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Top-down effect of fish presence on mosquito assemblages in the city of Padova

Relatore:
Prof. Alberto Barausse

Diplomando:
Alessandro Rosa

“Non si può restare soli,
se sei in Gali qui”
(Studio 206)

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ABSTRACT

Throughout history mosquitoes always represented a problem to humans for their ability to act as a vector for many diseases. Due to a combination of globalization and global warming new species of mosquitoes are expanding their geographic range posing a threat to people. In Italy tropical diseases such as West Nile (1998) and dengue (2020) are spreading. In particular in the province of Padova a total of 101 cases of West Nile were recorded in summer 2022. Also, concurrently, a mass fish die-off connected to drought conditions occurred in the city of Padova. To investigate a possible causal connection between the two phenomena, given that fish can be insectivorous, two on-field experiments were set up to explore the presence and extent of a top-down regulatory effect of fish on mosquito assemblages in the urban canals of Padova. The experiment in the wild failed in detecting mosquito larvae, but interesting insights were obtained from the controlled experiment, regarding the species assemblages (in particular, the presence of *Culex pipiens* was detected even in the city center, together with the expected *Aedes albopictus*) and their oviposition preference to different degrees of fish cue exposure. A total of 2771 mosquito larvae were collected through the three weeks of the experiment and 2073 of them were identified to the genus or species level. While *Aedes* individuals were found in all the locations, the *Culex* mosquitoes appear to be more common farther away from the city center, but where present they showed a marked preference for the water containing dead fish, where they were the most abundant species, but this preference faded as time passed. Conversely *Aedes* females avoided all kinds of fish cues, with a preference for the tap water environment which continued to be very productive for them throughout the study period. In conclusion it appears to be possible for the city canals to rapidly generate *Culex* mosquitoes outbreaks after an episode of mass fish death, causing potential public health emergencies in case of drought events and/or channel mismanagement.

1. INTRODUCTION

1.1 Biology of mosquitoes

Mosquitoes are insects belonging to the family Culicidae, a group of Nematoceran Diptera. The family Culicidae comprises about 3500 species found all over the world except permanently frozen areas and with the greater diversity found in the tropics (Rueda, 2008). The family demonstrated to be very successful, being found in forms similar to the contemporary ones since the Mesozoic era (Becker et al., 2010). Like all Diptera, mosquitoes undergo a complete metamorphosis. All mosquitoes require aquatic habitats to complete their development, but some species of the genera *Aedes* and *Ochlerotatus* are able to develop in humid soil (Becker et al., 2010).

1.1.1 Larvae and Pupae

Mosquito eggs are divided into two main categories: floating eggs either singly (Anophelinae) or in rafts (e.g. *Culex, Coquillettidia, Culiseta*) that hatch soon after being laid (2-7 days, depending on the temperature) and eggs that do not hatch immediately (e.g. *Aedes, Ochlerotatus, Culicella*), are laid in moist soil or in containers just above the water level, because they do not possess floating adaptations and would sink and drown if submerged. After the embryo is developed it hatches as soon as the egg is submerged again (Becker et al., 2010).
After the eggs hatch the larvae are released into the water. The larvae possess a head with mouth parts, eyes and antennae, a thorax and ten abdominal segments, seven of them are almost identical, while the last three are specialized for osmotic regulation (papillae) and breathing (siphon). The segment eight bears a siphon, in the subfamily Culicinae, or a spiracle, in the subfamily Anophelinae. These structures possess retractable lobes to break the surface tension and glands that produce a hydrophobic substance to prevent water to flow in the respiratory system. An exception are the larvae of the genera *Coquillettidia* and *Mansonia* which possess saw-bladed siphons that are used to pierce the stems of aquatic plants and breath through their aerenchyma (Becker et al., 2010).
There are two main feeding strategies among mosquito larvae: some of them are filter-feeders (most Culicini) and hang straight down the water surface and use their head brushes to create a current that brings the organic particles towards the mouth, while others are browsers that use their mouthparts to re-suspend and/or shred organic material from the bottom (most *Aedes* and *Ochlerotatus* species) or the water-air interface (Anophelini) (Becker et al., 2010).

Larval development is usually very fast (from few weeks to few days), but highly dependent on water temperature with the optimum being very different between species. After four molting events, the larvae reach the pupal stage when the imaginal disks start proliferating to form the adult tissues at expenses of the energetic reserves stored in the body of the larva (Becker et al., 2010).

Pupae possess two respiratory trumpets on the thorax to breathe air, they do not feed and their development is usually very short (one or two days). Differently from most of other insect species, mosquito pupae are very mobile, being able to swim to the bottom of the water source where they are present by twitching their abdomen that has two paddle-like appendages at the tip (swimming to the bottom and waiting for the disturbance to pass is also the only defense mechanism of the larvae) (Becker et al., 2010).

1.1.2 Adults

The adults emerge from the pupae at the water surface and are able to fly as soon as the exoskeleton has hardened in few minutes. They possess the general adult insect anatomy, with the body divided into a head with antennae, eyes and mouthparts, a thorax with six legs, two wings and two halteres (characteristic of Diptera), and an abdomen, carrying the reproductive apparatus (Becker et al., 2010).

Male mosquitoes become sexually mature one day after emergence so they usually develop faster as larvae in order to be sexually mature at the time of female emergence. Mating occurs soon after the emergence of the females, which mate only once during their lifetime, storing the sperms for several egg batches. Males after copulation secrete a substance called matronae from reproductive glands that makes the female unreceptive. On the contrary, males can mate several times and can recognize a receptive female by the frequency of their wingbeat, which is detected by the plumose antennae (Becker et al., 2010).

Both sexes possess specialized piercing/sucking mouthparts. They feed on plant juices as a carbohydrate source, while the females of almost all species, being anautogenous, have to obtain blood from an external host as a source of proteins for egg maturation (Becker et al., 2010). Once the female mosquito penetrated the skin of the host with its stylets, saliva is injected in the wound to prevent coagulation, since it contains anticoagulants similar to hirudin (from leeches) (Parker & Mant, 1979). The saliva elicits an immune response from the body of the host, causing itching in the area. ADP and ATP found in the blood act as stimulants to begin sucking. The total amount of blood can get up to three times the mosquito body weight (Becker et al., 2010). Some species can be autogenous, with females being able to develop the first batch of eggs without a blood meal, even though these eggs show reduced hatch rate (O'Meara, 1985; Weitzel et al., 2009). Regarding feeding and resting behaviour, females can be divided into four categories: endophagic or exophagic if they prefer biting indoors or outdoors and endophilic or exophilic if they prefer to rest indoors or outdoors after the blood meal (Becker et al., 2010). This behaviour is tightly connected to the ability of dispersion of the different species, which can span from few hundred meters to several kilometers during their lifetime, for example species related to urban environments such as *Aedes albopictus* or *Aedes aegypti* tend to move around 150-300 meters on average, while other species that prefer rural areas like *Culex pipiens* or *Anopheles atroparvus* tend to spread for more than 1500 m on average (Tsuda et al., 2008; Guerra et al., 2014; Moore & Brown, 2022). Females tend to spread farther than males and can sustain the flight longer (Cui et al., 2013), even though the flight performance is dependent on the temperature (Reinhold et al., 2018).

