Integrated Control of *Aedes Albopictus* (Diptera: Culicidae) in Southwest Germany supported by the Sterile Insect Technique

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Abstract

Background

The invasive species *Aedes albopictus*, the Asian tiger mosquito, has undergone an extreme expansion by steady introductions as blind passengers in vehicles from the Mediterranean to South-West Germany. The more than 15 established populations in the State of Baden-Württemberg and Palatine (South-West Germany) have become a major nuisance and public health threat. *Aedes albopictus* deserves special attention as vector of arboviruses like dengue, chikungunya or Zika virus. In Germany, control of *Ae. albopictus* is implemented under the auspice of health departments and regulatory offices.

Methods

The control strategy comprised three components or pillars: a) community participation (CP) based on the elimination or sanitation of breeding sites with the use of fizzy Bti-tablets (Culinex Tab plus); b) Door-to-Door (DtD) control by trained staff applying high doses of a Bti-water-dispersible granular formulation (Vectobac WG) aimed for a long-lasting killing effect; and c) the Sterile Insect Technique (SIT) to eliminate remaining *Ae. albopictus* populations. Prior to large scale routine city-wide treatments, the efficacy of the three elements was evaluated in laboratory and semi-field trials. Special emphasis was given to the mass release of *Ae. albopictus* sterile males.

Results

More than 60% of the local residents joined the Community Participation within the large-scale control program. It was shown that the most effective element was the DtD intervention including the application of Vectobac WG (2700 ITU/mg after radiation with 25 kGy) to potential breeding sites (10 g/rainwater container, max. 200L ≙ 13,500ITU/L and 2.5g/container <50L) with a persistence of at least about three weeks. The average time required for the inspection and treatment per property was 27 minutes. In Ludwigshafen the larval source management resulted in a container index for *Ae. albopictus* below 1% in 2020 compared to 10.9% in 2019. The mean number of *Aedes* eggs/ovitrap were 4.3 in Ludwigshafen and 18.23 in Freiburg-Metzgergrün (SIT areas); while 22.4 in Freiburg-Gartenstadt (Control area). After the strong reduction of the *Aedes* population by Bti-application, the weekly release of 1,013 (Ludwigshafen) and 2,320 (Freiburg) sterile *Ae. albopictus* males/ha from May until October resulted in a high percentage of sterile eggs. In the trial area of Ludwigshafen the sterility of eggs reached 82.61% (mean: 60.52%; SD: 42.88%) and in Freiburg 62.68% (SD 28.21%). The natural sterility in the control area was 16.93±13.5%. The field results were in line with data obtained in cage tests under laboratory conditions where wild females mated with sterile males showed sterility rates of 87.53±9.15%. The sterility of eggs laid by females mated with unirradiated males was only 3.3±2.8%. The overall sterility of about 83% in Ludwigshafen indicates that our goal to almost eradicate the *Ae. albopictus* population could be achieved.

Conclusions

It is shown that an integrated control program based on a strict monitoring scheme is most effective when it comprises three components, namely a) community participation, b) DtD intervention including long-lasting Bti-larviciding to strongly reduce *Ae. albopictus* populations and c) the release of sterile males to reduce the

remaining *Ae. albopictus* population to a minimum or even to eradicate it. The combination of the use of Bti with SIT are most effective and selective tools against *Ae. albopictus*, one of the most dangerous mosquito vector species.

1. Background

Among the more than 3,500 known mosquito species, about 30 have spread beyond their original geographical borders [1, 2]. Several invasive species have a severe impact on public health, not only in the tropics but also in temperate climates. *Aedes aegypti*, *Ae. albopictus*, *Ae. japonicus, Ae. koreicus*, *Ae. atropalpus* and *Ae. triseriatus* belong to this group.

Aedes albopictus deserves special attention as a vector of at least 22 arboviruses, including dengue, chikungunya, Zika and yellow fever viruses [3–8]. Since 1990, *Ae. albopictus* took a permanent foothold in Europe. It had been introduced to Italy by the international trade of used tires. From Genoa [9, 10], *Ae. albopictus* was spread as a blind passenger via road, rail and boats over Italy and further along the Mediterranean coast to France, Spain, the Balkan, Greece and Turkey [57]. Today, the species is firmly established in the whole European Mediterranean basin. It has started spreading across the Alps into central Europe. Autochthonous transmissions vectored by *Ae. albopictus* of chikungunya, dengue and Zika viruses have flared up in the Mediterranean region [6, 7, 8].

Realizing the risk of passive transport of *Ae. albopictus* from Italy to Germany by road and rail, especially during the summer holiday season, the German Mosquito Control Association (KABS) started a monitoring program in 2005 [2, 19]. Surveillance of resting stations and camping grounds along the highway A5 leading from Italy revealed during the summer months

a regular and increasing appearance of *Ae. albopictus* along the Upper Rhine area [2]. A first established population was reported in an allotment garden in Freiburg in September 2014. By 2019 at least twenty cities along the upper Rhine valley were infested by overwintering populations of *Ae. albopictu*s (Fig. 1) [55].

Figure 1: **Recorded** Aedes albopictus **populations in 2019 (red: not under control; green: eradicated; green-red: strongly reduced; yellow: population size not known).**

In all colonised areas, immediate surveillance was initiated by means of ovitraps, larval sampling and human bait collections to assess the size of the infested area and the abundance of *Ae. albopictus*. The goal was to detect and eliminate the breeding sites and to start eradication programmes.

After an in-depth evaluation of control tools in a previous study [24], it was decided to base the control strategy on three pillars: 1) community participation (CP) including distribution of Bti-fizzy-tablets containing the protoxins of *Bacillus thuringiensis israelensis* (Bti), 2) Door to Door (DtD) activities including the removal or treatment of all breeding sites with a Bti-suspension by trained staff with Bti at three weeks intervals, and 3) the integration of the Sterile Insect Technique (SIT), an environmentally-friendly insect pest control method, to wipe out remaining *Ae. albopictus* populations originated from cryptic and/or non-accessible breeding sites.

1.1. Community participation (CP)

Community participation (CP) focuses on the increase in public awareness to prevent mosquito breeding and to record the occurrence of *Ae. albopictus* as an “early warning system”. The CP includes detailed information provided 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. Thus, public awareness was strengthened by detailed information on the characteristics, distribution, and the biology of the Asian tiger mosquito. In addition, measures are communicated to prevent the proliferation of the mosquito. This included the elimination of breeding sites, environmental sanitation, e.g. by the use of firmly fitting lids to water containers and the treatment with Bti tablets (Culinex Tab plus). Bti tablets are distributed to the public in support of the DtD activities conducted by the expert teams. Additionally, in heavily infested areas, mosquito nets were distributed free of charge to thoroughly cover rainwater containers preventing access to female mosquitoes thus the proliferation of vectors.

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

Due to the lack of professional know-how and active involvement CP alone was not enough to reach the goal of strongly reducing or even eliminating the Asian tiger mosquito [59]. Therefore, DtD control of *Ae. albopictus* by trained staff was implemented. This measure along with long-lasting Bti-treatments was highly effective. *Ae. albopictus* populations could be strongly reduced or even eliminated.

