e) how to approach the residents, especially under the consideration of Covid-19.

3. The creation and distribution of flyers with detailed information and Bti-tablets for self-help (11th and 12th of May, 2020, 1820 households, 2 people, each 8 working hours).
4. Deploying 30 ovitraps (≈1 trap/2ha) more or less equally distributed in the district and inspected on bi-weekly intervals. Eggs were counted and checked for embryogenesis according to the description above (Fig. 2).
5. DtD activities from May to September 2020 in five different rounds to make the action effective and cost-efficient.

a. 1st round from 18th of May to 7th of June carried out by one team of two people: visiting all properties to carefully map and treat all potential breeding sites. Recording the occurrence of breeding sites, mosquito larvae and species in a Q-GIS-database is the basis for the decision-making process of the following control steps focusing on hotspots of breeding sites and the number of eggs in the ovitraps. The control of hotspot areas based on an efficient surveillance system contributes to a cost-effective reduction of the *Aedes*

b. 2nd round from 23rd of June to 7th of July carried out by one team of two people: hotspot treatment for all properties where breeding sites of *albopictus* were recorded in the first round.

c. 3rd round from the 28th to 31st of July carried out by one team of two people: hotspot treatments of breeding sites in properties over a 100 m radius of the 6 ovitraps (2A, 4A, 5A, 1B, 8B, 9B) in which eggs of *Aedes albopictus* were found in ovitrap collections at 29th of May, 15th of June, 29th of June and 13th of July.

d. 4th round from the 17th to 21st of August (one team): similar to the 2rd round, inspections and treatments of properties with rainwater containers and small breeding sites.

e. 5th round: Similar to the first round. All houses were inspected, and breeding sites were controlled for larvae and treated. Larvae were collected and determined by species in the laboratory. Additionally, a questionnaire was prepared and those residents who agreed to participate were asked about their own contribution to combat tiger mosquitoes and their view of the success of the ongoing control activities (three teams each of two people (14th to 22nd of September).

Metzgergrün in Freiburg: From June to October 2020, more or less in a three-weekly interval, accessible gardens were inspected and treated with Vectobac WG in 4 rounds as was performed over the rest of the infested area in the City of Freiburg.

2.2.3. Application of the Sterile Insect Technique (SIT) against *Aedes albopictus*

One of the major goals of the study was the assessment of the effectiveness and efficiency of the SIT approach against *Ae. albopictus* under routine conditions, including two different transport systems in a routine delivery practice, the quality control of the released male populations and the effect on the sterility of the eggs laid by the wild population.

In this cooperative program, *Ae. albopictus* derived from our German *Aedes albopictus* population in Heidelberg was mass-reared at the Centro Agricoltura Ambiente "G. Nicoli" (CAA) in Crevalcore, Italy, and irradiated in the nearby hospital in Ferrara. Several hundred eggs were collected in ovitraps at the Heidelberg population in 2017 and sent to CAA to establish a stock colony. This approach was adopted to prevent the introduction of mosquitoes with different genetic backgrounds from the local one.

2.2.3.1. Mass production of *Aedes albopictus* at CAA

An effective mass-rearing procedure is essential to ensure the large-scale production of quality sterile males over a long period [31,32,33]. The rearing process should not lead to the artificial selection of genetically homogeneous individuals whose competitiveness with wild individuals is significantly reduced due to the loss of fitness. Therefore, with the support of the Insect Pest Control Laboratory (FAO-IAEA) in Seibersdorf, Austria, mass-rearing technologies have been developed and employed in our program.

Following authorisation obtained from the local governments (Regierungspräsidien) in Neustadt (State of Palatine) and Freiburg (State of Baden-Württemberg), the mass production of *Ae. albopictus* was started at the CAA facility from eggs collected in Heidelberg. Mass production was conducted by applying procedures as previously published without using any animal as a blood supply [32-35].

2.2.3.2. Sex sorting

Sex sorting was performed by the Fay-Morlan glass sorters on the pupal stage in water [50]. This technique takes into account that the male pupae are significantly smaller that the female pupae. CAA aims for no more than about 1% of females per released batch of sterile males.

2.2.3.3. Sterilisation by pupal irradiation

Sterilisation was conducted at the Medical Physics Department of St. Anna Hospital (Ferrara, Italy) by an IBL 437 irradiator (CIS Bio International, Bagnols-sur-Cèze, France) with a Cs-137 linear gamma ray source, exposing male pupae aged 24-32 h in water [30]. After dose-response studies, the pupae were routinely radiated at a dose of 35 Gray (1.85 Gy/min for 19 minutes). In previous tests it was shown that this dosage provides the best results concerning the male sterility and competitiveness of sterile males versus fertile, wild males [30,36].

After irradiation, the pupae were kept in a climatic room at 28 ±1°C for adult male emergence, after which adults were chilled at 8-10°C and packaged for delivery to the field site by DHL flight express service. Time from exiting the production facility to the release areas was always below 24 hrs.

2.2.3.4. Quality control of the sterile *Aedes albopictus* males

Random samples of thirty sterile males were withdrawn from three different batches (SIT-Batches: 7, 8, 9; see Tab. 1) and released each in a Bug Dorm rearing cage (BioQuip, CA, USA). Virgin *Ae. albopictus* females were derived from our colony (Heidelberg strain) by separating individual pupae in small glass vessels covered with a net to allow single specimens to emerge, thus guaranteeing that females are not exposed to males and inseminated before the test. The emerged females in each glass vessel were checked macroscopically by the less plumose antenna, shorter palps and proboscis. In each of the three cages, 30 unirradiated virgin females were introduced to the 30 radiated males and kept at 25±2°C and 70±5% RH (dark: 8 h/light: 16 h). Three cylindrical dark containers (diameter: 7cm; height: 6 cm) were positioned in each cage, half filled with water and holding a wooden board (size: length: 8 cm; width: 3cm) as a substrate for egg-laying. In addition, a vessel with cotton soaked with a 10% sugar solution, 10 raisins and a piece of apple was offered as a carbohydrate source. Three similar cages with the same number of unirradiated males and females derived from the laboratory colony were kept in parallel under the same conditions as the control. After 24, 48, 72, 96, 120 and 144 hours, the lower arm of the PI was offered for 20 minutes in each cage to allow the females to take a blood meal until *ad libitum*. The number of biting females per offering session was recorded. The females were kept for another six days in the cages for oviposition. The wooden boards were marked and removed and kept for five days in chambers with wet cotton (>90% humidity) to allow for complete embryogenesis. Then, the wooden boards were transferred into a hatching container (size: 22x7x4.5cm) and flooded with tap water. The hatched larvae were counted after 24 hours and removed from the container. Then, containers with wooden boards were filled with a 10% hydrogen-peroxide solution and kept for 48 hours at a temperature of 25±2°C to bleach the exochorion of the eggs [52]. The single boards were removed and all eggs (including the egg shells of the hatched larvae) were counted using a binocular (Motic, SMZ-171, Germany); the embryogenesis of each single egg was assessed. Due to the resulting transparency of the exochorion, in eggs with fully developed embryos, the eyes of the embryo and the “hatching tooth” could be easily recognised as dark spots on the head capsule at the anterior part of the embryo. Eggs showing no embryonic structures were addressed as “sterile”. The sterility was also tested by disrupting or bursting the egg-shell with a needle to identify the segmentation of an existing embryo or non-segmented whitish egg masses. Non-embryonated egg-shells burst easily when touched with the needle.

2.2.3.5. Effect of the ratio between sterile and fertile males in cage experiments

A sufficient reduction of an established wild *Ae. albopictus* population is essential for the successful employment of the SIT technique. In the mating process, the sterile males have to compete with the wild males, which are supposed to be more vitally performing than the radiated males; even the dose of radiation is known to damage the sperm but not the somatic cells [30]. In this experiment, the effect of different ratios of wild males versus sterile males was tested. The test design was the same as in the previous test series. In bug dorm cages, the following ratios of wild to sterile males together with 30 unirradiated females were evaluated: a) 1:1 (15 wild and 15 sterile males/cage); b) 1:5 (5 wild and 25 sterile males/cage); c) 1:10 (3 wild and 30 sterile males/per cage). In each cage, 30 unirradiated females were released after the release of males. In each cage, three cylindrical dark containers (diameter: 7cm; height: 6cm) half-filled with water and holding a wooden board as substrate for egg-laying, were positioned. In addition, a vessel with cotton soaked with a 10% sugar solution, 10 raisins and a piece of apple was offered as a carbohydrate source. Following the release of the females after 24, 48, 72, 96 120 and 144 hours, the lower arm of the PI was offered for 20 minutes in each cage to allow the females to take a blood meal *ad libitum*. The number of biting females per blood feeding session was recorded. After the last blood meal, the females were kept for another six days in the rearing cages to allow full oviposition. The wooden boards were removed after six days, marked and kept for another five days in chambers with wet cotton (>90% humidity) to allow complete embryogenesis. The rate of embryogenesis was assessed as described in the previous experiment by bleaching and bursting the egg shell to control the segmentation or non-segmentation of the embryo or egg mass.