Mosquito movements can be divided mostly in three categories: passive dispersal, host-seeking and oviposition. The passive dispersal occurs mostly in the first hours or days of adult life, with the mosquitoes being carried away by the dominant winds, with high concentrations of mosquitoes that can appear in areas with no breeding spots (Bidlingmayer 1985). The host-seeking behaviour kicks in after encountering host stimuli and is characterized by a directional flight: this is achieved by a zigzag motion going to the source of the chemical compound, which is usually carbon dioxide (and also lactic acid) (Smith et al., 1970; Price et al., 1979). Different mosquito species target different hosts so their distribution is different too, for example *Culex* and *Culiseta* are more ornitophilic and found mainly in the canopy of trees, while *Aedes* and *Ochlerotatus* prefer mammals as hosts and are found at ground level (Becker et al., 2010), but in general spreading vertically does not seem to be a problem even for species usually found near the ground, being able to colonize high urban environments such as skyscrapers with no issues (Liew & Curtis, 2004). The last dispersion mechanism of the females is oviposition. The females, not providing parental care to the offspring, try to find the most suitable environment to lay the eggs. The factors that determine the suitability of a site are multiple, for example the physical and chemical properties of the water such as pH, salinity (Medeiros-Sousa et al., 2020), dissolved oxygen and conductivity (Yadav et al., 2012), volatile compounds exhalating from the water that seem to have a big influence: from conspecifics (Dhileepan, 1997), plant communities (Becker et al., 2010, Yadav et al., 2012), organic substance degradation (Becker et al., 2010) and predators, such as fish (Angelon & Petranka, 2002; Kraus & Vonesh, 2010), water bugs, Odonata and Caudata larvae (Vonesh & Blaustein, 2010). Finally other factors influencing the oviposition are the size of the breeding spot which can be linked partially with predator avoidance or the usual size of breeding spots present in the environment where they evolved into (Sunahara et al., 2002) and the flow condition of the environment, with mosquitoes preferring still waters because larvae are weak swimmers (Yadav et al., 2002).

1.2 Ecology of mosquitoes

Mosquitoes are found throughout the world and exhibit a wide range of adaptations, so there is almost no water source that at least a species cannot colonize. Looking at the life cycle, mosquitoes can be divided roughly into three categories: tropical species, floodwater mosquitoes and snow-melt mosquitoes (Figure 1).

The tropical species live in environments that are always hot and wet, so they can breed throughout the year, but can survive the dry season as dormant embryos (Minakawa et al., 2001).

Floodwater mosquitoes are mostly summer species adapted to hot temperatures. These species are able to quickly colonize occasionally inundated areas after heavy rains, due to the eggs, laid previously in moist areas, and a very rapid larval development. These species are usually multivoltine and the last generation before winter lay eggs that are able to undergo dormancy as an overwintering strategy (Becker et al., 2010). Given the fact that at the beginning of the spring there are no adults present, the peak of presence of these species is delayed to late summer (August/September) (Marini et al., 2017).

Snow-melt species are usually univoltine and lay eggs in wet areas formed by the spring melting water, thus they are adapted to very cold temperatures. In these mosquitoes are usually the adult females that survive the winter entering diapause in repaired shelters (Becker et al., 2010). The females are able to lay eggs as soon as the temperatures rise enough to fly, so great abundances of these species can be found earlier in the season (June/July) (Marini et al., 2017).

Some of these species are known to overwinter also as larvae, that, with reduced metabolism, are able to survive even some days under a frozen surface (Becker et al., 2010).

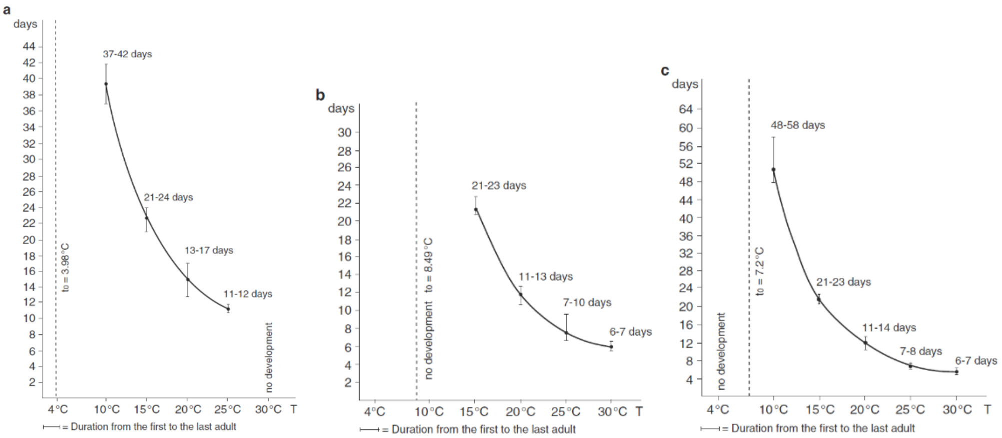

Figure 1. Larval development of three ecologically distinct species in relation to temperature: a) *Ochlerotatus cantans*, a snow-melt mosquito species, b) *Aedes vexans*, a floodwater mosquito, c) *Culex pipiens*, a common widespread species. Image from Becker et al. (2010)

1.3 Mosquitoes as disease vector

Mosquitoes are vectors for many medically relevant pathogens and parasites such as protozoans, nematodes, bacteria and viruses causing diseases such as malaria, yellow fever, West Nile fever, Chikungunya fever, dengue and more. The transmission can be mechanical, with the direct passage of the pathogen from the mosquito to the individual, or biological, with the mosquito as an intermediate host for pathogen development (Becker et al., 2010). The transmission of these pathogens can occur both horizontally, between an infected mosquito, a host and a healthy mosquito, and vertically, between the infected female mosquito and the eggs, giving the possibility to certain mosquito-borne diseases to overwinter (Becker et al., 2010).

Now a brief description of the main mosquito-borne diseases is provided.

-Malaria is caused by organisms of the genus *Plasmodium* spp. that use mosquitoes as hosts for sexual reproduction, while reproducing asexually in vertebrates (Becker et al., 2010). The disease is transmitted by mosquitoes of the genus *Anophele* and was endemic in Europe until WWII, after which better sanitation and agricultural practices (such as widespread use of DDT) eradicated the problem (last indigenous case in 1975 in Greece) (Bruce-Chwatt et al., 1975; Jetten & Takken, 1994).

-Chikungunya fever is of arboviral origin (Togaviridae) and is transmitted by mosquitoes of the genus *Aedes* spp. It is usually distributed in Asia and Africa, but in 2007 an outbreak occurred in the Ravenna province in Italy caused by a tourist returning from India (Becker et al., 2010).

-Yellow fever is of arboviral origin (Flaviviridae) and is transmitted by mosquitoes of the genus *Aedes* spp., in particular *Aedes aegypti*. It caused severe epidemics in Western Europe in the 1700s, but now thanks to mass vaccination and vector control programs it is concentrated mostly in Africa and South America (Becker et al., 2010).

-Dengue is of arboviral origin (Flaviviridae), is transmitted by *Aedes* spp. mosquitoes (mostly *aegypti* and *albopictus*). It is caused by four sierotypes of Dengue virus, tropically distributed worldwide, with the first epidemics in the late 1700s in Asia, Africa and North America. Nowadays is spreading rapidly due to increased abroad tourism and global warming, with the first cases in Europe that occurred in Athens in the early 1900s (Becker et al., 2010). The first case reported in Italy was in 2020.

-West Nile virus is of arboviral origin (Flaviviridae), is transmitted mainly by *Culex pipiens*, but also by other *Culex* spp., *Ochlerotatus* spp. and *Anopheles* spp. species and the principal host are birds. The virus was first isolated in Africa in the 1930s and the first outbreaks in Europe occurred in the following years (Camargue), the it spread though entire Europe in the late 1990s. The first case was reported in Italy in 1998 (Becker et al., 2010), but in recent years the cases multiplied getting up to almost 300 in 2022 (101 only in the Padova province) (Istituto Superiore di Sanità, 2022).

-Filariasis is caused by nematodes of the genera *Wuchereria* spp. and *Brugia* spp. found in the tropics and subtropics worldwide. The most important vectors are *Culex pipiens* and *Mansonia* spp. Differently to other mosquito-borne diseases, filariasis is still well confined in the tropical area and is of no medical importance in Europe (Becker et al., 2010).