1.3. The Sterile Insect Technique

The final goal was a significant reduction or ideally the elimination of *Ae. albopictus* populations. Therefore, the Sterile Insect Technique (SIT) was added as third pillar to the integrated control strategy using gamma-irradiated sterile males. *Ae. albopictus* is particularly suitable for employing SIT, as the species is easily mass-reared, has a limited flight range, does not reproduce in enormous masses within a very short period like floodwater mosquitoes, and breeding sites are well defined in urban areas mainly [29].

Therefore, the SIT method is an excellent tool to access also those breeding sites which are out of reach of standard control measures. This applies also to property owners refusing entry permission.

Preceding the release of sterile males, the natural *Ae. albopictus* population has to be low or strongly reduced by CP and DtD control. The sterile males have to outcompete their wild counterparts resulting in a large majority of wild females laying sterile eggs [30].

1.4. Final goals of the three-pillar approach

The goal of the pilot program was to assess the effect of the three-pillar control strategy against *Ae. albopictus* starting in the laboratory, extending to semi-field tests and ending in routine field applications. Ovitraps served as the main monitoring tool. A cost-analysis is presented to serve as a guideline for further planning of community-based control activities.

2. Methods

2.1. Study areas

The large-scale study was conducted in three *Ae. albopictus* infested large areas in Southern Germany:

a) The **"Melm"** district (65 ha) within the City of Ludwigshafen (Palatine). This is a well-defined residential area, developed at the end of the last century. It consists of about 950 family homes with gardens and some apartment buildings. Abundant breeding sites of *Ae. albopictus* were present. The 65 ha were subdivided into three sectors of almost equal size, each with specific mosquito control scheme: Sector A: 23 ha (CP + DtD); Sector B (CP + DtD + SIT): 17 ha; and Sector C: 25 ha (CP + DtD) (Fig. 2).

b) the **Metzgergrün** area within the city of Freiburg (Baden-Württemberg). The selected site has a size of 4.5 ha comprising mostly apartment buildings for social housing (Fig. 3). It contains in adjacent gardens a large number of potential breeding sites such as used tires and water catching garbage. In this area, the SIT technique was employed in addition to CP and DtD.

c) **Gartenstadt** (Freiburg): This site served as control area (4 ha) and is about 1 km away from Metzgergrün. The infestation by *Ae. albopictus* was comparable to the Metzgergrün.

2.2. Design and layout

The large-scale field study was based on a three-pillar strategy, namely community participation (CP), door-to-door control (DtD), and sterile insect technique (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 helping the residents to control the Asian tiger mosquito on their properties. Each household received a flyer with instructions and attached to each flyer was a card board box containing 10 Bti-tablets in a blister pack (Culinex® Tab plus, Lot: 0604783, activity: 1000 ITU/mg, Culinex GmbH, Ludwigshafen).

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 efficacy of CP. Furthermore, the residents were interviewed regarding their knowledge on mosquito control. During the last control round (no. 5) in September, all available residents were asked if they had implemented the proposed control activities on their property.

2.2.2. DtD control

The most powerful tool to control *Ae. albopictus* was the DtD approach by trained staff applying long-lasting Bti-treatments. Therefore, in the first step it was crucial to assess the optimum dosage for the Bti-treatments in large and small water collections.

2.2.2.1. Assessment of the optimum effective dosage for Bti-treatments

Whereas the control of floodwater mosquitoes requires only a single effective dosage of Vectobac WG (strain AM5265, Valent Biosciences, Libertyville, USA) [56], the control of container-breeding species needs a long-term residual activity due to the constant follow-up of generations. Thus, the sequence of retreatments and the costs for manpower could be strongly reduced when a long-term effect of several weeks (at least 3 weeks) is achieved.

In a first series of tests, the effect of Bti-treatments in rainwater containers was simulated. The Bti mixture was prepared with Vectobac WG with an activity 2700 ITUs/mg by thoroughly mixing 250 g of Vectobac WG with 1.5 l of tap water (pH: 7.8; conductivity: 680 µS; $0.675x10^9$ ITU/1500 ml). Aliquots of the suspension were taken with an Eppendorf pipette by continuous stirring and applied to plastic buckets filled with 20 litres of tap water (1/10 of the usual water volume of a regular rainwater container). From the stock suspension 6 ml, 0.6 ml and 0.06 respectively were added to the 20 l of container water. This corresponded to 6 g, 0.6 g and 0.06 g of Vectobac-WG. Each dosage was tested in four replicates at a constant temperature of 24°C ± 1°C, with 4 buckets serving as a control. Twenty third instar larvae of laboratory-reared *Ae. albopictus* were added to each bucket. The mortality reading was taken at 24 and 48h post-treatment. At weekly intervals, 5 l of water was removed and replaced with the same amount tap water to simulate natural conditions when water is removed for watering the garden. Then, 20 third instar larvae were again added to each container. At each 48h reading larval cadavers were removed. The experiment was run until the mortality rates fell below 60%.

In a second series, the efficacy of Vectobac WG was tested in four different small water containers typically present in garden areas: a) terracotta flowerpots with rough surfaces (volume: 1,400 ml); b) terracotta pots with smooth walls (volume: 1,400 ml); c) plastic flowerpots (volume: 950 ml); d) zinc pots (volume: 800 ml); and e) terracotta flowerpot saucers (volume: 200 ml). Before application, the recipients were scrubbed with flower soil (Compo Sana potting soil), cleaned with water and dried for 24 h to simulate natural conditions. The inside of four empty containers of each type were homogenously sprayed with a Mesto pressurised sprayer (Mesto BUGSI 1.5L), with 15 ml of Vectobac WG stock solution (250 g Vectobac WG mixed with 1.5 L of tap water; 0.675x 109 ITUs/1500 ml) resulting in 2.5 g Vectobac WG/small container ($6.75x10^6$ ITUs/container). The containers were dried for 48 hours and filled with tap water. Twenty *Ae. albopictus* third instar larvae were added to each container. The mortality reading was done at 48 h post-treatment. The containers were emptied after the mortality reading and dried again for 48 hours, refilled with water and stocked with a new batch of larvae. The procedure was repeated until the mortality rates fell below 60%. Four containers of each type served as untreated control. The test was conducted at 24°C ± 1.5°C and 80% RH.

2.2.2.2. Routine Bti-treatments

The field staff consisted of six persons with in-depth knowledge on mosquito biology and taxonomy, especially breeding habits of *Ae. albopictus*. The staff had to handle the application of Bti at predetermined dosages to each potential breeding site as well as evaluate the mosquito traps at regular intervals for surveillance purposes. All accessible properties were inspected

from June to October at intervals of three weeks. All potential breeding sites were treated with

Vectobac WG. Finally, keeping close contact with the residents of nearly 2000 households was

essential, providing information and Bti-tablets. Between May and September, the program included five rounds of surveillance, control and data collection. The data were entered into a geographic information system (GIS).