2.2.3.6. Shipment of sterile *Aedes albopictus* males

The shipment of the sterile males has to be cost-effective and timely, and the stress (mortality rate) of the caged males has to be as low as possible. In the course of our study, we tested two types of small containers for the shipment of radiated males: a) shipment in small round plastic containers (diameter: 5.2cm; height: 4.7cm) covered with a dark plastic lid; or b) shipment in plastic tubes (height: 45 cm; diameter: 8cm) covered with nets and fixed with a rubber band. Each small container holds about 1000 radiated males. The small round plastic containers were packed in an outer styropor box (size: 49x36x36cm, volume 63.5 L) which contained 11 gel-cooling elements (Blu Ice, DRYCE; www.dryce-pharma.com) and two frozen (Green Ice, DRYCE) to keep the temperature in the styropor box at approximately 10°C. The frozen cooling elements were in bubble foil to avoid straight contact with the containers holding the sterile males. The outer styropor box (size: 48x99x39cm: 185.3 L) holding the plastic tubes had to be bigger because of the larger size of the plastic tubes compared to the small round plastic containers. According to the size of the treated area, the styropor box contained up to 30 small plastic containers or plastic tubes, respectively. The styropor boxes were shipped on a weekly basis from May until October by DHL on an overnight service. The cost of each shipment with DHL was recorded.

2.2.3.7. Assessment of the accurate number of the sterile males and females per container and shipment

Out of the 18 shipments from CAA, Italy, one plastic container holding approximately 1,000 radiated males was transferred to a refrigerator and kept for 2 hours at -15°C to kill all mosquitoes. With the aid of a binocular (Motic, SMZ-171, Germany), the number of males and females per container was determined. Thus, the average number of released males could be determined. Furthermore, it was our goal to keep the contamination of the samples with *Aedes* females as low as possible with the appropriate use of the sexing technique [50]. The percentage of females per small container should not exceed 1% in order to not contribute to a nuisance situation caused by released radiated *Aedes* females.

2.2.3.8. Field application of SIT

a) Sector B (CP+DtD+SIT) in Melm, City of Ludwigshafen

During the course of the study, in sector B, in addition to the DtD activities and the application of Bti, the SIT approach was applied. The results were validated in comparison to districts A and C, which served as a control. The styropor boxes containing the sterile males were shipped weekly overnight on Mondays by DHL and arrived in our laboratory on Tuesday at midday. The release took place in the late afternoon at around 7pm at 13 release spots (Fig. 2). Before the release, the containers holding the sterile males were kept at room temperature to acclimatise the males.

Altogether during 18 releases between the 29th of May and the 7th of October, more than 310,000 sterile males amounting to 320 containers were released. The number of containers per release varied between 12 and 30 depending on the availability of radiated males at CAA (Table 1). Considering the size of sector B (SIT area) of 17 ha, a mean number of 1,013 sterile males/ha was released from 29th of May until 7th of October on a weekly basis.

a) Metzgergrün, City of Freiburg

From the 15th of June to 7th of October 2020, 136,000 sterile males were released homogenously over the 4.5 hectares, which amounts to 2,320 sterile males/hectare altogether (Tab. 1; Fig. 3). The higher number of released sterile males was chosen because of the larger wild population of *Ae. albopictus* in the Metzgergrün district compared to the Melm district in Ludwigshafen.

2.2.3.9. Assessment of the efficacy of the implemented control strategy

The surveillance of the *Ae. albopictus* population was based on inspections of the breeding sites including larval sampling as well as the employment of ovitraps.

a) Larval sampling

Breeding sites holding water were inspected for mosquito development stages, either by employing a plankton net in larger water containers or pouring the water through a plankton net when small breeding sites were examined. In larger water containers like rainwater barrels, the net was drawn through the water in a figure of “8” pattern to sample the larvae. By the aid of touch, the mosquito developing stages could be easily identified in the water column.

For transportation to the laboratory, a plastic container with a close-fitting cap was 3/4 filled with water from the site. The containers were carefully marked by recording the date and location of sampling for the determination of container indices. The third and fourth instar larvae were identified to the species level by using the keys in Becker et al. [38]. Earlier larval instars were reared to the 4th instar by a mosquito breeder for species determination.

b) Employing Ovitraps

Ovitraps were the main tool with which to monitor the presence, phenology and abundance of *Ae. albopictus* and to assess the effect of control activities as well as to estimate the population density based on the number of deposited eggs [53]. The ovitraps consist of a dark plastic container with a total volume of 1.5 litres. They were positioned on the ground or hung on shaded places at a maximum height of 1.5 metres. They were filled with hay infusion (3 g hay pellets dissolved in 5 l of tap water) up to a level of 3/4. A wooden board (length: 17 cm; width: 3 cm) was added to support oviposition. The board or wooden stick had a rough and smooth side and was positioned with the rough side facing upwards to provide small depressions on the surface of the board which are ideal for eggs to be deposited in. In order to prevent the potential development of larvae to adults, 10 grains of Vectobac G (activity: 200 ITU/mg, Valent BioSciences, Libertyville, USA) were added to the water. The wooden boards were replaced at a bi-weekly interval and the water in the ovitraps was refilled. The collected wooden boards were clearly marked with a permanent marker at the dry end of the stick to refer to the location of the ovitrap and the date of collection. They were kept in paper foil and stored in a refrigerator until they were checked by means of a binocular (Motic, SMZ-171, Germany) for the presence of eggs.

A skilled person is able to distinguish between eggs of the indigenous species *Ae. geniculatus* and the exotic species *Ae. albopictus, Ae. koreicus* and *Ae. japonicus*. The results have been validated by hatching some of the eggs and rearing the larvae to the fourth instar for morphological determination [37,38].

The sterility of eggs was checked as described above by bleaching the exochorion of the eggs using a 10% hydrogen peroxide for 48 hours and disrupting the egg-shell to prove existing embryos or unsegmented whitish egg masses.

Employment of the ovitraps in the test areas

a) Melm: on 18th of May across the whole area of 65 ha, 30 ovitraps were positioned at almost equal distances at half-shadowed spots on the ground, namely in sector A: 10 traps; B (SIT): 10 traps; and C: 10 traps (Fig. 2). Each of the ovitraps covered about 2 ha of the test area. The wooden boards were collected in bi-weekly intervals from the 1st of June until the 19th of October 2020. The number of eggs and embryogenesis were assessed as described above (Fig. 2).

b) Metzgergrün (Freiburg): on 14th of July, across the area of 4.5 ha, 21 ovitraps were installed as described above. The district was heavily infested with *albopictus* and, therefore, chosen as the SIT test area. Beside the DtD application of Bti (Vectobac WD) in this district, the sterile insect technique was additional applied from the middle of July onwards (Fig. 3).

c) Gartenstadt (Freiburg): this district is similarly infested by *albopictus* area as Metzgergrün; therefore, this area was chosen as a control area without the employment of SIT. Eighteen ovitraps were set up the 15th of August and the wooden boards were collected in a fortnight interval until the 13th of October. The number of eggs and the sterility were assessed as described above.

2.3. Statistical analyses

For statistical analyses, the Student's *t*-test was applied by Microsoft Excel, version 16.45 (21011103).

3. Results

3.1 Community participation

The distribution of Bti-fizzy tablets, which were fixed to flyers, took place on the 11th and 12th of May. During two working days (16 working hours), a total of 1,820 households received the information packet in their mailboxes (less than 1 minute/household). The positive effect of this activity and the active cooperation of the residents is documented by the high rate of accessed houses during the first DtD round at the end of May/early June amounting to 78.38% access; only 9.13% refused entry to their properties, while 12.49% were absent. More than half of the properties (55.42%) did not contain any breeding sites (Tab. 2). During the questionnaire survey in September, 517 estate owners answered the questions as follows: 298 people implemented the proposed control actions given on the flyer (57.64%), while 28 people (5.4%) claimed that the control of the Tiger mosquito is not important for them. Surprisingly, the acceptance of the DtD activity declined during the 5th DtD activity; only 70.36% allowed access (724 out of 1029 property owners) and 9.72% refused entry. The residents claimed that they did not feel comfortable with the frequency of DtD visits and stated that it was annoying.

3.2. DtD activities and Bti-treatments

3.2.1. Assessment of the optimum dosage of Bti by application with pressurised hand sprayers during DtD activities

3.2.1.1. Efficacy of Bti-treatments in large water containers (rainwater barrels)

Out of the three dosages tested, the 1 g Vectobac WG/20 litres (corresponds to 10 Grams Vectobac WG per rainwater container of 200 litres) showed 100% mortality up to the 8th week and 98.75% up to the 12th week, despite the fact that 25% of the water volume was exchanged on a weekly basis to simulate natural conditions in a garden situation. At the 15th week, the mortality dropped below 90% and in the 21st week below 60% (Fig. 4). At a rate of 0.1 g Vectobac WG, the mortality rates were slightly lower than with the previous dosage, namely 92.5% until week 12 and below 90% in the 14th week. The mortality at a dosage of 0.01 g Vectobac WG dropped already after the 3rd week to 91.25%. As expected, the dosage of 1 g/20 l of tap water provided a sufficient mortality of 100-97.5% up to 12 weeks, whereas the mortality at the lowest dosage dropped below 95% after 3 weeks.