1.4 Mosquito species in Italy

Almost 60 species belonging to the family Culicidae are reported for Italy (Italian Fauna Checklist, 2024), but the most common in urban areas are the Asian Tiger mosquito (*Aedes albopictus*) and the common house mosquito (*Culex pipiens*) (Zamburlini et al., 2019; Toma et al., 2020).

1.4.1 *Aedes albopictus*

The Asian tiger mosquito (*Aedes albopictus*) is probably the most common species in urban environments in the Po river valley and Veneto-Friulana plain. It is native to the Southeast Asia but thanks to the eggs being resistant to desiccation they have been transported with tropical plants or tires for long distances colonizing almost all continents. In Italy the species was firstly described in 1990 in Genova, but in few years spread throughout the country (Becker et al., 2010). It is highly adapted to colonize urban environments, choosing as breeding spots every small natural or artificial container (tree holes, bamboo stumps, flower pots, tin cans, broken glass bottles, manholes), but usually avoiding larger water sources (Huang, 1972). The eggs are laid on the walls of the container just above the water level and have to complete the embryonic development before being submerged again and hatch, but after that the development is relatively fast (12-15 days from hatching to the emergence) (Becker et al., 2010). The females prefer biting mammals (especially humans) at dusk or in daytime in shaded areas (Becker et al., 2010). They do not spread much, with most studies indicating a mean flight distance of few hundred meters at most (Guerra et al., 2014). It is a competent vector for some arboviral diseases like Chikungunya and dengue, so the populations have to be monitored to avoid spreading of these diseases by tourists coming back from tropical countries (Becker et al., 2010).

1.4.2 *Culex pipiens* complex

There are several subspecies that make up this complex with very subtle morphological differentiation, especially among females, but the main three taxa are *Culex pipiens pipiens*, *Culex pipiens pipiens* biotype *molestus* and *Culex pipiens quinquefasciatus* (Becker et al., 2010).
The last one is spread worldwide in the tropical area, can reproduce all year long, does not undergo diapause and is strictly anautogenous. It is very common in urban environments, resting and biting indoors. The main difference between the first two taxa is the adaptation to urban environment. The *Cx. pipiens pipiens* females always enter diapause in winter and lay eggs in semi-permanent or large

water sources, are mainly ornitophilic and usually anautogenous. On the other hand the females of the biotype *molestus* are able to reproduce overwinter in warm, humid, protected areas, such as sewage systems or cellars, always autogenous, being able to bite soon after the first batch of eggs have been laid, and prefer to bite mammals instead of birds (Becker et al., 2010). Both of the biotypes are found across Europe, but the biotype *molestus* have been reported only for some of the largest cities (Becker et al., 2010).

All of the taxa in this complex prefer biting at night and laying eggs in natural or artificial water containers, usually larger than those chosen by *Aedes albopictus*. Given the fact that these environments are relatively rarer than the small containers, the females of this species spread more than the *Aedes albopictus* counterparts, with a mean flight distance between 800 and 1800 meters (Cui et al., 2013; Guerra et al., 2014). The eggs are organized in rafts of 150-240 eggs, floating on the water surface and the development is very fast (8-15 days from the egg to the adult) (Becker et al., 2010). The species of this complex are the main vectors of the West Nile virus that is now endemic in northern Italy, so the control of the populations is of great importance (Becker et al., 2010).

1.5 Top-down effects on mosquitoes

The top-down effect refers to the control exerted by a higher trophic level on a lower trophic level, mainly through predation (Carpenter et al., 1985). This effect has been well documented in freshwater environments for example with the depletion of phytoplankton by freshwater mussels (Caraco et al., 1997; Schol et al., 2002) or to study the effect of fish release for fishing purposes (Rosenfeld, 2000; Nystrom et al., 2003; Meissner & Muotka, 2006), but the results are not always obvious and can be influenced both by the specific characteristics of the study site (flow regime, turbidity, nutrient availability) and by the omnivory degree of the system, because it is difficult to see a clear top-down effect on the food web if the top predator feeds on more trophic levels (Meissner & Muotka, 2006).

Mosquito larvae in their environment occupy a low-intermediate trophic level, feeding indiscriminately on a vast array of microscopic organisms such as bacteria, protozoans and rotifers effectively reducing their density in a water source (Mitchell, 2009). They are in direct competition mostly with other filter feeders especially cladocerans, ostracods and copepods with whose abundance there is a negative correlation (Ranasinghe & Amarasinghe, 2020). Given the fact that are not very mobile, mosquito larvae (and adults while ovipositing or emerging) are easy prey for many freshwater predators and in particular water bugs (Notonectidae and Gerridae) (Sih, 1986), Cyclopoid copepods (Baldacchio et al., 2017), Odonata, Coleoptera and Caudata larvae and fishes (Onyeka, 1983; Sunahara et al., 2002). Some fish species have also been employed for mosquito control in certain areas, even though not always with the hoped results given the fact that they caused an excessive predatory pressure also on other invertebrate taxa (Chandra et al., 2008). In general, fish have proven very effective in mosquito predation in various occasions, with in some cases with an almost perfect exclusion (Marti et al., 2006), but in other cases the effect was more species dependent (Louca et al., 2009). But from various studies it seems that the main effect of the fish presence on mosquitoes is indirect, because fish cues (or predator cues in general) appear to discourage oviposition (Angelon & Petranka, 2002; Louca et al., 2009; Kraus & Vonesh, 2010), even though the response is taxa dependent, with a marked avoidance for fish, water bugs and Odonata larvae, less avoidance for Notostracans, larvivorous Diptera larvae and Caudata larvae and a marked preference for Cyclopoid copepods (this is because this kind of larvivorous copepods actively produce volatile substances to induce female mosquito oviposition) (Vonesh & Blaustein, 2010). Moreover it seems that the larvae are also affected negatively by predator cues, showing reduced growth and survival (Beketov & Liless, 2007).

2. OBJECTIVE

The mosquitoes pose a significant threat to human health being carriers of many diseases, and being able to spread them very efficiently due to the blood-sucking behaviour of the females. As a result of this, measures to contain their populations, especially in very densely populated areas, are continuously implemented and updated.

Despite this, in 2022 the province of Padua recorded the highest number of West Nile cases in Europe (European Centre for Disease Prevention and Control, 2023), a disease carried by mosquitoes of the *Culex* genus. Interestingly, in the case of the city of Padova, this phenomenon was concurrent with a mass fish death in the urban canals, connected to the effect of a prolonged drought (Faccin et al., 2023).

In normal conditions the ability of mosquitoes to proliferate is controlled by the presence of various predators, particularly fish, that act both directly (active predation on al the stages) and indirectly (discouraging oviposition by the females), reducing the potential water sources available for mosquito reproduction. While some species evolved to reproduce in very small water sources and are very abundant also in the city centers, other, preferring larger bodies of still water, are forced to the urban outskirts.

The aim of this study is to test how determinant is the presence of predator fish to discourage the female oviposition in a certain area, if this pattern is the same across different species, and how these species are distributed throughout the urban area. This goal was accomplished by both a manipulatory experiment under natural conditions, during which was it tested if the absence of fish from a section of a canal was sufficient to allow mosquito proliferation, and a mesocosm experiment, where suitable environments for oviposition (containers filled with water) were exposed to a gradient of fish cues, also with a condition that mimics an episode of mass fish death.

3. MATERIALS AND METHODS

3.1 Study area

The experimental study was carried on in the city of Padova which has an intricate network of urban canals (Figure 2). From the "Ponte dei Cavai" upstream, the water runs in the "Tronco Maestro" canal, then it divides a first time in the "Alicorno" canal (which supplies water to the moat in Prato della Valle square) and a second time in the "San Michele" and then "Santa Chiara" canal, before continuing north and changing name in "Piovego" canal after receiving the wastewaters from the "Fossa Bastioni" moat. The Piovego canal runs through and outside of the city until it flows in the Brenta river. Alicorno and Santa Chiara canals flow through the city center and merge just before running in a subterranean section and emerging as "San Massimo" and then "Roncajette" canal, which then flows east and south, merging with the Bacchiglione river outside of the city. Moreover throughout the city various wet areas are present such as public and private ponds, swimming pools, fountains and manholes that can act as possible breeding sites for mosquitoes.