Melm in Ludwigshafen: Biting *Ae. albopictus* were signalled for the first time in early August 2019. The county health department and the city authorities were informed. *Ae. albopictus* populations were found along four streets. In addition, scattered across the district, more breeding sites (unused flowerpots, saucers, rainwater barrels) were identified. Gullies were not functioning as breeding sites because all the water collected, runs off directly into the sewage plants. In total, 55 potential 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 and to coordinate further actions. In a first step, the residents were informed via media, and a website was created as a platform to extend reporting. In the last week of August 2019, all households received flyers and Bti-tablets for self-help. Several citizens reported severe nuisance caused by *Ae. albopictus* in their garden area. An action plan was designed together with authorities serving as a concept for an integrated control strategy in 2020. The plan included:

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 (six people) in early May including: a) the biology and taxonomy of mosquitoes to be able to distinguish between *Ae. albopictus* and other container-breeding mosquitoes such as *Cx. pipiens* s.l./*Cx. torrentium*, *Culiseta annulata*, *Cs. longiareolata* or *Ae. japonicus*; b) the breeding habitats of *Ae. albopictus* and how to identify and enter the breeding sites in a database; c) the correct application of the biological larvicide Bti; d) the handling of mosquito traps; and e) how to approach the residents, especially under the restrictions imposed by Covid-19.
3. Flyers with detailed information along with Bti-tablets for self-help were distributed to 1820 households on 11th and 12th of May, 2020.
4. Deploying 30 ovitraps (≈1 trap/2 ha) distributed within the district and inspected on bi-weekly intervals (Fig. 2). Eggs were counted and checked for embryogenesis according to the description in paragraph 2.2.3.3.
5. DtD activities from May to September 2020 in five rounds to make the action effective and cost-efficient. 1) 1st round from 18th of May to 7th of June. All properties were visited, breeding sites carefully mapped and treated with Bti. All data were entered into Q-GIS supporting the next control steps focusing on hotspots of breeding sites and the number of eggs in the ovitraps. 2) 2nd round from 23rd of June to 7th of July: hotspot treatments of all properties where breeding sites of *Ae. albopictus* were recorded during the first round. 3) 3rd round from the 28th to 31st of July: 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. 4) 4th round from the 17th to 21st of August: similar to the 2rd round, inspections and treatments of properties with rainwater containers and small breeding sites. 5) 5th final round from 14th until 22nd of September: Similar to the first round. All houses were re-inspected. Breeding sites were checked for larvae and treated. With a questionnaire residents who agreed to participate were asked about their own contribution to combat tiger mosquitoes and their view of the success of the campaign.

Metzgergrün in Freiburg

From June to October 2020, at three-weekly intervals, accessible gardens were inspected and treated with Vectobac WG in five rounds.

2.2.3. Integration of the Sterile Insect Technique (SIT)

The third pillar along with CP and DtD control was to challenge *Ae. albopictus* populations by sterile males under field condition. This included the logistic of a steady supply with sterile males, the quality control and their effect on field populations.

In this context, *Ae. albopictus* eggs collected in Heidelberg in the 2017, were used to start the mass-rearing at the Centro Agricoltura Ambiente “G. Nicoli” (CAA) in Crevalcore, Italy. This approach was chosen to prevent the use of mosquitoes with different genetic backgrounds.

Shipping from Italy to Germany was started following approval by the authorities.

2.2.3.1. Mass production of *Aedes albopictus*

An effective mass-rearing technology is essential to ensure sustainable large-scale production of high quality sterile males [31, 32, 33, 60]. In our program, the mass-rearing methods developed by the Insect Pest Control Laboratory (FAO-IAEA) have been adopted [32–35].

Sex sorting was performed by the Fay-Morlan glass sorters at the pupal stage in water [50]. This technique exploits the difference in size between male and female pupae. The aim was to keep the number of residual females to no more than 1% of the released sterile males.

2.2.3.2. Sterilisation by pupal irradiation

Pupal irradiation 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] to a dose of 35 Gray (1.85 Gy/min for 19 minutes). This dosage provides the optimal combination between male sterility and their competitiveness [30, 36].

After irradiation, the pupae were kept in a room at 28 ± 1°C for adult emergence. The young adults were chilled at 8–10°C and packaged for delivery to the field site by DHL flight express service. The time span between leaving the production facility and field release was always in the range of 20–24 hrs.

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

In each of three BugDorm rearing cages (BioQuip, CA, USA) thirty sterile males, randomly sampled from three different batches (SIT-Batches: 7, 8, 9; see Table 1) were individually released. Then, 30 virgin *Ae. albopictus* from our colony (Heidelberg strain) were introduced into each cage and kept at 25 ± 2°C and 70 ± 5% RH (dark: 8 h/light: 16 h). Three cylindrical dark containers (diameter: 7 cm; 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 support for oviposition and egg collection. In addition, a receptacle with cotton, soaked with a 10% sugar solution, 10 raisins and a piece of apple was offered as a carbohydrate source. Three cages with the same number of unirradiated males and females were included as control. At 24, 48, 72, 96, 120 and 144 hours, the forearm of the PI was offered for 20 minutes in each cage to allow the females to take an ad libitum blood meal. 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 removed and kept for five days in chambers with wet cotton (> 90% RH) to allow for complete embryogenesis. Then, the wooden boards were transferred into a hatching container (size: 22x7x4.5 cm) 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 25 ± 2°C to bleach the exochoria [52]. The boards were removed and all eggs (including the egg shells of the hatched larvae) were counted using a binocular (Motic, SMZ-171, Germany) and 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. It cannot be excluded that some of the developed embryos have chromosomal damages which doesn’t allow hatching or normal development. Thus, the sterility may had been underestimated. Eggs showing no embryonic structures were rated 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.

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

Reduction of wild *Ae. albopictus* populations by standard control measures prior to the application the SIT technique is essential to increase the efficacy. Even though the dose of radiation is known to damage the sperm but not the somatic cells of the sterile males [30], the sterile males could be less competitive than the wild males in the mating process. Thus, the effect of different ratios of wild males versus sterile males was tested. The design was the same as in the previous test series. In BugDorm cages (size: 30x30x30 cm) (BioQuip, USA), 30 females per cage were challenged with the following ratios of wild to sterile males: a) 1:1 (15 wild and 15 sterile males); b) 1:5 (5 wild and 25 sterile males); c) 1:10 (3 wild and 30 sterile males. In each cage, three cylindrical dark containers (diameter: 7 cm; height: 6 cm) half-filled with water and holding a wooden board for oviposition. As mentioned above, the same carbohydrate source was offered. Alike, after 24, 48, 72, 96 120 and 144 hours, an *ad libitum* blood meal was offered by the forearm of the PI. 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 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. Each trial was conducted in three replicates.

2.2.3.5. Shipment of sterile *Aedes albopictus* males

The shipment of the sterile males has to be cost-effective and timely, with the mortality rate of the caged males being as low as possible. In the course of our study, we tested the shipment in small round plastic containers (diameter: 5.2 cm; height: 4.7 cm) covered with a dark plastic lid. Each small container holds about 1000 radiated males. The small round plastic containers were packed in a second plastic container and an outer styropor box (size: 49x36x36 cm, 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 ± 2°C. The frozen cooling elements were in bubble wrap to avoid straight contact with the

containers holding the sterile males. According to the size of the area to be treated, the styropor box contained up to 30 small plastic containers. 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.

2.2.3.6. 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 approximate 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]. A percentage of 1% females per small container seems acceptable for us in order not to contribute to a nuisance situation caused by released *Aedes* females even if fully sterile.

2.2.3.7. Field application of SIT

a) Sector B in Melm, City of Ludwigshafen

The sterile males were released within trial area B (Melm, City of Ludwigshafen) after standard interventions by CP and DtD control. The release took place early evening at 7pm in 13 sites (Fig. 2; Table 1). Eighteen recurrent weekly releases between the 29th of May and the 7th of October, with more than 310,000 sterile males were carried out. This corresponds to a mean number of 1,013 sterile males released per week/ha within the 17 ha trial area.