3.2.1.2. Efficacy of Bti treatments in small water containers

With the exception of the zinc flower pot, in all other small vessels, the mortality rate reached 100% until the 9th drying-out and refilling process (36 days) when a dosage of 2.5 g Vectobac WG/vessel was applied. In the zinc flower pot, the mortality dropped to below 95% by the 10th week. Overall, the applied dosage of 2.5 g Vectobac WG guaranteed a total killing effect for more than one month (Fig. 5).

The dosage of 250 g Vectobac WG mixed with 1.5 l of tap water was chosen for the routine treatment of breeding sites. The results achieved in the meso-cosmos served as a guideline for Bti applications during the routine treatments. The Mesto pressurised sprayers (Mesto BUGSI 1.5L) were calibrated to release 15 ml of the Vectobac WG solution per second resulting in a level of 2.5 g Vectobac WG. One second of treatment was enough to achieve the desired dose of 2.5 g Vectobac WG/small container. Rainwater barrels with a capacity of 200 litres are treated for 4 seconds to release 60 ml of the suspension and to achieve the desired dosage of 10 g Vectobac WG/rainwater barrel.

3.2.1.3. Efficacy of routine DtD treatments in Melm, City of Ludwigshafen

The routine treatment in Melm was carried out in five DtD treatment rounds (Tab. 2):

Round 1: In the period from 18th of May until 12th of June, two people visited 953 properties, taking 540 working hours. On the visited properties, 747 (78.38%) could be entered, 119 owners (12.49%) were absent and 87 (9.13%) refused entry. Overall, 414 properties (55.42%) were free of any container, 333 properties had containers, including 1497 small containers and 38 rainwater barrels. Only six containers were proven positive for mosquito development stages (five rainwater barrels with *Cx. pipiens* s.l./*Cx. torrentium* developing stages and one earthen jar with larvae of *Ae. albopictus*). The HI was 0.8% and the Container Index CI_{total}: 0.39%; CI_{albo}: 0.07%. The consumption of Vectobac WG was 4 kg.

Round 2: From the 23rd of June until 7th of July, hotspot visits were conducted for 318 properties which contained rainwater barrels, and numerous potential breeding sites were documented in round one. In this round, 261 (82.07%) were entered, 23 (7.23%) owners were absent and 34 (10.69%) refused entry. Overall, 240 properties (91.9%) showed 741 potential breeding sites, including 32 rainwater barrels. None of the breeding sites contained larvae. The Vectobac WG consumption for the prophylactic treatment during 140 working hours was 2 kg.

Round 3: Hotspot treatment was conducted from the 27th to the 31st of July in a circle of 100 m around ovitraps that were positive for *Ae. albopictus* eggs in June and July (positive ovitraps: 2A, 4A, 5A, 1B, 7B). The inspections of 110 properties revealed 340 potential breeding sites including 19 rainwater barrels. In 5 houses, a total of 15 breeding sites were inhabited by mosquito developing stages (HI: 4.55%). Twelve sites were infested by *Cx. pipiens* s.l./*Cx. torrentium* and three (one rainwater barrel and two plastic bags) with developing stages of *Ae. albopictus*). The CI_{total} was 4,41% and CI_{albo} was 0.88%. During 84 working hours, 1 kg of Vectobac WG was consumed.

During this round it was noted that two irrigation bags fixed on trees for watering trees by the constant release of water droplets were infested by *Ae. albopictus* larvae. Hundreds of irrigation bags are refilled with water on a bi-weekly basis. The company commissioned to fill the bags with water mixed 100 g Vectobac WG into a 1000 l tank before refilling the bags. In the following inspections from July onwards, no more mosquito larvae could be found and the application of Vectobac WG did not have a negative impact (clocking) on the watering process.

Round 4: From the 17th to the 21st of August, hotspot visits were conducted for 273 properties with rainwater containers and numerous breeding sites similar to those in round 2. In this round, 193 (70.7%) could be entered, 71 (26%) owners were absent and 9 (3.3%) refused entry. Out of the 193 properties, 147 (76.17%) contained 1044 potential breeding sites (995 small breeding sites and 49 rainwater barrels). However, in 19 houses, a total of 26 breeding sites (17 small breeding sites and 9 rainwater barrels) were infested with larvae (CI_{total}: 2.49%; HI: 9.84%), of which 25 were infested by *Cx. pipiens* s.l./*Cx. torrentium* associated with *Culiseta. longiareolata* and *Anopheles maculipennis* s.l.; only one flower pot had larvae of *Ae. albopictus* (HI_{albo}: 0.52%; CI_{albo}: 0.1%). During 80 working hours, 3 kg of Vectobac WG were consumed.

Round 5: In the final round from the 14th to the 22nd of September, all properties were visited by four teams each of two people, who were assigned to one sector area for final evaluation. In total, 1029 properties were visited, of which 724 (70.36%) owners allowed entry to the houses, 205 (19.92%) were absent and 100 (9.72%) refused entry. From the 724 properties inspected, 517 (71.4%) contained 2668 potential breeding sites (2585 small breeding sites and 83 rainwater barrels). In 58 properties, 82 (3.07%) breeding sites were infested with mosquito larvae (14 rainwater barrels and 68 small water collections). Sixty-nine breeding sites were inhabited by *Cx. pipiens* s.l./*Cx. torrentium* or/and *Cx. hortensis*, *Cs. longiareolata* and *Cs. annulata*, and 13 breeding sites contained larvae of *Ae. albopictus,* either as only *Ae. albopictus* (six breeding sites) or in association with *Cx. pipiens* s.l./*Cx. torrentium* (seven breeding sites) (HI=8.01%). The total CI_{total} is 3.07, however when only the breeding sites with *Ae. albopictus* are considered the CI_{albo} is 0.49%. During 325 working hours, 7.3 kg of Vectobac WG were consumed (see Tab. 2)

3.2.1.4. Efficacy of routine DtD treatments in Metzgergrün, City of Freiburg

The routine treatments in Metzgergrün, City of Freiburg were performed over five rounds of DtD activities and Bti treatments. Starting in the second half of June, the routine treatments were conducted in a three-week rhythm. The Bti application was carried out until all available properties were treated, usually over five consecutive days. After each application round, the accessibility of the area was subjectively evaluated and classified. Compared to other areas in the city of Freiburg, the accessibility of the properties was classified ranging from poor to moderate. Throughout the season, Bti tablet blisters were handed out to residents, including to those whose properties could not be inspected.

3.3. Application of the Sterile Insect Technique (SIT) against *Aedes albopictus*

3.3.1. Laboratory evaluation of the SIT

3.3.1.1. Quality control of the sterile *Aedes albopictus* males

The mean egg sterility of females which were inseminated by sterile *Ae. albopictus* males amounted to 87.53±9.15%, whereas the sterility of eggs derived from unirradiated males and females was only 3.3±2.8% (Tab. 3). According to the Student's *t*-test, all results are significant ($p \leq 0.001$). In the contrary, 96.70±2.8% of the eggs derived from unirradiated individuals were embryonated, while only 12.47±6.49% of the eggs laid by females inseminated by radiated males developed into embryos (Tab. 3).

Tab. 3: Assessment of the sterility and number of eggs laid by females mated with radiated males or with wild males.

3.3.1.2. Assessment of the accurate ratio between sterile and fertile males in cage experiments

The results of this experiment document the importance of an appropriate ratio between wild and radiated males (Fig. 6). The egg sterility of the unirradiated populations amounts to 5.9±6.6%. When the ratio between radiated and un-radiated males is 1:1 (15 radiated and 15 un-radiated males per cage), the sterility is 30.05±13.13%, but when the ratio is 1:5 (five fertile males to 25 sterile males), the sterility is 85.95±3.18%. The ratio 1:10 (3 fertile males to 30 sterile males) results in a sterility of 84.57±4.00%, without differing much from the ratio of 1:5.

3.3.2. Field Application of SIT

3.3.2.1 Delivery and release of *Aedes albopictus* sterile males

A total of 310,000 radiated males were send to Ludwigshafen (Melm) in 18 shipments and a total of 136,000 males were sent in 13 shipments to Freiburg (Metzgergrün) by DHL overnight services (Table 1). All shipments were delivered within 24 hours. The average costs for the shipments of the small plastic containers in the smaller styropor outer box (volume: 63 L) to Ludwigshafen amounted to 158 Euros/shipment and the costs for the shipment of the plastic tubes in the larger outer styropor boxes to Freiburg was 238 Euros/shipment. The average mortality of the radiated males transported in small plastic containers was 7.4% (min.: 0.5%; max: 30.0%). The mortality of the radiated males in the plastic tubes was not assessed regularly but, according to estimations, was less than 5%.

In Table 1, the exact numbers of released sterile males are recorded, which amount to the release of about 1000 sterile males on a weekly basis from the 29th of May to the 7th of October in Ludwigshafen (Melm) and of 2300 sterile males from the 15th of July to the 7th of October in Freiburg (Metzgergrün).