In order to have a good coverage of the urban environment while accounting for the water course through the city, the experiments were set up in three areas: the area where water enters the city under "Ponte dei Cavai", the waterways in the middle of historical city center and the outflow area from the city.

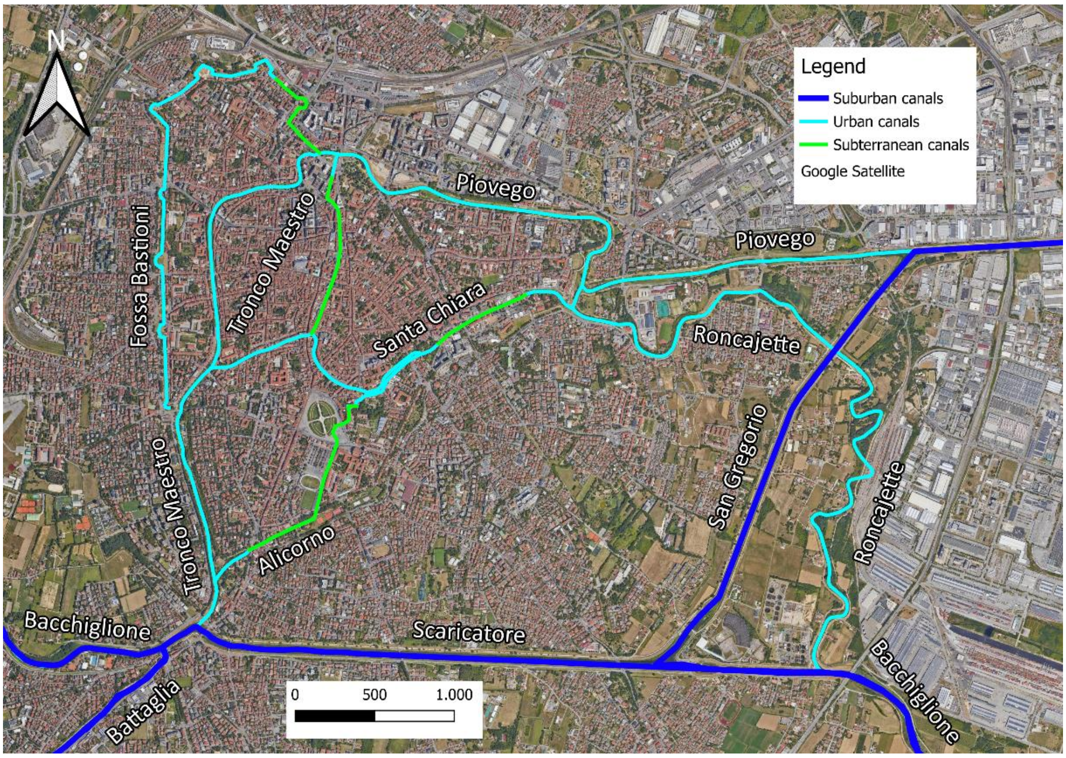

Figure 2. Padua city waterways.

3.2 Experimental setup

To assess the effect of fish presence on mosquito presence and oviposition in the area two experimental setups were implemented, one in the wild employing net barriers to create areas of the canal where the fish were excluded and another one in more controlled conditions with three sets of four water containers with a gradient of fish presence. The two experimental setups and sampling protocols are described below.

3.2.1 Net manipulative experiment

To assess if the lack of predators can explain a rapid increase in mosquito abundance in a given area, an exclusion experiment was conducted in three sites in the network of canals in Padova. The sites have been chosen in order to minimize environmental differences, while allowing an easy access from the riverbank on foot and being in not very frequented areas, to avoid disturbances by people (fishermen or curious people). The positions are reported in Figure 5: one at the “Torrione Ghirlanda” in the “Tronco Maestro” section, another upstream of the “Golena San Massimo” (Figure 4), in the “Piovego” canal, and the last one downstream of the “Ponte delle Gradelle” in the “Roncajette” canal. All of the sites were within *Ludwigia sp.* patches (although in “Golena San Massimo” site there was a conspicuous presence of *Vallisneria spiralis* and in “Ponte delle Gradelle” there was some *Potamogeton* sp.).

This experimental setup was inspired by the study performed by Gelwick et al. (1997) and is composed of triangular pen of 0.7 m^2 of surface with 2 m poles at least 50 cm buried in the sediment and at least 30 cm sticking out of water in order not to be submerged in case of changes in water level in the canals. Between the poles a net with a mesh size of 1 mm is stretched (the mesh size is chosen to exclude all the fish from the pens, even *Gambusia* sp. juveniles which are few mm long at birth).

Two pens per site were placed, with the top against the current, one opened to the fish on the downstream side, in order to check for the effect of slowdown of the river flow caused by the presence of the net, while the other was closed, after vigorously perturbing the water inside the area to make the fish flee (Figure 3).

The triangular nets were placed on 16th August and left in place until 4th September and the animal community was examined weekly (20th and 27th August and 4th September), in order to not to cause excessive disturbance to the area. One scoop with a macroinvertebrate hand net was performed within the plant coverage and its content analyzed in situ: one per each triangular net and one in a point outside the nets. The organisms were assigned to broad taxonomic groups and then released back into the environment.

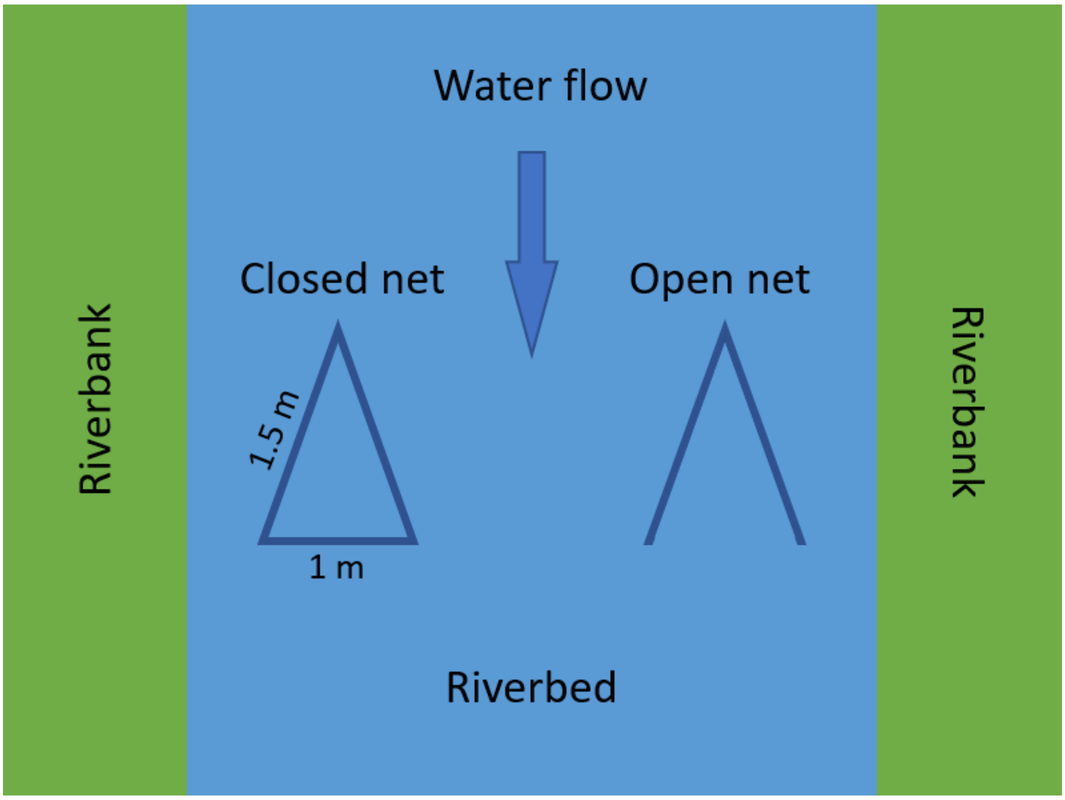

Figure 3. Scheme of a site of the net manipulative experiment.