Table 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**		**8,5±10%**

b) Metzgergrün, City of Freiburg

From 15th of June to 7th October 2020, 136,000 sterile males were released over the 4.5 hectares, which amounts to a total of 2,320 sterile males/ha (Table 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.

2.2.4. 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 egg counts and embryogenesis check on ovitraps.

a) Larval sampling

Breeding sites holding water were inspected for mosquitoes by aid of a torch and samples were collected with a plankton net and identified to the species level [38].

b) Ovitraps management

Standard ovitraps to determine the number of deposited eggs were the main tool to assess the effect of the intervention, also allowing an estimate of the population density [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 and 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. In order to prevent the 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 bi-weekly intervals and the water was replenished. The boards were marked with the date and site of collection, wrapped in paper foil and stored in a refrigerator until egg count. Random morphological determination was done by hatching some of the eggs and rearing to the fourth larval instar [37, 38]. Sterile eggs were identified as described above.

Deployment of the ovitraps in the test areas

a. Melm: Starting on 18th of May 30 ovitraps were evenly positioned across the 65 ha area, ten in each of the three sectors A, B and C (Fig. 2). The wooden boards with the eggs were collected bi-weekly between the 1st of June and 19th of October 2020. The number of eggs and embryogenesis were assessed as described above (Fig. 2).
b. Metzgergrün (Freiburg): Across the area of 4.5 ha, 21 ovitraps were positioned as described above. This district was heavily infested with *Ae. albopictus*, and therefore chosen as the SIT test area. Following DtD control and application of Bti (Vectobac WG), the release of sterile males started in middle of July (Fig. 3).
c. Gartenstadt (Freiburg): this district was chosen as control area without SIT application. It was similarly infested by *Ae. albopictus* as Metzgergrün. Eighteen ovitraps were installed. The wooden boards were collected bi-weekly between 15th of August and 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 (two sample assuming unequal variances) was applied to the quality control of sterile males test to evaluate the sterility of the eggs and to the cage experiments to assess the effect of the ratio sterile to fertile males (Microsoft Excel, version 16.45, 21011103)

3. Results

3.1. Community participation

A total of 1,820 households received written information on the control of *Ae. albopictus* and Bti-tablets in their mailboxes (less than one minute/household). The positive effect of this activity and the active cooperation of the residents is documented by the high rate permitting access to the 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 (Table 2). During the questionnaire survey in September, 517 estate owners answered the questions as follows: 298 residents 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.

Table 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

3.2. Door-to-Door control and Bti-treatments

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

Water containers were treated with a dose of 1 g Vectobac WG/ 20 litres ($1.35x10^5$ ITUs). The excellent persistence of mosquitocidal activity is shown in Fig. 4. Mortality of 100% was achieved during eight weeks, followed by a small decrease (of 1%) over the next four weeks. Only after 15 weeks mortality started to drop below 90%. These results were obtained with a weekly exchange of 25% of the water to simulate the use of collected rainwater to irrigate gardens.

Larval control of *Ae. albopictus* in large containers was achieved for a whole month with an initial application rate of ($6.75x10^6$ ITUs/container) as described in 2.2.2.1 (Fig. 5).

3.2.2. Efficacy of the DtD treatments

3.2.2.1. Efficacy of the DtD treatments in Melm, Ludwighafen

During the five DtD rounds, including two complete rounds (round 1 and 5) and three hotspot rounds (rounds 2–4) at properties and the surroundings when positive ovitraps or mass breeding sites have been observed, a total of 2.683 properties have been inspected. Detailed results are given in Table 2. Out of the 2.683 properties, 2.035 (75.85%) could be entered, while 418 (15.6%) owners were absent and 230 (8.57%) refused permission to enter. A total of 6.328 breeding sites including 83 rainwater containers were treated with a high Bti dosage to achieve a long-lasting effect and recorded in a Q-Gis programmes. Out of the 6.328 breeding sites 129 were infested with mosquito larvae (CI: 2%) and 18 were infested with *Ae. albopictus* (CI: 0.28%). The highest CI values were recorded end of August with 4.41% for all mosquitoes and 0.88% for *Ae. albopictus* (Table 2). The positive effect of the control strategy is documented with a CI of 0.49% for *Ae. albopictus* end of September. A total of 1.169 working hours of the two to six inspectors have been invested which results in an average inspection time/property of 26 minutes. Altogether 17.3 kg of Vectobac WG were applied to the 2.683 breeding sites.

3.2.2.2. 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 properties were treated, lasting five consecutive days. After each application round, the accessibility of the area was evaluated and recorded. Compared to other areas in the city of Freiburg, the accessibility of the properties was classified as ranging from poor to moderate. Throughout the season, residents were provided with blisters of Bti-tablets for self-help including 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, inseminated by sterile *Ae. albopictus* males amounted to 87.53 ± 9.15%, whereas the sterility of eggs derived from non-irradiated males and females was only 3.3 ± 2.8% (Table 3). The real sterility might be even higher because the bleaching method may fail in detecting embryos going through delayed mortality (data not published). According to the Student's *t*-test, all results are significant ($p \leq 0.001$). In contrary, 96.70 ± 2.8% of the eggs derived from non-irradiated individuals were embryonated, while only 12.47 ± 6.49% of the eggs laid by females inseminated by radiated males developed into embryos (Table 3).

Table 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%

3.3.1.2. 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 (Table 1). These data indicate a release of about 3,700 radiated females in Ludwigshafen (Melm) and 1,620 radiated females in Freiburg (Metzgergrün).

3.3.1.3. Determination of the most effective ratio between sterile and fertile males in cage experiments

The results of this experiment document the importance of the determination of the ratio between wild and radiated males (Fig. 6). The egg sterility of the non-irradiated populations amounts to 5.9 ± 6.6%. When the ratio between radiated and non-irradiated males is 1:1 (15 radiated and 15 non-irradiated 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 increases to 85.95 ± 3.18% (results are highly significant: $p \leq 0.001$). At a ratio of 1:10 (3 fertile males to 30 sterile males)

the sterility remained at a nearly unchanged level of 84.57 ± 4.00% (results are not significant different: p = 0.6649).

3.3.2. Field Application of SIT

3.3.2.1 Release of *Aedes albopictus* sterile males

A total of 310,000 radiated males were sent to Ludwigshafen (Melm) in 18 shipments, while a total of 136,000 males were sent in 13 shipments to Freiburg (Metzgergrün) by DHL overnight services (Table 1). All shipments reached its destination within 24 hours. The mortality of the irradiated males during transport averaged 8.46 ± 10.07%.

In Table 1, the exact numbers of released sterile males are recorded, which amount to the release of about 1000 sterile males/ha on a weekly basis from the 29th of May to the 7th of October in Ludwigshafen (Melm) and of 2300 sterile males/ha from the 15th of July to the 7th of October 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 of *Ae. albopictus* from 16th of May to the 5th of October 2020 (Table 5). The mean number *of Ae. albopictus* eggs per trap/two weeks was 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 with a total of 301 eggs (Table 4).