3.3.2.2. Accuracy in sex sorting

The number of radiated *Aedes*-females in the delivered batches slightly exceeded 1%. The number of 1.13±0.64% recorded by CAA is almost identical to the numbers (1.19±0.63%) evaluated by IfD (Tab. 1). These data indicate a release of about 3,700 radiated females in Ludwigshafen (Melm) and 1,620 radiated females in Freiburg (Metzgergrün).

3.4. Assessment of the efficacy of the implemented integrated control strategy

3.4.1. Ludwigshafen

Of the 30 installed ovitraps, 17 exhibited a total of 919 eggs in the time period from 16th of May to the 5th of October 2020 (Table 5). During the study period, no eggs of *Ae. geniculatus* and/or *Ae. japonicus* were found. The mean number *of Ae. albopictus* eggs per trap/two weeks is 4.4 eggs. The highest number per trap was 110 eggs on the 13th of July in trap 2A. The highest number of positive traps per sample date occurred on the 24th of August with 10 traps being positive for a total of 301 eggs (Tab. 4).

The 18-week release of about 1,000 sterile males/ha from early May until early October in the SIT area resulted in an overall sterility of 82.62% (when the absolute numbers of eggs are considered) or 60.52±42.88% (av. sterility/sampling date), when the sterility of each sampling date is considered (Tab. 4). The sterility of the 274 eggs collected in section A was 43.8% (av. sterility/sampling date: 31.04±42.9%), and of the 220 eggs collected in section C was 38.64% (av. sterility/sampling date: 22.98±38.29%). The relatively high sterility in the adjacent non-SIT sections A and C of 31% and 23% can be explained by the migration of sterile males and/or females mated with sterile males into the non-SIT control areas. Not only is the generally low number of eggs in the ovitraps an indicator of a successful control strategy, but so is the fact that no eggs were recorded in all ovitraps after the 7th of September.

In the second half of September, 2,668 breeding sites were inspected and only 82 (CI: 3.07%) were infested with mosquito larvae. Out of these, 69 inhibited developmental stages of *Cx. pipiens* s.l./*Cx. torrentium* or/and *Cx. hortensis*, *Cs. longiareolata* and *Cs. annulata*, and only 13 breeding sites contained the larvae of *Ae. albopictus* either alone (6 breeding sites) or associated with *Cx. pipiens* s.l./*Cx. torrentium* (7 breeding sites) resulting in a very low CI_{albo} of 0.49%.

3.4.2. Freiburg

The 21 ovitraps employed during the six bi-weekly sampling period in the SIT area in Freiburg (Metzgergrün) from the 14th of July (first collection date: 28.07.2020) until the 13th of October collected a total of 2,298 eggs (Tab. 5), which was about 6-times higher when compared to almost the same time period in the SIT area in Ludwigshafen. A significant number of eggs of other species including *Ae. japonicus* and *Ae. geniculatus* were found throughout the sampling period on the wooden boards. The release of about 2,320 sterile males per hectare on a weekly basis from the 15th of July to the 7th of October produced sterility rates increasing from 14.9% on the 28th of July to 51.6% on the 11th of August, 79.1% on the 29th of August, 74.6% on the 15th of September, 59.3% on the 29th of September, and 96.6% on the 13th of October (Fig. 7) The mean sterility is 77.4±15.35%.

In the control area without SIT on the collection dates from 29th of August to the 13th of October (4 x bi-weekly sampling), 1,615 eggs were collected (Tab. 5). The mean sterility amounted to 14.95±8.46%. In the first half of September, random breeding sites were inspected for the larvae of *Ae. albopictus* in a designated part of the control area. The samples were taken from around 35 different properties covering an area of 3.5 hectares. Of the 105 inspected breeding sites, 29 contained developmental stages of *Ae. albopictus,* resulting in a CI_{albo} of 27.7%. Other species found included developmental stages of *Ae. japonicus, Ae. geniculatus, Cx. pipiens or Cs. annulata*.

4. Discussion

The control of *Ae. albopictus* is a great challenge for authorities and the scientific community in Germany and elsewhere. When an established population of the Asian tiger mosquito is assessed by employing a suitable surveillance program, success in the containment of *Ae. albopictus* can only be guaranteed when all stakeholders are focusing on the immediate implementation of suitable control strategies [39]. This requires sufficient financial resources and the close cooperation between health departments, regulatory offices and field operators. The importance of comprehensive control efforts to combat the Asian tiger mosquito, one of the most dangerous vectors for arboviruses, is also reflected by the close cooperation between scientific institutions on a federal state and state level in Germany. Programs for efficient surveillance activities, risk assessment and the evaluation of control tools were initiated for instance by the Federal Agency for Environmental Protection (UBA) in cooperation with the Friedrich Löffler Institute (FLI) and the Bernhard Nocht Institute (BNI). The Sate of Baden-Württemberg, which is currently the most affected by *Ae. albopictus* in Germany, financed comprehensive research programs for the risk assessment concerning the establishment of *Ae. albopictus* on a community level and for evaluation of the efficiency of different control strategies to combat the Asian tiger mosquito [24]. Most of the research programs are guided by a national expert committee for invasive mosquito species located at the FLI (www.fli.de/de/kommissionen/nationale-expertenkommission-stechmuecken). In 2017, the Tri-national Initiative Group of Entomology in the Upper Rhine Valley (TIGER) was initiated under the auspice of the Institute for Parasitology in Strasbourg, France, as an EU-funded INTERREG-V project on the regional level including scientists from France, Switzerland and Germany. The project goals are to strengthen the cooperation between the authorities dealing with the risk management related to the occurrence of *Ae. albopictus*. At the European level, a “Practical Management Plan for Invasive Mosquito Species in Europe: I. Asian Tiger Mosquito (Aedes albopictus)” was prepared in the frame of the LIFE CONOPS project “Development & demonstration of management plans against – the climate enhanced – invasive mosquitoes in S. Europe. The project was co-funded by the EU Environmental Funding Programme LIFE+Environment Policy and Governance supported by COST (European Cooperation in Science and Technology) [39]. This plan includes public health assessment, monitoring by employing ovitraps, standard control measures in public and private areas, community participation, DtD activities in private areas, emergency control measures in response to the detection of imported cases of dengue, chikungunya or Zika, quality control of treatment efficacy, and the prevention of resistance to insecticides [39]. The toolkit to combat container-breeding mosquitoes includes powerful techniques and strategies like community participation, microbial control tools (e.g. Bti), insect growth regulators, surface layers, the sterile insect technique (SIT) e.g. based on radiation, the insect incompatibility technique through the endosymbiont *Wolbachia*-induced incompatibility (IIT), or the combination of SIT and IIT [29,38,40]. Last but not least, chemical products such as methoprene, diflubenzuron, pyriproxyfen (IGRs) or pyrethroids as adulticides can be applied during outbreaks. Space spraying of adulticides should be only applied in the case of health emergencies [41]. Modern genetic approaches as in site-specific gene editing with

CRISPR/Cas9 can augment the currently existing toolbox. Cas9-mediated gene-editing can be an efficient platform for gene-driven strategies to introduce suppression and pathogen-blocking genes into wild mosquito populations [38,42].

In our study, we evaluated a model-like integrated vector management strategy (IVM) based on a three-column strategy: Community participation (CP); Microbial control by application of Bti during DtD activities; and the Sterile Insect Technique (SIT) in the cities of Ludwigshafen and Freiburg, both with urban areas infested by *Ae. albopictus*. The majority of *Ae. albopictus* breeding sites occur on private properties. Therefore, CP with the goal of implementing environmental management targeting the breeding sites and use of Bti-tablets should be an essential part of each control programme because it is cost-effective and sustainable [25,26,43]. Thus, the public is transformed from “spectators” to “actors” by increasing public awareness through the provision of detailed information on the biology and control of *Ae. albopictus.* Water containers can be treated by residents with Culinex Tab plus (Bti fizzy tablets) to eliminate the larvae where breeding sites cannot be modified by sanitation. The citizens are given the tablets free of charge in the frame of community participation. The WHO/WHOPES recommends the use of sterilised Bti tablets in potable water containers because of their safety aspects [28]. The Culinex Bti-fizzy tablets are sterilised by Gamma radiation, where only the protein crystals produced by the bacilli as an active ingredient and no spores or living bacilli are applied [44]. In the preparation phase of the DtD activities as the core implementation of the project in Ludwigshafen, we informed the citizens in the affected areas through the media and in consultation with the city and health authorities which activities are planned in the frame of the project and how the citizens can contribute to the containment of *Ae. albopictus*. The distribution of Bti-tablet blisters which were fixed with glue to flyers took place in the middle of May in Ludwigshafen. A total of 1820 households received the information packets into the mailboxes in less than 1 minute/household. It is cost-effective when we take the total cost per household (including the tablets and flyers) of about 2.50 Euros into consideration. The awareness and active cooperation of the residents is documented in the results of the first DtD activity and the questionnaire which revealed that 78.38% of the household owners allowed access and more than half of the properties (55.42%) did not contain any breeding sites during the first round. According to the questionnaire (517 residents were responding), 57.64% of the residents (298) implemented the proposed control activities. On the other hand, 28 people (5.4%) claimed that the control of the Tiger mosquito is not important for them. This experience was shared with many other similar studies [39]. The public reacts only when they see an immediate advantage of their actions by reducing the nuisance or when they are controlled by intensive DtD inspections combined with written messages to the owners who refuse by the regulatory authorities. On average, during all five DtD rounds, 8.21±3,4% of the property owners refused entry to their property, which represents a problem when the elimination of a population is aimed for. According to the practical management plan for invasive mosquitoes, a minimum access threshold of ≥95% should be aimed for [39]. Another problem is that an average of 16.41±8.24% of the people were absent during the first visit during the course of the intervention. In this case, the field staff dropped a written message in the mailbox and asked for an appointment during the next round, so that those properties could be inspected. However, this procedure is time-consuming.