Figure 4. Nets at the “Golena San Massimo” site.

3.2.2 Container experiment

The effect of fish presence on mosquito proliferation was also tested with a mesocosm experiment consisting of three sets of four containers with a gradient of presence of fish cues. The containers were 60 liters cilindrical blue barrels (60 cm tall and with 34 cm diameter) filled with approximately 45-50 liters of water, modified to have a double bottom, with a 1mm mesh in order to let the two environments communicate. The opening of the barrel was of intermediate dimension in order to be eligible for oviposition by the majority of mosquito specie as evidenced by Sunahara et al. (2002).

One of the containers was filled with tap water and closed with fine mesh for a few days in order to avoid oviposition while waiting for the chlorine to evaporate. The other three were filled with river water collected in situ, then in one of these about 25 adult specimens of *Poecilia reticulata* collected from a colony found in the "Orto Botanico" were placed. In another one of these containers about the same quantity of dead fish were placed. So, the four treatments contained increasing levels of fish cues, from none to most. The live fish were fed with pellets (Tetra MICRO Pellets) each two days and in the occasion also a partial water change was performed with river water (about 20 liters at a time).

The three sets of containers were placed at the "Bastione Alicorno", close to Tronco Maestro and towards the outskirts of the city, where the water enters the urban canals, inside the "Orto Botanico" botanical garden in the historical city center, close to the Alicorno canal, and at the "Golena San Massimo", towards the outskirts of the city, where the water flows out. All containers were very close to canals (10-20 m).

All the sets of containers were left evolving for three weeks, the first one between 20th August and 10th September 2024, because of issues in the access to the "Bastione Alicorno" during the Assumption week, and the other two between 12th August and 2nd September 2024.

3.2.3 Mosquito rearing

The barrels were checked as often as possible (usually every 1-2 days) and mosquito larvae present were removed with a small fish net, counted and reared at the laboratory inside 200 ml plastic glasses, at densities of maximum 50 larvae per glass (Figures 6 and 7). The glasses were marked with data about the site and day of collection and covered with a double mosquito net to prevent the escape of the adults. The glasses were checked daily during the working days, the adult specimens were captured with tweezers and preserved in 80% ethanol for further identification in 1.5 mL or 2 mL eppendorfs marked with the same information of the glasses and the day of emergence. The dead larvae and pupae that might be present were also collected and preserved in 80% ethanol. At the same time, to avoid issues connected to degraded water quality, a partial water change was performed and a small quantity of fish pellets (Tetra MICRO Pellets) added as food for the larvae. The larvae and adults were identified to the genus or species level using the dichotomous key present in the book "Mosquitoes and their control" (Becker et al., 2010).

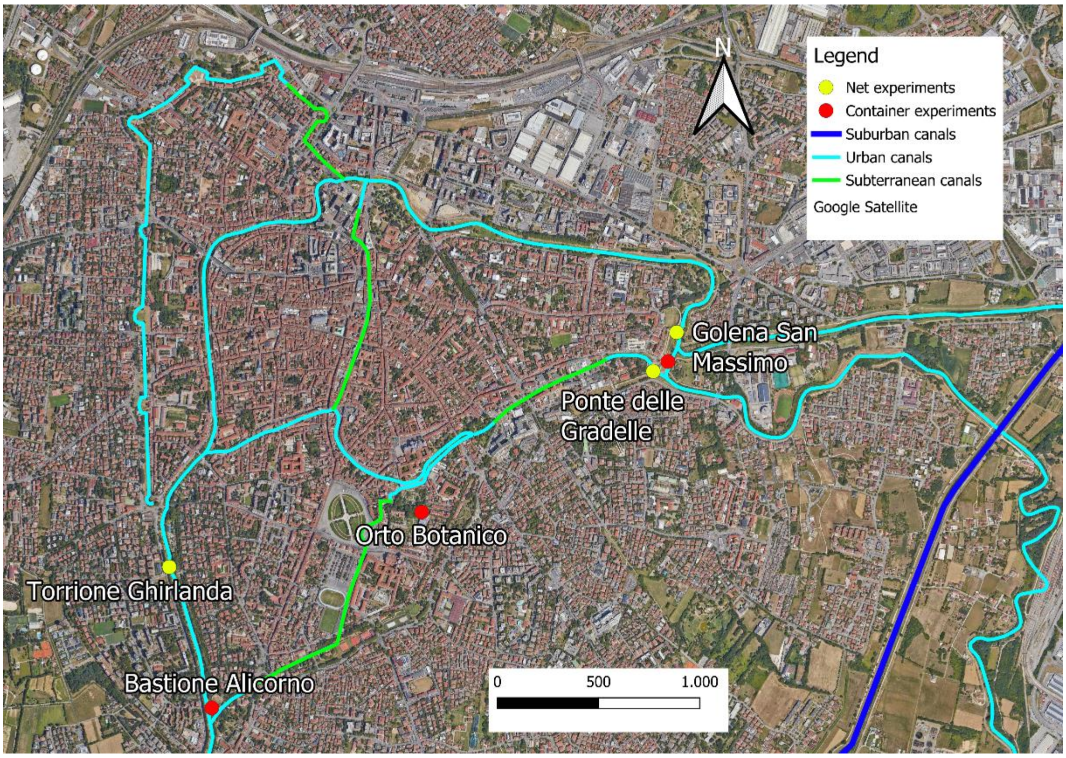

Figure 5. Position of the experimental setups through the Padua city.

Figure 6. Mosquito larvae collected at the “Bastione Alicorno” site.

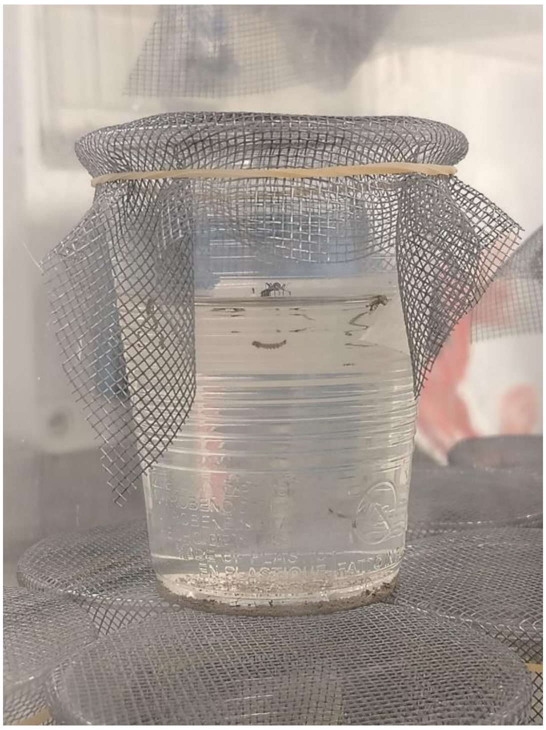

Figure 7. Mosquito larvae rearing setup, mosquito larvae can be seen in the water column and an adult has just emerged on the water surface.