Table 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.**	**10.8.20.**	**24.8.20.**	**7.9.20.**	**Total**
total no. of eggs	45	88	216	173	301	96	919
Section A							
total no. of eggs	45	64	120	8	34	3	274
no. of sterile eggs	2	6	103	0	6	3	120
sterility %	4.44%	9.37%	85.83%	0	17.65%	100%	43.8%
Section B-SIT							
total no. of eggs	0	24	91	101	114	90	420
no. of sterile eggs	0	23	59	84	91	90	347
sterility %	0	95.83%	64.84%	83.17%	79.82%	100%	82.62%
Section C							
total no. of eggs	0	0	0	64	153	3	220
no. of sterile eggs	0	0	0	8	74	3	85
sterility %	0	0	0	12.5%	48.37%	100%	38.64%
See also Supplement to Table 4							

Table 5
Number of *Aedes albopictus* eggs and percentage of sterility of the 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%

The weekly release of about 1,000 sterile males/ha from early May until early October in the SIT area (section B) resulted in an overall sterility of 82.62% (Table 4). The sterility of the 274 eggs collected in section A was 43.8%, and of the 220 eggs collected in section C was 38.64%. The relatively high sterility in the adjacent non-SIT sections A and C can be explained by the migration of sterile males and/or females mated with sterile

males into the non-SIT control areas. The fact that no eggs were recorded in any of the ovitraps after the 7th of September indicates the successful control strategy.

In the second half of September, 2,668 breeding sites were inspected and only 13 breeding sites contained the larvae of *Ae. albopictus.* 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 (Table 5), which was about six-times higher when compared to almost the same time period in the SIT area in Ludwigshafen. 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 96.6% on the 13th of October (Fig. 7) The mean sterility was 77.4 ± 15.35%.

In the control area (without SIT) (Freiburg Gartenstadt) on the collection dates from 29th of August to the 13th of October (4 x bi-weekly sampling), 1,615 eggs were collected (Table 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 4 hectares. Of the 105 inspected breeding sites, 29 contained developmental stages of *Ae. albopictus*, resulting in a CI_{albo} of 27.7%.

4. Discussion

The goal of this large-scale integrated field trial was the maximum reduction or even the elimination of established populations of *Ae. albopictus*. In addition to the two pillars CP and DtD control by trained staff with long lasting Bti-treatment, the SIT was used as the third pillar. CP and DtD are important to reduce *Ae. albopictus* in obvious and easily accessible breeding places. Sterile males are able to find remaining females in hidden mating places. The selected trial sites, infested by *Ae. albopictus* in the urban environments of the cities of Ludwigshafen and Freiburg, were ideal for the three-pillar approach. The majority of *Ae. albopictus* breeding sites are located on private properties, thus it must be in the own interest of the residents to prevent breeding of *Ae. albopictus* in close proximity. If properly instructed and using Bti, the collaboration with residents is cost-effective and sustainable [25, 26, 43]. The support by DtD activities conducted by trained staff proved to be the backbone of the integrated control approach. The cooperation between the residents and the DtD teams still leaves room for improvement since the minimum of 95% of accessible properties was not achieved [29]. The mean costs involved in the control measures by CP and DtD control amounted to € 2.50 per property.

The control of the container-breeding *Ae. albopictus* requires a long-lasting larvicides to make it cost effective and efficient. This goal was achieved with increased doses of Bti [45]. The results obtained in Ludwigshafen were convincing. As a result of the stringent Bti application the container index of *Ae. albopictus* was pushed down from 10.9% to below 1%. (Table 2). In contrast to the previous year, no nuisance by residents was reported. This is also underlined by the low number of eggs found in the ovitraps in the Melm area (mean number/ovitrap 4.3). In the control area Freiburg Gartenstadt six ovitraps contained considerably more than

100 eggs/ovitrap and a maximum of 340 eggs/ovitrap/two weeks. Carrieri et al. [49] calculated the epidemic risk threshold for a Chikungunya transmission based on the mean egg density/trap/week. Infections can already occur when the average number of eggs/trap/week is above 44 eggs. In the control area of Gartenstadt this threshold was exceeded. The climate change with rising temperature will very likely accentuate this problem.

Risk-benefit parameters do not apply to the integrated approach for the control of *Ae. albopictus*. The use of Bti even in higher doses is safe and the release of sterile males do not bear any negative impact. The cost-benefit factor needs of course special consideration. In general, the costs are an up-front investment to prevent spread and high density of *Ae. albopictus* populations. Protection from autochthonous viral infections must have the highest priority. Thus, the costs to protect from *Ae, albopictus* amount to an estimated € 2 per person per season. This is highly cost effective considering the severe potential implication for public health.

Germany, like other countries north of the Alpes, are at the beginning of the invasion by *Ae. albopictus*, and thus confronted with a big challenge. Containment of *Ae. albopictus* will only succeed by close collaboration between all the stakeholders, from the directly affected residents to the field workers, researchers, up to government agencies. [39]. The importance and urgency to combat the Asian tiger mosquito has been recognized by all levels of the Federal Republic of Germany. Programs for surveillance, risk assessment and the evaluation of control tools are actively supported by the Federal Agency for Environmental Protection (UBA) in cooperation with the Friedrich Löffler Institute (FLI) and the Bernhard Nocht Institute (BNI). The State of Baden-Württemberg, which is currently most affected by *Ae. albopictus*, financed risk assessment programs concerning the establishment of *Ae. albopictus* on a community level [24].

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 both 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 in case of outbreaks only. 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].

Conclusions

This field pilot trial shows that an integrated control program is most effective when it comprises three pillars, namely a) community participation, b) DtD activities including long-lasting Bti-larviciding of all accessible larval sites to strongly reduce wild *Aedes albopictus* population as a precondition a successful application of SIT, and c) SIT to strongly reduce or even eradicate *Ae. albopictus* populations. The combination of Bti and SIT, two highly effective, selective and safe measures may allow the achievement of *Ae. albopictus* suppression without any negative impact on the public health and the environment.

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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Figures