Beside environmental sanitation and the application of Bti tablets by the residents, larviciding by trained personal during DtD activities is the strongest way in which to reduce mosquito populations. The control of container-breeding mosquitoes like *Ae. albopictus* requires a long-term effect of the larvicide to minimise the costs of intensive manpower. The tests with the Bti formulation Vectobac WG at high dosages of 10 g for larger water containers or 2.5 g for small water collections applied by a pressurised hand sprayer revealed a long-term effect of 10-13 weeks (Fig. 2 and 3). This proves the long-term persistence of the protein crystal of microbial control agents, especially in defined artificial containers [45]. The crystal structure of the parasporal protein body obviously prohibits the fast decomposition of the toxins by microbes [45]. Beside the advantage of the unique selectivity and ease of handling of Bti-formulations, the lack of resistance is a unique feature of Bti [46]. The selectivity and safety for users also means that Vectobac WG can be used for wide-scale space applications, e.g. with a mist blower to cover a large area in a short period of time [47,48].

The second column of the strategy, namely the DtD inspection and treatment of all potential breeding sites with a high dosage of Bti (Vectobac WG) by trained personnel resulting in a long-term effect for at least one month, has to be the core intervention in an IVM program against *Ae. albopictus*. The importance of DtD interventions is also highlighted in the practical management plan developed by LIFE CONOPS/COST [39]. The results during the intervention in Ludwigshafen are convincing taking into account that the container index was always below 4% when all mosquito species and less than 1% when only *Ae. albopictus* populations are considered (Tab. 2). Before the DtD campaign, during the random sample survey in 2019, the CI_{albo} was 10x higher (10.9%). However, a strict comparison of these data is not possible because only a small number of breeding sites were inspected in 2019. Although the CI_{albo} increased 7 times when the first full round in May/June 2020 was compared with the last full round in September 2020, the number of 13 positive breeding sites out of more than 2,600 potential breeding sites in September (CI_{albo}: 0.49%) is very low. This is also reflected in the low number of only two records of *Ae. albopictus* reported by the residents in 2020 and no nuisance, as in the previous year 2019, was recorded. The extremely low number of positive breeding sites is due to the long-lasting effect of Vectobac WG treatments. The results achieved by employing 30 ovitraps underline the strong reduction of the *Ae. albopictus* population. Over the entire intervention period in Ludwigshafen, an average of 4.3 eggs/ovitrap/2 weeks was found. Only three ovitraps (2A, 8B and 5C) showed more than 100 or almost 100 eggs per sampling period in July and August. In the districts Metzgergrün and Gartenstadt in Freiburg, much higher numbers of eggs were collected in the ovitraps. In the SIT-area Metzgergrün, the average number of eggs was 18.2 eggs/ovitrap/2 weeks and even higher in the non-SIT area Gartenstadt, reaching 22.43 eggs/ovitrap/2 weeks. In this area, six ovitraps contained considerably more than 100 eggs/ovitrap and a maximum of 340 eggs/ovitrap/2 weeks. Carrieri et al. [49] calculated the epidemic risk threshold for a Chikungunya transmission based on the mean egg density/trap/week. Infections with virulent virus strains can already occur when the average number of eggs/trap/week is above 44 eggs, and when less virulent strains are involved above 146 eggs/trap/week. In one area in Gartenstadt (locations of trap 15-18), the average number of eggs/week is 87 eggs/trap/week. According to Carrieri et al. [49] this area could be defined as a risk area for a possible virus transmission when individuals with viraemia return from endemic areas and when the climate conditions are appropriate for transmission. The climate extremes (temperatures above 30°C for weeks) in the Upper Rhine Valley over the last few years would definitely favour possible transmission. The higher number of eggs in some areas of Freiburg can be explained by the late start of control activities in 2020. In Ludwigshafen, where regular DtD inspections and Bti treatments took place, the number of eggs is permanently below the epidemic risk threshold. The control strategy involving the residents from the beginning of the operation via the distribution of flyers and Bti tablets and DtD activities, either in full rounds or hot spot control defined by careful monitoring programs by mapping the breeding sites and a suitable network with ovitraps, seems to be an appropriate strategy. In the first full round at the beginning of the reproduction period of *Ae. Albopictus,* the potential breeding sites can be prophylactically treated with a sustainable effect of Bti; in the following hot spots rounds, two and four, only those properties are inspected which provide many breeding sites or where the surveillance program with ovitraps indicate an increased number of *Ae. Albopictus* individuals. Thus, the costs of the DtD activities can be strongly reduced in cases where a full round every three weeks cannot be afforded financially. This strategy also has the advantage that the residents do not feel annoyed by the frequent visits of field staff. The inspection time per house can be calculated between 43 (1^{st} round) and 27 minutes (5^{th} round), which depends on the skills of the trained assessors. On average, about half an hour has to be invested for the inspection and treatment of one property according to the data achieved in the model-like intervention in Ludwigshafen. A total of 6,328 breeding sites have been treated during all rounds, which amounts to the consumption of 7.3 kg of Bti (Vectobac WG) and total costs of less than one Euro per breeding site (Tab.2).

The strongest constraint of the DtD activities to achieve the almost complete eradication of tiger mosquitoes is that about 9% of the residents (full rounds 1 and 5) refused entry to their property. This frequent circumstance in addition to the sporadic occurrence of cryptic breeding sites hinders reaching the goal of a strong reduction of the *Aedes* population to a critical number of individuals to eliminate the population. The traditional genetic control by the use of γ-radiated sterile *Ae. albopictus* males is an excellent tool to overcome this constraint, especially when the specific biological features of *Ae. albopictus* such as limited flight range and a slow build-up of the population during the beginning of the reproduction period are taken into account. The prerequisites to employ SIT are successful mass production, accurate sex sorting, optimal sterilisation and the rapid shipment of *Ae. albopictus* males. Over the course of the study, the CAA facility was able to provide sufficient numbers of sterile males. The sterilisation with a dose of 1.85 Gray for 19 minutes (total of 35 Gray) maintains the competitiveness of the released sterile males versus the wild type males [30]. This could be proven by the cage experiments with wild and radiated males resulting in a sterility of 87.53±9.15% when wild females were exposed to sterile males, while the mating of wild males and wild females amounted in the embryonation of 96.70±2.8% (natural sterility of 3.3%) (Tab. 3). The sexing procedure by Fay-Morlan [50] provided acceptable results of an average of less than 1.2% residual females per batch. Also, the shipment procedure in outer styropor boxes containing sterile males in small plastic containers is greatly improved by using suitable gel-cooling elements to keep the temperature at around 10°C and to ship them overnight by DHL on a weekly basis. The mortality of radiated males per shipment resulted in an average of below

8%. When dealing with large numbers, sterile males must be packed in small containers which may cause higher mortalities and possibly less adequate adaptation to the field temperatures. The mortality could be significantly reduced by keeping the males for about two hours at room temperature (22°C) after arriving in Ludwigshafen to adapt slowly to the higher temperature after shipment in cool boxes. The costs per shipment for the smaller boxes are reasonable, with an average amount of 175 Euros/shipment.

The efficacy of the SIT is proven by the high number of sterile eggs recorded in the application areas compared to the control areas. In Ludwigshafen, the 18 releases each of 1,013 sterile males/ha resulted in a sterility of more than 80% when the absolute numbers of eggs or 61% when the sterility rate of each collection date is considered. The sterility rates of about 40% (absolute no. of eggs) or 31% and 23% observed in the adjacent non-SIT areas in Ludwigshafen can be explained by the migration of the sterile males and/or the sterilised females. The majority of ovitraps in the adjacent non-SIT areas is not further away than 250 metres from the sterile male release sites. This effect is underlined when the results in Freiburg are compared, where the distance between the SIT area and the control area is more than 1 km. In this area of 4.5ha, during 13 releases, an average of 2,320 sterile males/ha were released. The sterility of the eggs in the application and control area for comparable collection dates from the 29th of August to the 18th of October is 75.5% (absolute number of eggs), or 77.4% when the average sterility of each sampling date is considered. In the non-SIT area, the sterility is 17.28% (absolute no. of eggs) and 14.95±8.46% when the average sterility of each sampling date is considered. The lower rate of sterility in Freiburg can be explained by the higher number of eggs in both areas in Freiburg compared to Ludwigshafen where the wild population was strongly reduced by DtD activities from the beginning of the campaign. However, in both areas, it could be shown that the sterile insect technique offers an effective strategy with which to reduce tiger mosquito populations when it is accompanied by a successful control campaign based on the application of larvicides. The unavoidable release of about 1% of radiated females together with sterile males did not cause any complaints from the residents. However, it was very important to inform the public about the release ahead of the operation.