3.3 Data analysis

The data analysis were performed using the software R 4.3.3 and Microsoft Excel. A PERMANOVA analysis was performed to test for differences at the community level both in the “Net manipulative experiment” and the “Container experiment”. Both analyses were carried on with 9999 permutations and using a Bray-Curtis dissimilarity matrix. For the analysis of the “Net manipulative experiment”, the matrix of taxa, with relative abundances, was tested against the fixed parameters “Treatment” (Closed net, Open net or Outside of the nets) and “Time” (First, Second or Third week of sampling) and the random parameter “Site” (Ponte delle Gradelle, Golena San Massimo or Torrione Ghirlanda). In the “Container experiment”, the abundance of both *Culex* and *Aedes* mosquito larvae collected in the three weeks was used to create the dissimilarity matrix and was tested against the fixed parameter “Treatment” (Control, River Water, Live Fish or Dead Fish) and the random parameter “Site” (Golena San Massimo, Orto Botanico or Bastione Alicorno).

One-way ANOVA tests were employed to detect significant differences between the means of abundances in one taxa against the same combination of parameters as above both for the “Net manipulative experiment” and the “Container experiment”. Afterwards a Tuckey posthoc test was performed to investigate the pairwise comparisons.

4. RESULTS

4.1 Net manipulative experiment

4.1.1 Community composition

A total of 791 animal individuals belonging to 14 taxa were identified from the hand net scoops inside and outside the nets, but no mosquito larvae were found in any of the treatments (Figure 8). The closed nets revealed to be not fully effective in excluding all the fish from them given the fact that juveniles of *Gambusia* spp. were found both in “Golena San Massimo” and “Ponte delle Gradelle” sites. In any case, a certain degree of exclusion of the main fish activity has presumably been reached. Only five taxa, namely mosquitofish (*Gambusia holbrooki*), leeches (Hirudinea), mayflies (Ephemeroptera), damselflies (Zygoptera) and true shrimps (Caridea) were found in all the sites.

The site that showed higher richness in species and abundance was “Ponte delle Gradelle” with 485 individuals and 11 taxa. Among them gobies (Gobiidae), amphipods (Amphipoda), nonbiting midges (Chironomidae), beetles (Coleoptera) and planarians (Tricladida) were found only here.

The “Golena San Massimo” site had exclusive taxa too, namely crayfish (*Procambarus clarkii*), dragonflies (Anisoptera) and true water bugs (Nepomorpha), on the other hand this was the only site where isopods (Isopoda) were not collected. It had intermediate values regarding both individual (252) and taxa (8) abundances.

Finally the “Torrione Ghirlanda” site had the fewest taxa (6) and abundances in general (54 individuals) (Table 1 in Appendix).

Figure 8. From left to right, a freshwater shrimp (*Atyaephira desmarestii*), a mayfly (*Cloeon sp.*) and an isopod (*Asellus sp.*), some of the most common taxa found during the survey.

4.1.2 Community development

Looking at the communities in their complex (Figure 9), the PERMANOVA analysis revealed that the only significant effect is the experiment site (p-value < 0.001), while no significant differences (at the 0.05 level) can be observed looking at the different treatments and along the temporal axis. The diversity of the communities, measured using the Shannon biodiversity index, did not reveal any significant differences looking either at the site, treatment or time of the sampling (Appendix).

Exploring the taxa individually only mayflies, damselflies and shrimps were found with enough consistency between the three sites to be compared, because both leeches and mosquitofish were found only in one treatment in one occasion in the "Torrione Ghirlanda" site, with two and one individuals respectively. A significant difference between individual abundances of these taxa was tested with the ANOVA one-way test for all of the three parameters: site, treatment and time. No common trends were found in total abundance because while both Caridea and Ephemeroptera presence differ significantly among sites (p-value < 0.05 for Caridea and < 0.01 for Ephemeroptera), with significantly less shrimps and mayflies collected in "Torrione Ghirlanda" site compared to "Ponte delle Gradelle" and "Golena San Massimo" respectively (Figures 10 and 11), Zygoptera larvae showed to be more influenced by the treatment (p-value < 0.05) with significantly less individuals collected outside the nets, than in the closed nets (Figure 13). Moreover Ephemeroptera showed a significant decrease in abundance through time (p-value < 0.05) with their numbers dropping from the first to the third week (Figure 12).

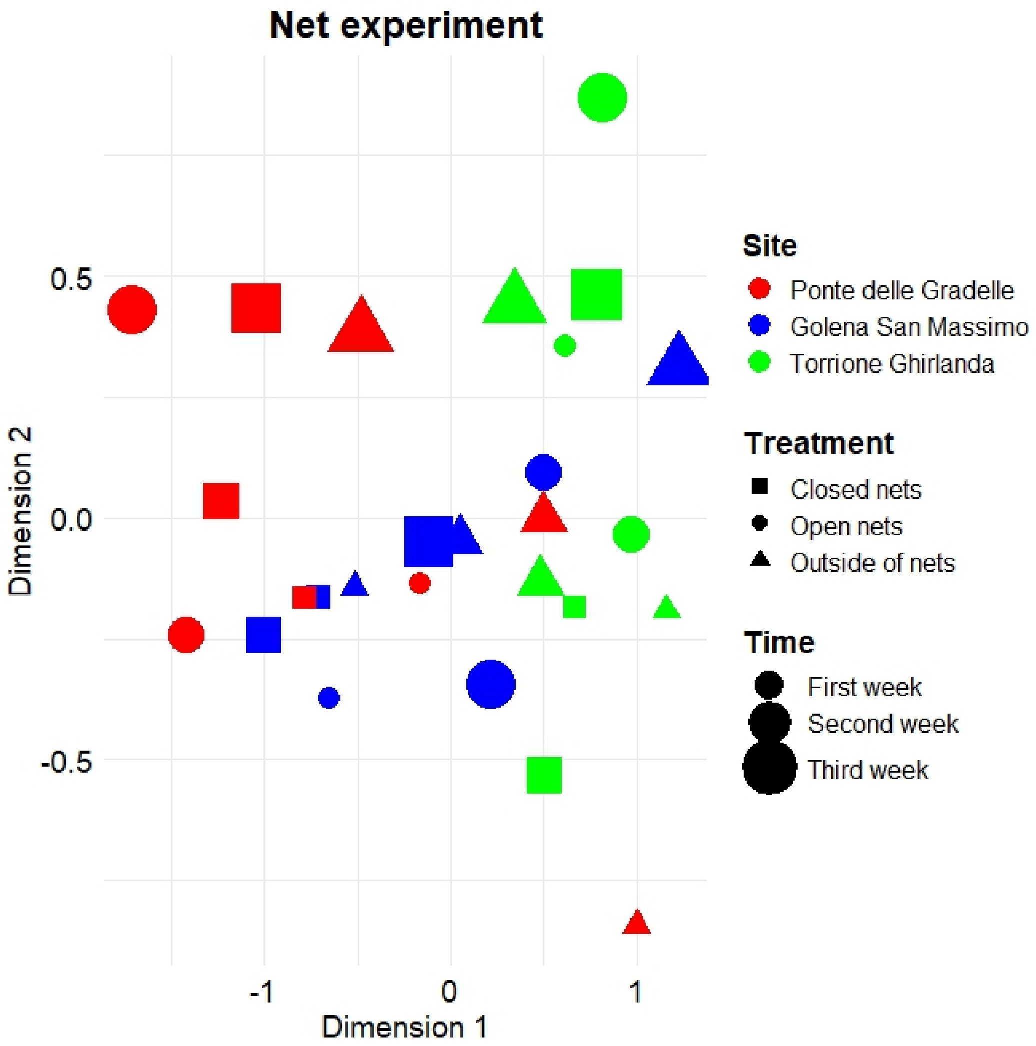

Figure 9. Multi-Dimensional Scaling (9.6% stress) of the communities found in the experimental sites, indicated by different colors. Different treatments are indicated by different shapes and the three temporal sampling points by different sizes of the symbols. No difference in the dispersion of the data were found.