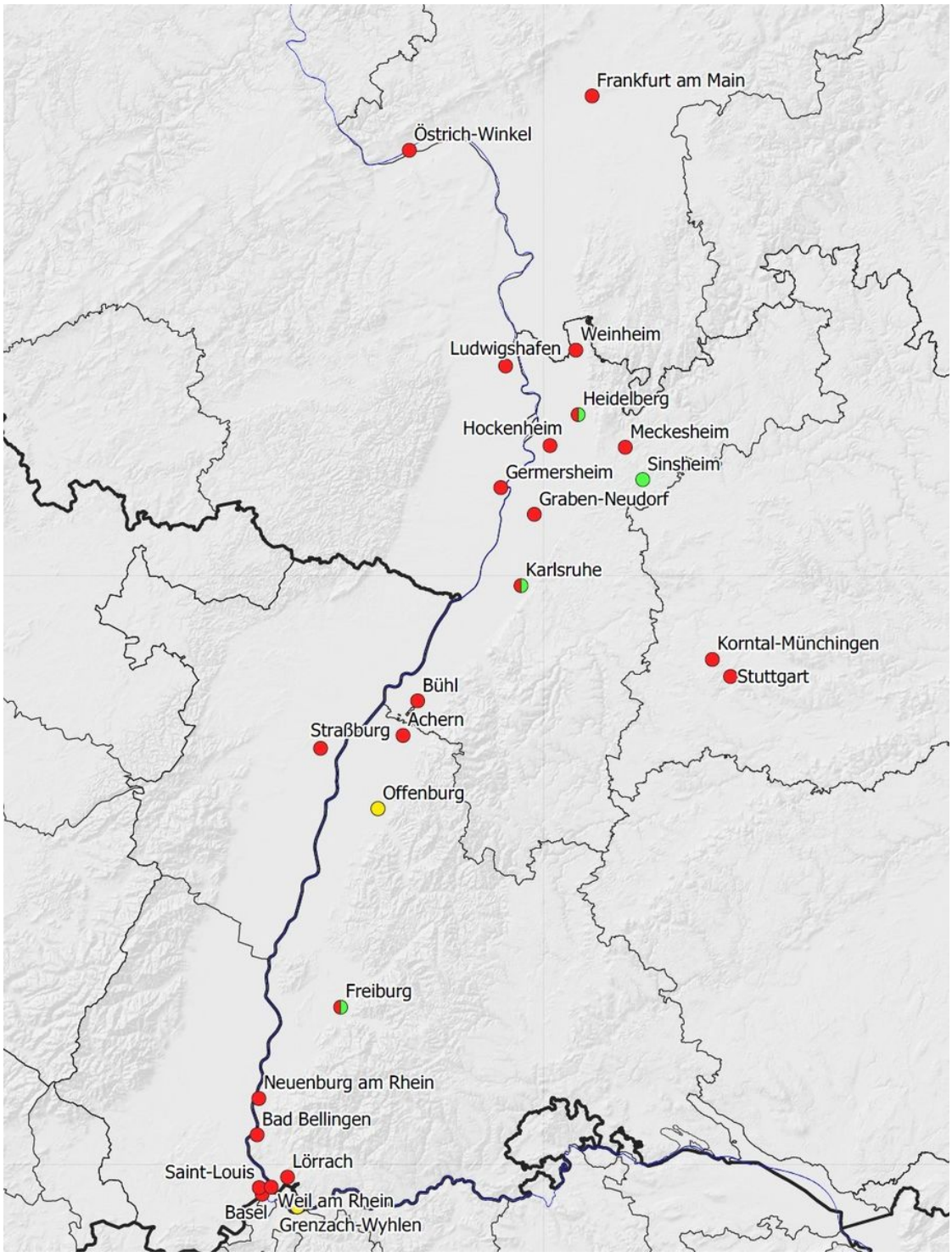

Figure 1

Recorded Aedes albopictus populations in 2019 (red: not under control; green: eradicated; green-red: strongly reduced; yellow: population size not known).

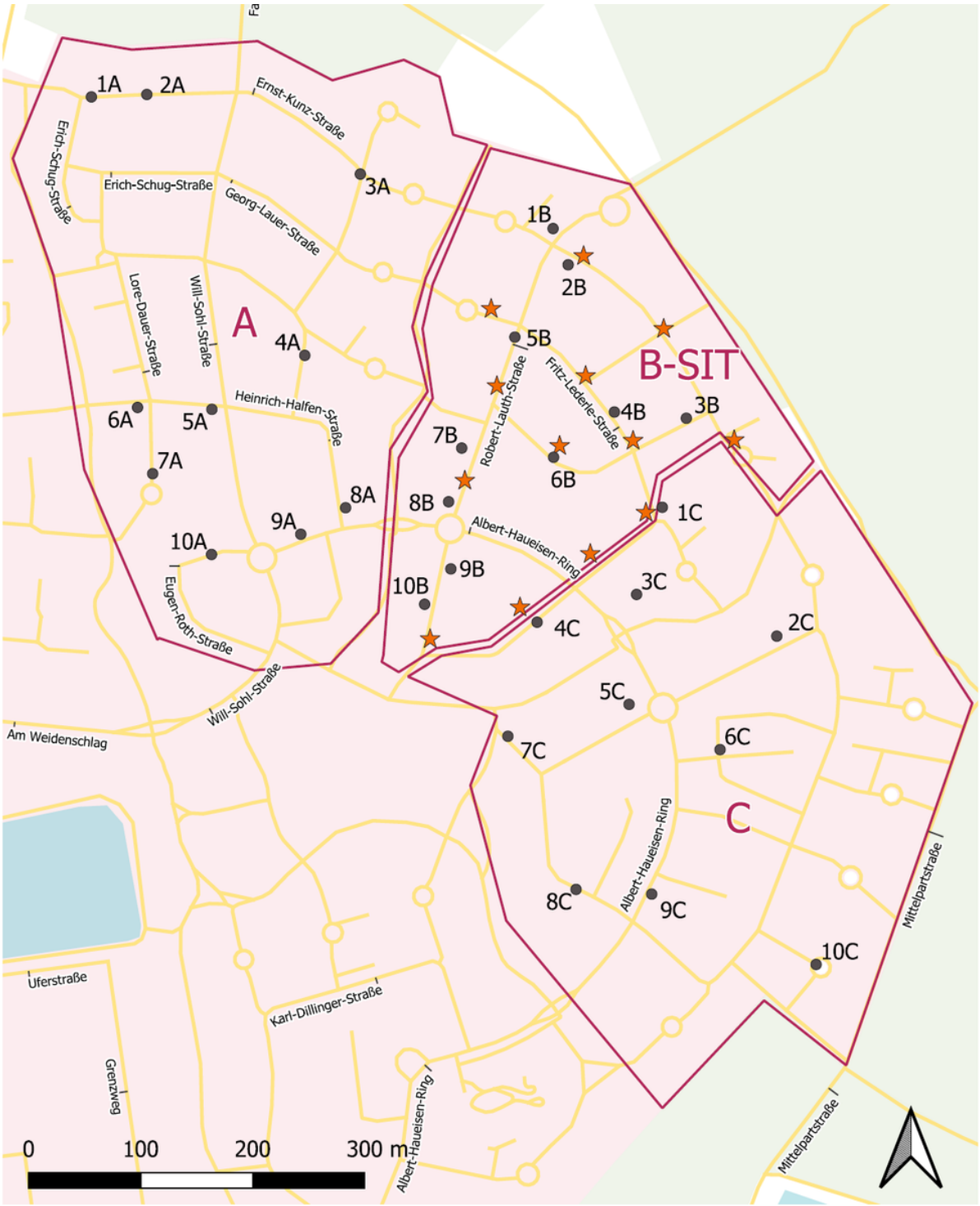

Figure 2

Study area Melm, positions of ovitraps and release spots for sterile males in Ludwigshafen