In our cage experiments focusing on the most effective ratio wild versus sterile males, the most effective ratio is 1:5. At this ratio, the achieved sterility was 85.95±3.18%. A 1:1 ratio results in a much lower sterility of about 30% and the higher rate of 1:10 did not increase the sterility. The effective ratio of 1:5 was also shown by Oliva et al. [51] in SIT simulation experiments in Reunion to suppress the wild *Ae. albopictus* population. This ratio allowed a two-fold reduction of the wild population’s fertility. Finally, the cost evaluation during the course of the SIT application in both cities revealed costs of about 1200 Euros/ha when 1000 to 2000 males were released on a weekly basis during the reproduction cycle. Altogether, 25,000 Euros have been spent on the provision and shipment of sterile males for about 21 ha, and we also had to calculate about 1 hour for the release action over 16 ha, which amounts to 5 minutes/ha. The total costs for the treatment of 1 ha with SIT were less than 1200 Euro.

Conclusions

In this study, we clearly prove that an integrated control program is most effective when it comprises three columns, namely a) community participation, b) DtD activities including long-lasting Bti larviciding of all possible breeding sites to strongly reduce the wild *Ae. albopictus* population as a basis for the successful application of SIT, and c) SIT to almost wipe out the *Ae. albopictus* population. The combination of Bti and SIT guarantees that two of the most selective, safe and effective tools are employed against one of the most dangerous mosquito vector species *Aedes albopictus*, the Asian tiger mosquito.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

NB designed the study and prepared the manuscript. SLK, AT, TH conducted field work and the improvement of the manuscript, DR, RL, JSC, AP and RB contributed to the improvement of the manuscript. All authors read and approved the final manuscript.

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Tables

Tab. 1: Overview of the released sterile males in Ludwigshafen (Melm) and Freiburg (Metzgergrün) in 2020, including the control of the released numbers, the number of females per batch according to CAA and IfD as well as the mortality of the radiated males after shipment.

No of Batch	no of males to Speyer	no males/ container	Corr. no of males	CAA-no of females (%)	Control-no of females (%)	no males to Freiburg	Time of release	Mortality in %
1	12.000	-	11.628	1,65	-	-	29.05.	n.a.
2	22.000	-	21.318	1,6	-	-	09.06.	n.a.
3	19.000	875	18.411	2,45	2,31	-	17.06.	n.a.
4	17.000	1070	16.473	1,75	2,42	-	30.06.	n.a.
5	13.000	865	12.597	1,54	1,15	-	07.07	1,4
6	19.000	968	18.411	0,22	1,5	5.000	15.07	8
7	12.000	943	11.628	0,35	0,1	5.000	21.07.	4,3
8	24.000	893	23.256	1,53	1,34	5.000	28.07.	30
9	20.000	930	19.380	0,7	0,86	8.000	04.08.	10
10	29.000	1050	28.101	0,62	0,99	8.000	11.08.	n.a.
11	21.000	968	20.349	1,34	1,34	8.000	18.08.	0,5
12	16.000	933	15.504	0,78	1,18	12.000	25.08.	0,5
13	9.000	969	8.721	1,64	1,32	7.000	01.09.	5
14	17.000	1173	16.473	0,74	0,34	12.000	08.09.	n.a.
15	18.000	1065	17.442	0,55	0,47	17.000	15.09.	n.a.
16	14.000	980	13.566	0,37	0,81	18.000	22.09.	n.a.
17	17.000	875	16.473	0,72	1,71	12.000	29.09.	n.a.
18	21.000	944	20.349	1,82	1,34	19.000	07.10.	n.a.
Total	**320.000**	**Ø969±84**	**310.080**	**Ø1,13±0,64%**	**Ø1,19±0,63**	**136.000**		**7,4%**

Tab. 2: Overview of the accessibility of the properties, occurrence of breeding sites and infestation rates (CI) with mosquito developmental stages, consumption of Bti (Vectobac WG) and working hours during the 5 rounds of control in Ludwigshafen, Melm.

Round/	Round 1	Round 2	Round 3	Round 4	Round 5
Date	18.5.20. - 12.6.20.	23.6.20. - 7.7.20.	27.7.20. - 31.7.20.	17.8.20.- 21.8.20.	14.9.20. - 22.9.20.
number of					
properties	953	318	110	273	1029
entered					
(%)	747 (78.38%)	261 (82.07%)	110	193 (70.7%)	724 (70.36%)
absent					
(%)	119 (12.49%)	23 (7.23%)	-	71 (26%)	205 (19.92%)
refused					
(%)	87 (9.13%)	34 (10.69%)	-	9 (3.3%)	100 (9.72%)
no. of properties					
with breeding sites	333 (44.58%)	240 (91.95%)	-	147 (76.17%)	517 (71.4%)
no. of properties					
without breeding sites	414 (55.42%)	21 (8.05%)	-	46 (23.83%)	207 (28.6%)
no. of potential					
breeding sites (all)	1535	741	340	1044	2668
positive breeding					
sites (all)	6 (0.39%)	no larvae	15 (4,41%)	26 (2.49%)	82 (3.07%)
positive breeding sites					
for *Ae. albopictus*	1 (0.07%)	no larvae	3 (0.8%)	1 (0.1%)	13 (0.49%)
CI total (%)	0.39%	-	4.41%	2.49%	3.07%
CI *Ae. albopictus*	0.07%	-	0.88%	0.1%	0.49%
Working hours	540	140	84	80	325
Bti comsumption	4	2	1	3	7.3

Tab. 3: Assessment of the sterility and number of eggs laid by females mated with radiated males or with wild males.

		Wild Type cages			SIT cages	
	No. of eggs	Embryonation (%)	Sterility (%)	No. of eggs	Embryonation (%)	Sterility (%)
Cage 1	490	459	31	944	54	890
		(93.67%)	(6.33%)		(5.73%)	(94.27%)
Cage 2	1000	992	8	939	122	817
		(99.2%)	(0.80%)		(13%)	(87%)
Cage 3	544	529	15	707	132	575
		(97.24%)	(2.76%)		(18.67%)	(81.33%)
Ø ± SD in %		96.70 ± 2.8%	3.3 ± 2.8%		12.47 ± 6.49%	87.53 ± 9.15%

Tab. 4: Number of *Aedes albopictus* eggs and percentage of sterility of the eggs in Melm, Ludwigshafen

Date	15.6.20.	29.6.20.	13.7.20.	27.7.20.	10.8.20.	24.8.20.	7.9.20.	Total
total no. of eggs	45	88	216	0	173	301	96	919
Section A								
total no. of eggs	45	64	120	0	8	34	3	274
no. of sterile eggs	2	6	103	0	0	6	3	120
sterility %	4.44%	9.37%	85.83%	0	0	17.65%	100%	43.8%
Section B-SIT								
total no. of eggs	0	24	91	0	101	114	90	420
no. of sterile eggs	0	23	59	0	84	91	90	347
sterility %	0	95.83%	64.84%	0	83.17%	79.82%	100%	82.62%
Section C								
total no. of eggs	0	0	0	0	64	153	3	220
no. of sterile eggs	0	0	0	0	8	74	3	85
sterility %	0	0	0	0	12.5%	48.37%	100%	38.64%

Table 5: Number of *Aedes albopictus* eggs and percentage of sterility oft he eggs in the SIT area (Metzgergrün) and the control area (Gartenstadt).

Date	28.07.20.	11.08.20.	29.08.20.	15.09.20.	29.09.20.	18.10.20.
SIT-area						
No. of eggs	623	475	555	481	135	29
No. of sterile eggs	93	345	439	359	80	28
Sterility %	14.9%	51.6%	79.1%	74.6%	59.3%	96.6%
Control area						
No. of eggs	-	-	498	902	80	135
No. of sterile eggs	-	-	132	129	8	10
Sterility %	-	-	26.5%	14.3%	10.0%	7.41%