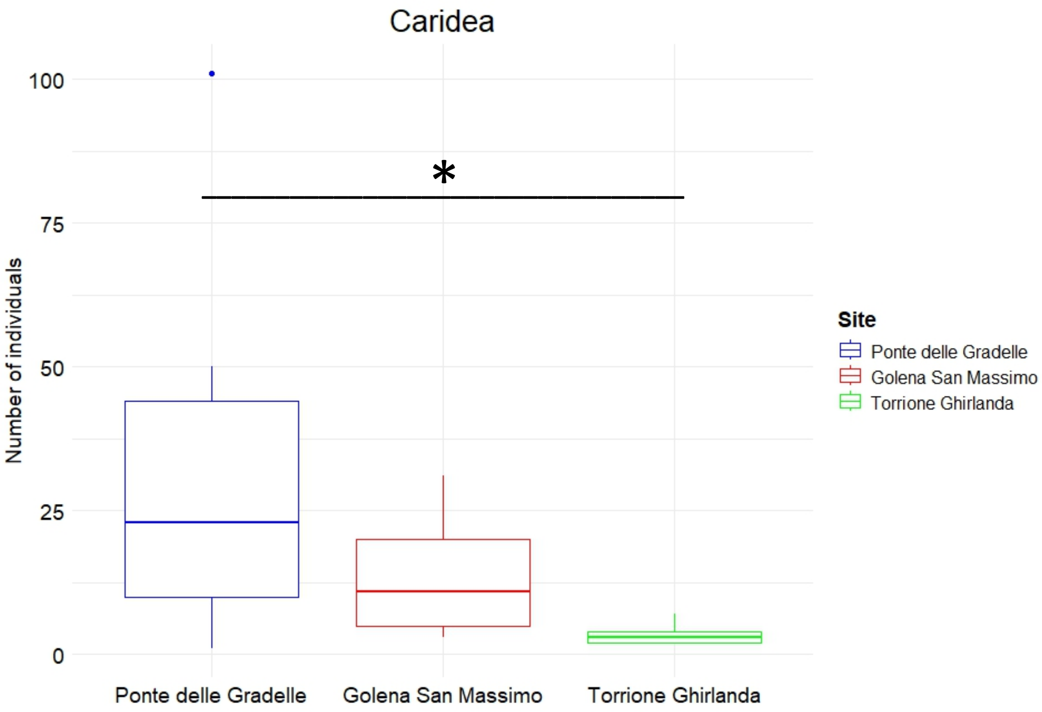

Figure 10. Total abundance of Caridea in the three sites.

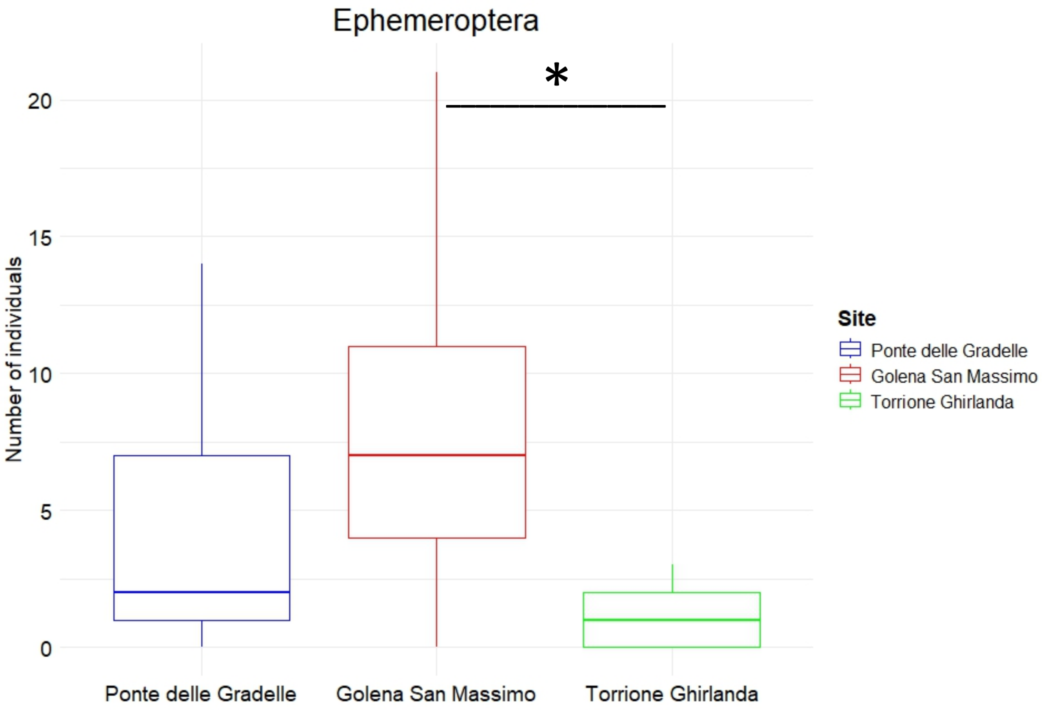

Figure 11. Total abundance of Ephemeroptera in the three sites.

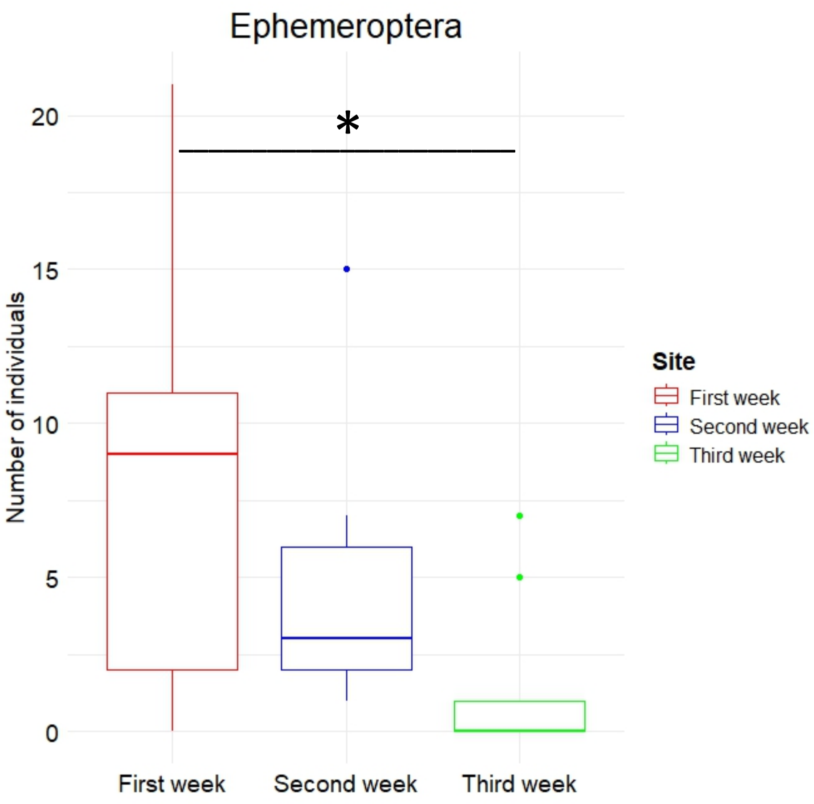

Figure 12. Total abundance of Ephemeroptera through the three sampling weeks.

Figure 13. Total abundance of Zygoptera in the three different treatments.

4.2 Containers

4.2.1 Larval abundance

Despite the time period of one set of containers was different, the climatic conditions during the three week period of the experiment were pretty similar, with two rainy days and similar temperatures, allowing to compare results.
The first mosquito larvae appeared almost at the same time in all three sets: the fifth day in the “Golena San Massimo” site, sixth day in the “Bastione Alicorno” site and on the seventh day at the “Orto Botanico”. A total of 2771 larvae were collected in the 12 containers in the three weeks of the experiment. The most of them in the “Bastione Alicorno” site (1253), followed by “Golena San Massimo” (998) and “Orto Botanico” (520) (Figure 14). The highest amount of mosquitoes was found in the “Control” treatment (1337), tightly followed by “Dead Fish” (1220) while “River Water” showed a very low abundance of larvae (207) (Figure 15). Only seven mosquito larvae were found inside the “Live Fish” treatment and only in the “Golena San Massimo” site. The one-way ANOVA revealed a significant effect of the “Site” factor (p-value < 0.001), due to the fact that all the treatments resulted significantly different from the “Live Fish” containers.