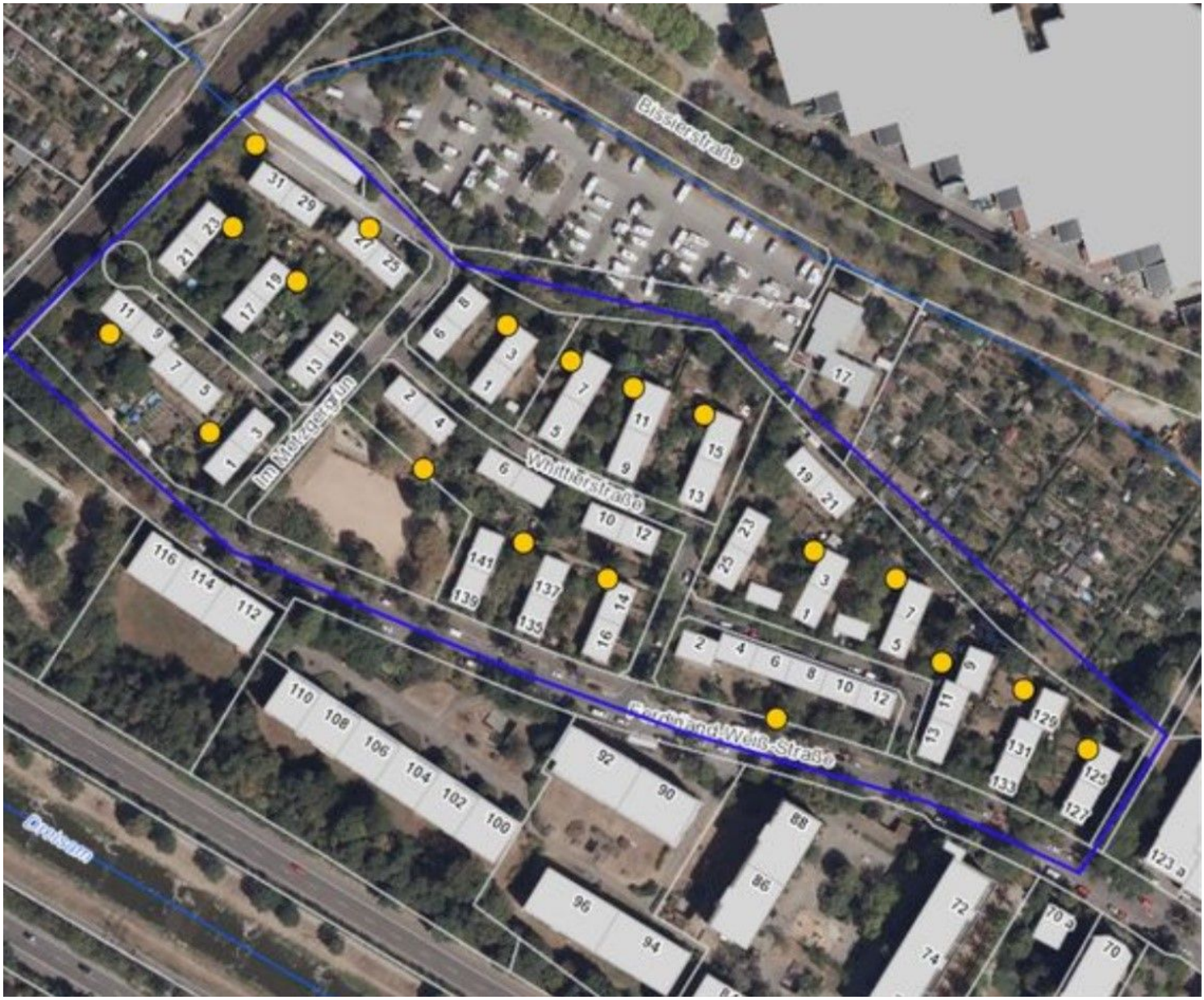

Figure 3

SIT area “Metzgergrün” in the city of Freiburg (purple: release area; yellow spots: location of the ovitraps).

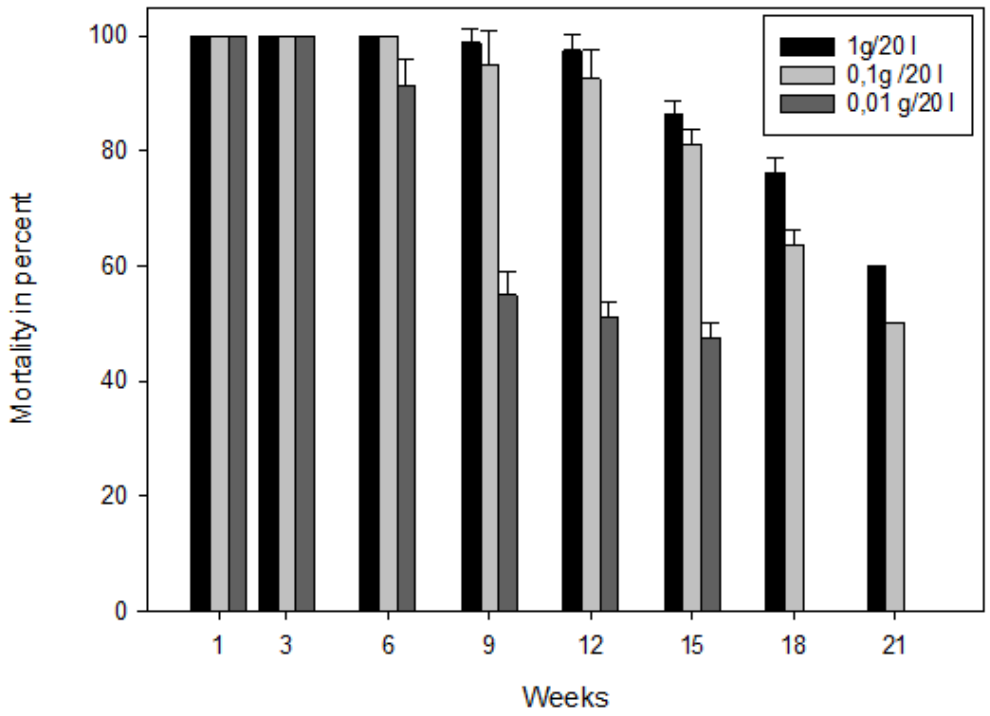

Figure 4

Long-term effect of Vectobac WG on Aedes albopictus applied to large water containers.