Figures

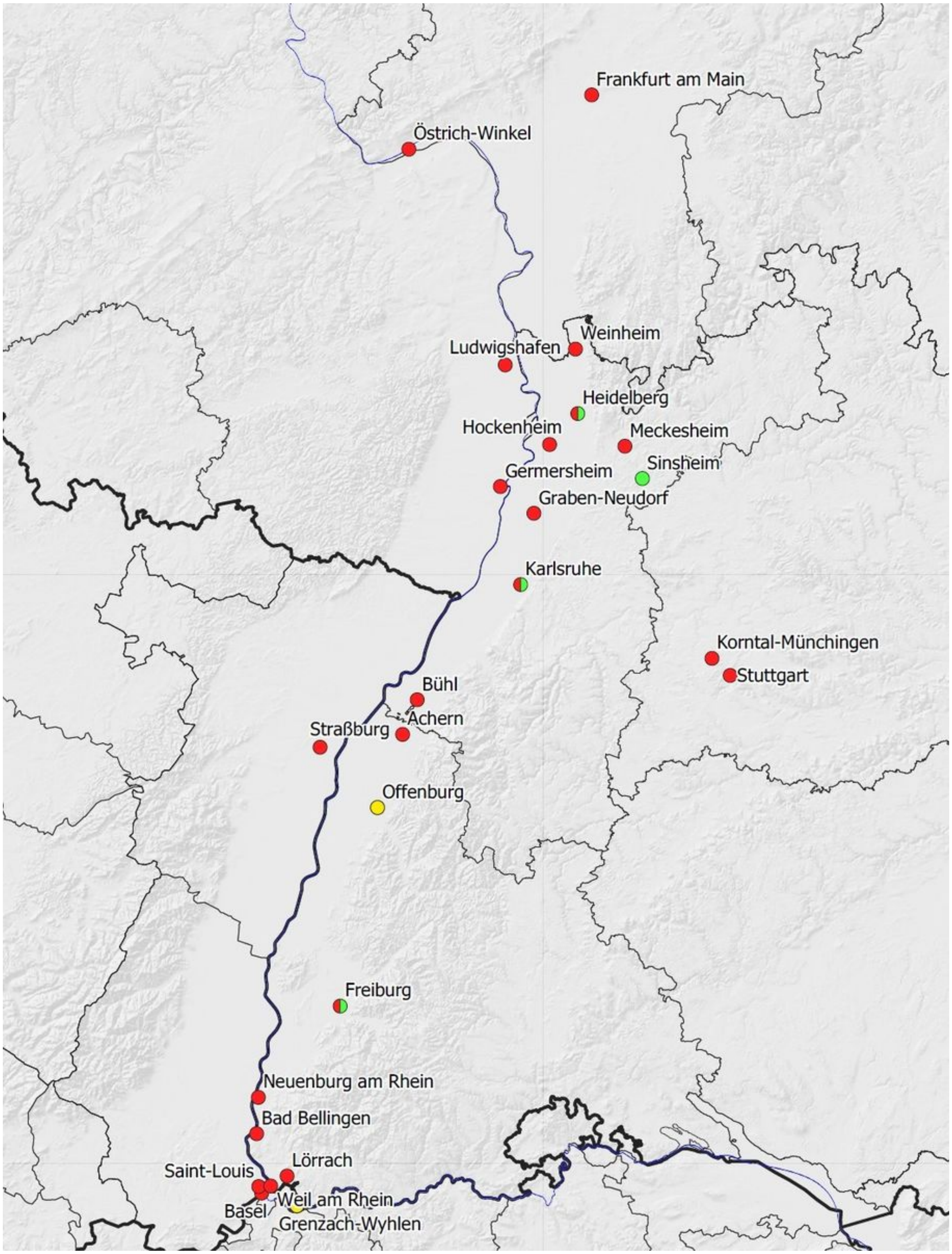

Figure 1

Recorded Aedes albopictus populations in 2019 (red: not under control; green: eradicated; green-red: strongly reduced; yellow: population size not known). Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

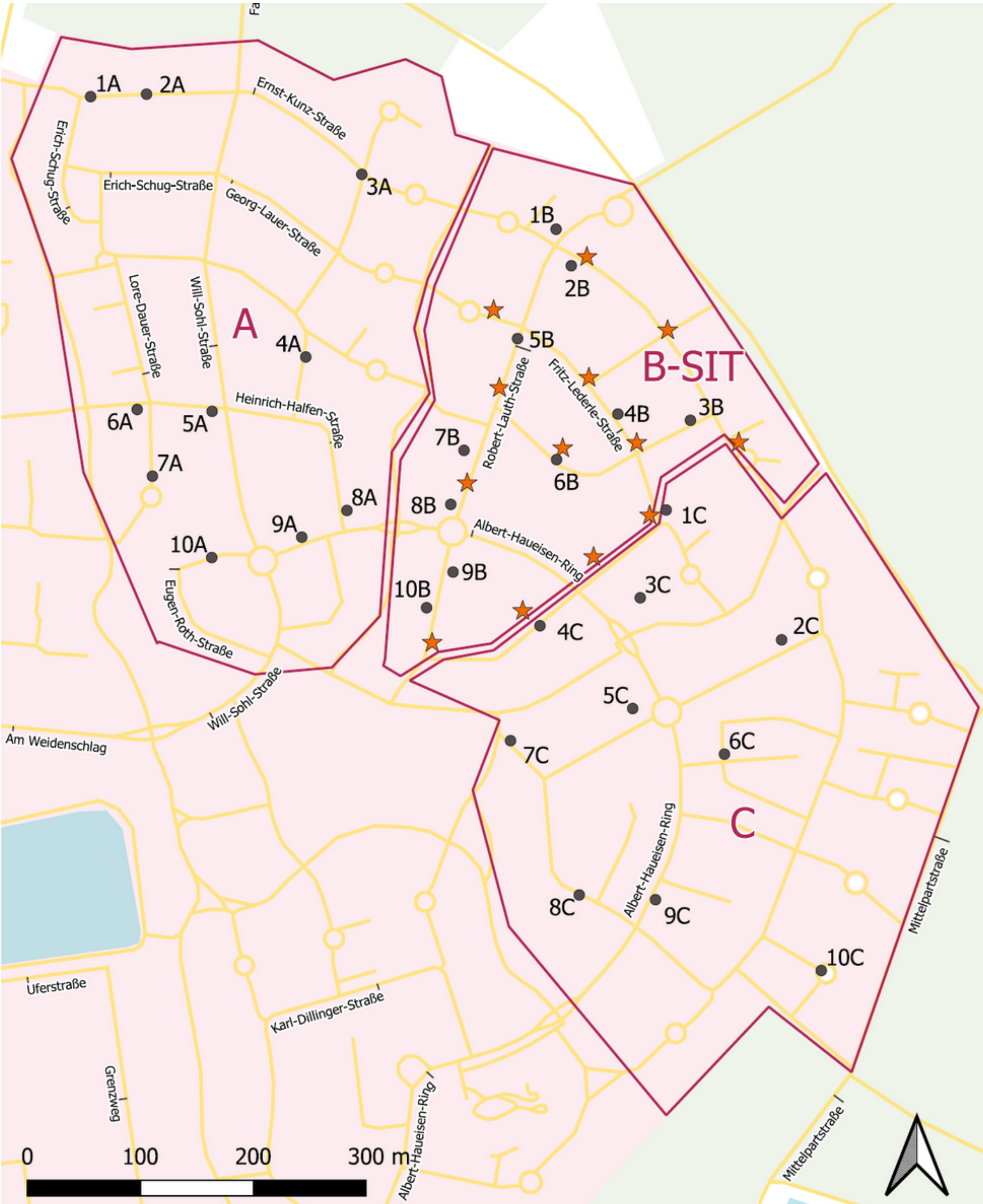

Figure 2

Study area Melm, positions of ovitraps and release spots for sterile males in Ludwigshafen (GeoBasis-DE/LVermGeoRP2020¸ 2018 Geofabrik GmbH and OpenStreetMap Contributors) Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Figure 3

SIT area “Metzgergrün” in the city of Freiburg (purple: release areas; yellow spots: location of the ovitraps). Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

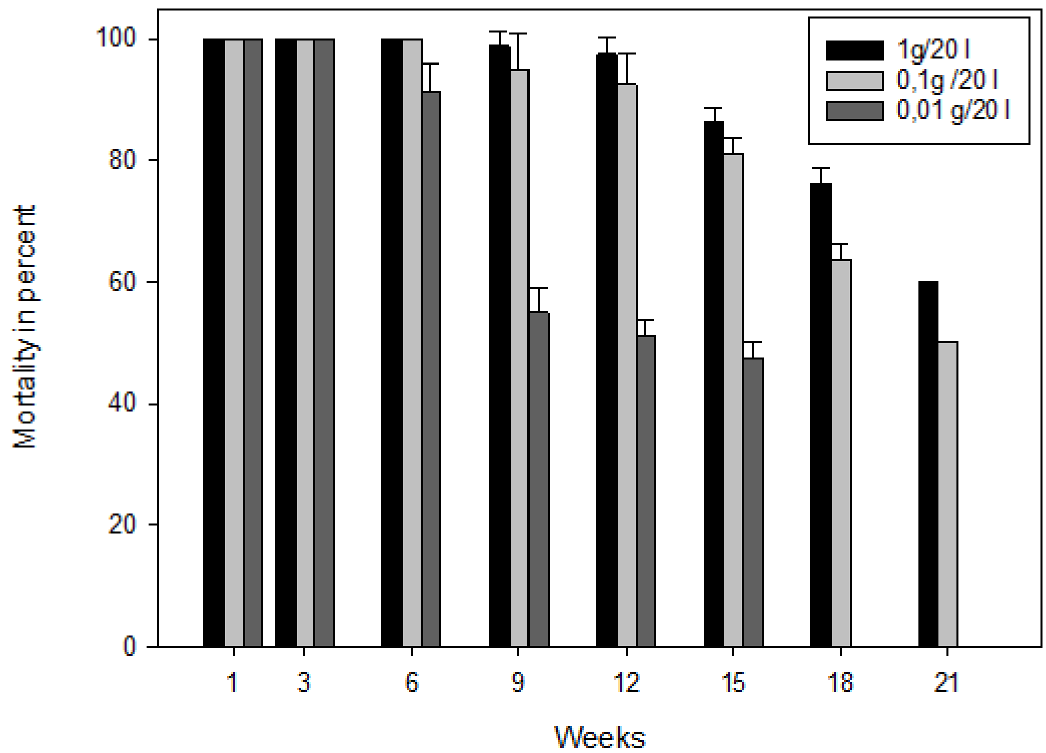

Figure 4

Long-term effect of Vectobac WG on Aedes albopictus larvae when high dosages are applied in larger water containers.

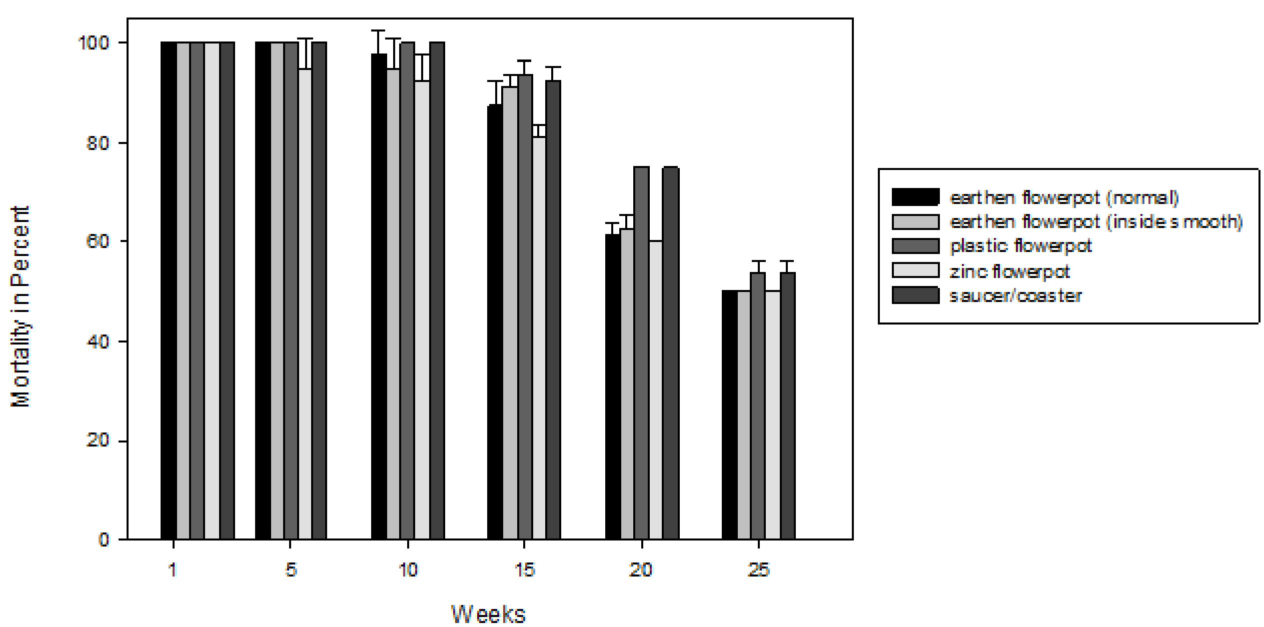

Figure 5

Effect of Vectobac WG at a dosage of 2,5 g/small container on third instar larvae of Ae. albopictus in different flower pots and coaster.

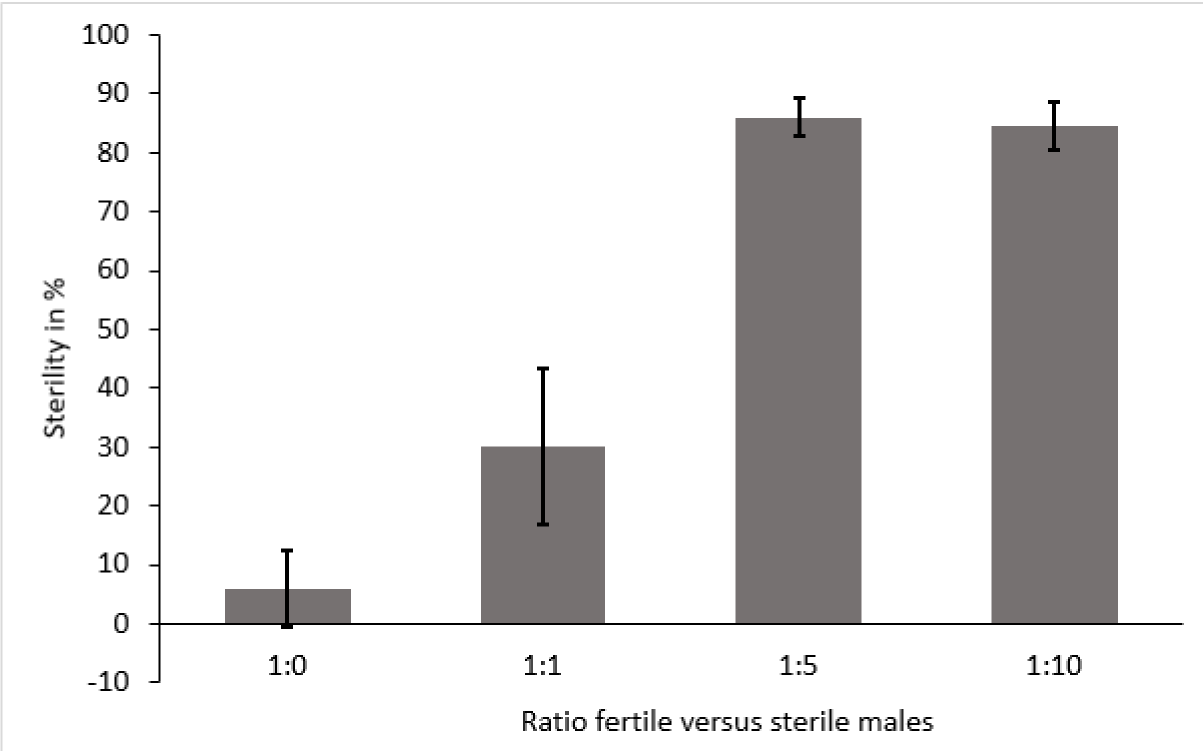

Figure 6

Effect of the ratio between radiated and unirradiated males on the egg sterility rate.

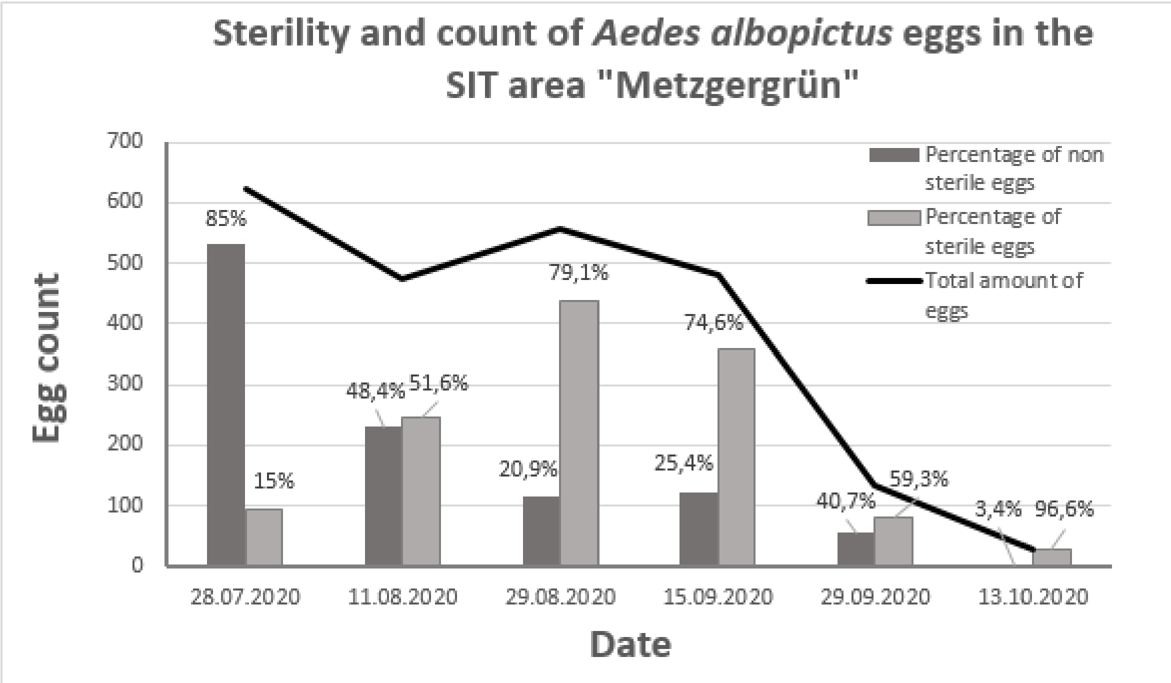

Figure 7

Sterility of Aedes albopictus eggs in the SIT area “Metzgergrün”

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