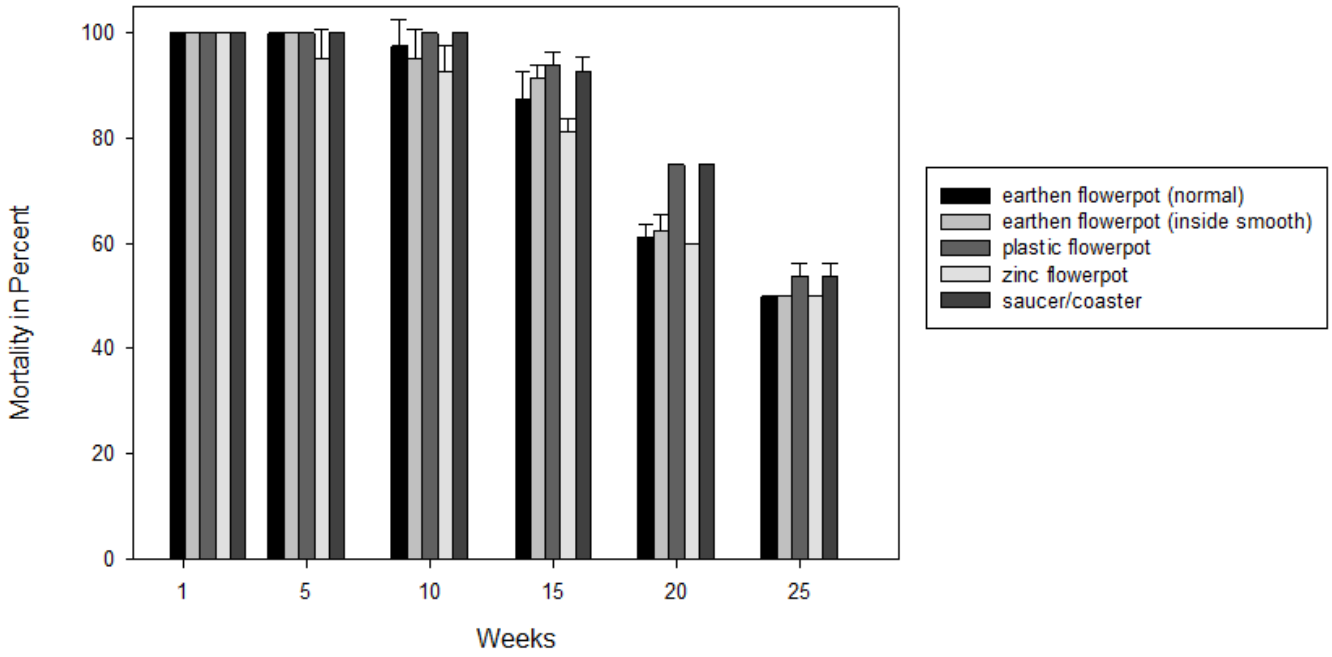

Figure 5

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 coasters

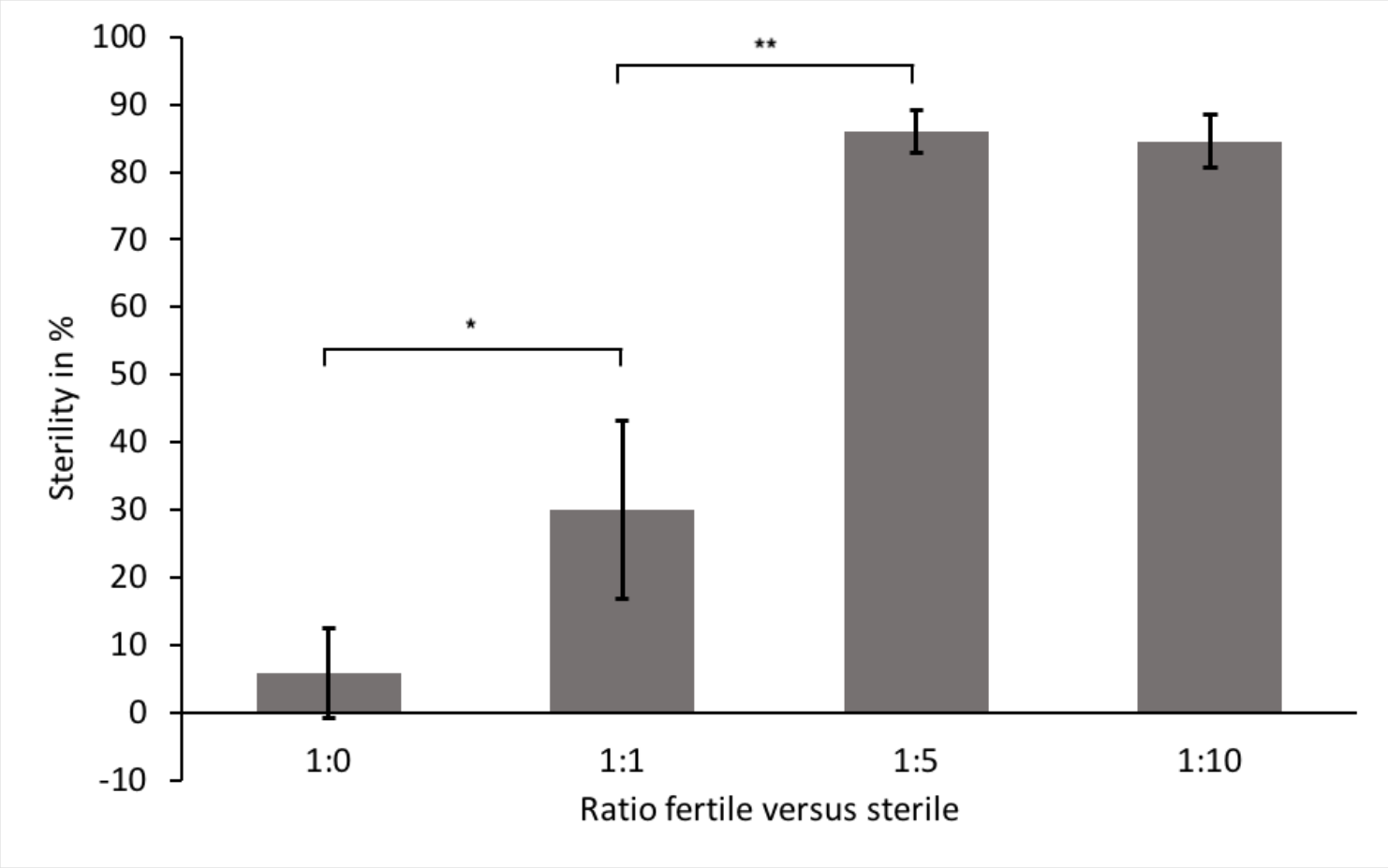

Figure 6

Effect of the ratio between radiated and non-irradiated males on the egg sterility rate. (see also Supplement 4 to Fig. 6)

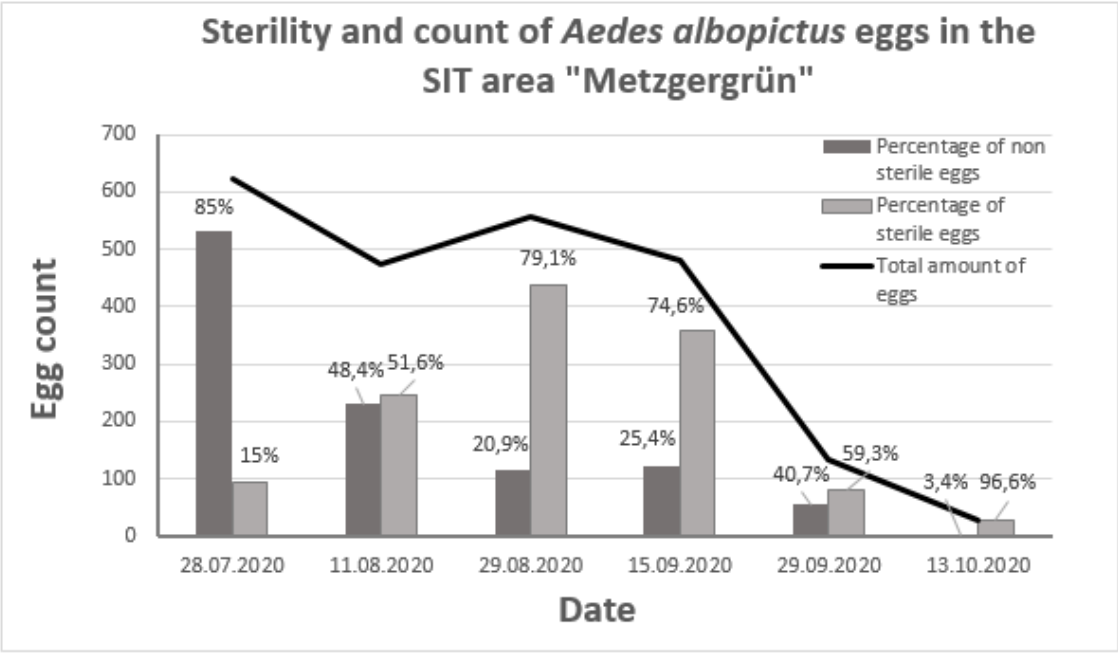

Figure 7

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

Supplementary Files

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