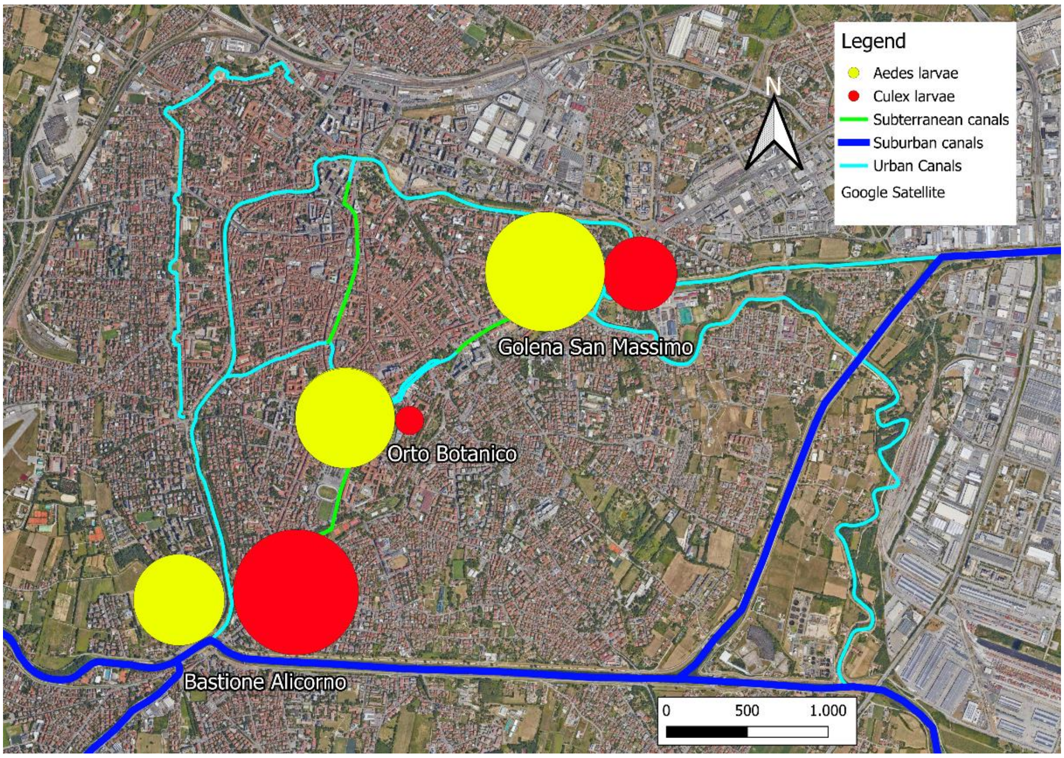

Figure 14. Total abundance of mosquito larvae in the containers in the three sites. The area of the circles is proportional to the number of mosquito larvae.

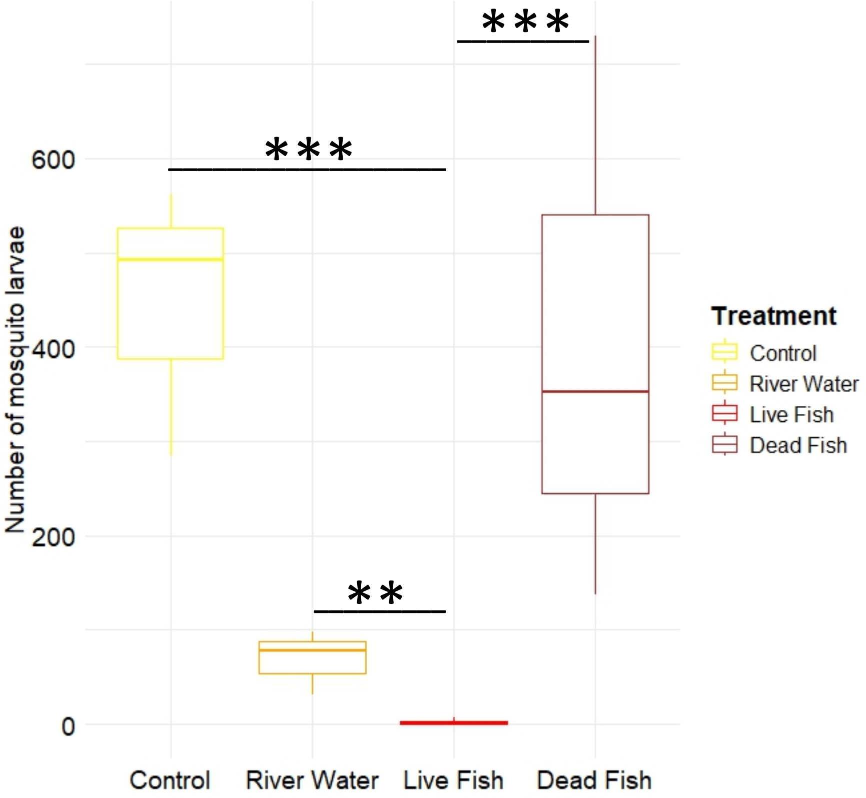

Figure 15. Total abundance of mosquito larvae in the four treatments.

4.2.2 Species distribution

All of the adults and fourth instar larvae were identified at least to the species level (2073 individuals). The identification revealed that females of only two genera were attracted by the containers: *Aedes* spp. and *Culex* spp.

Further identification to the species level was generally not possible for all the specimens because some of the diagnostic traits were degraded, but the only two species that were identified were *Aedes albopictus* and *Culex pipiens*, the most common species in urban environments in North Italy (Zamburlini et al., 2019), so for the following analysis are referred to the genus level.

Aedes mosquitoes were abundant in all of the three sites, but in particular at the “Golena San Massimo” set of containers (541), followed by “Orto Botanico” (381) and “Bastione Alicorno” (318), while *Culex* was clearly more abundant near the outskirts of the city, with 598 individuals at the “Bastione Alicorno” site and 204 at “Golena San Massimo”, with only 31 larvae found at the “Orto Botanico” in the city center (Figure 14).

The two species showed differences even more striking looking at their response to the treatments. *Aedes* females showed a general avoidance for every fish cue, preferring the “Control” treatment over the others in all three sites, for a total of 906 (73.1 % of total *Aedes*) larvae found in the “Control” containers, 172 (13.9 %) in the “Dead Fish” treatment and 155 (12.5 %) in the “River Water”. Only 7 larvae (0.5%) were collected in the water containing live fish.

This difference resulted to be significant with a one-way ANOVA test (p-value < 0.001). The posthoc analysis revealed that the difference is due to the avoidance of the “Live Fish” treatment, which is significantly different from all the others (Figure 16).

Culex mosquitoes on the other hand showed a great attraction for the “Dead Fish”, with 784 individuals (94.1 % of all *Culex*) collected mainly in “Bastione Alicorno” and “Golena San Massimo”, way more than the “Control” and “River Water” treatments with 28 (3.4 %) and 21 (2.5 %) individuals respectively. No *Culex* larvae were found in the “Live Fish” treatment. The difference was close to significance with a one-way ANOVA test (p-value = 0.0507), with a significant difference detected in the posthoc analysis between the “Dead Fish” and the “Live Fish” treatments (Figure 17).

In general only one genus per container was present in high abundances, with the other still present, but with fewer individuals.

The PERMANOVA analysis showed significant differences in the presence of the two species with respect to the “Treatment” parameter (p-value < 0.001) and no significance of the “Site” effect (Figure 18).

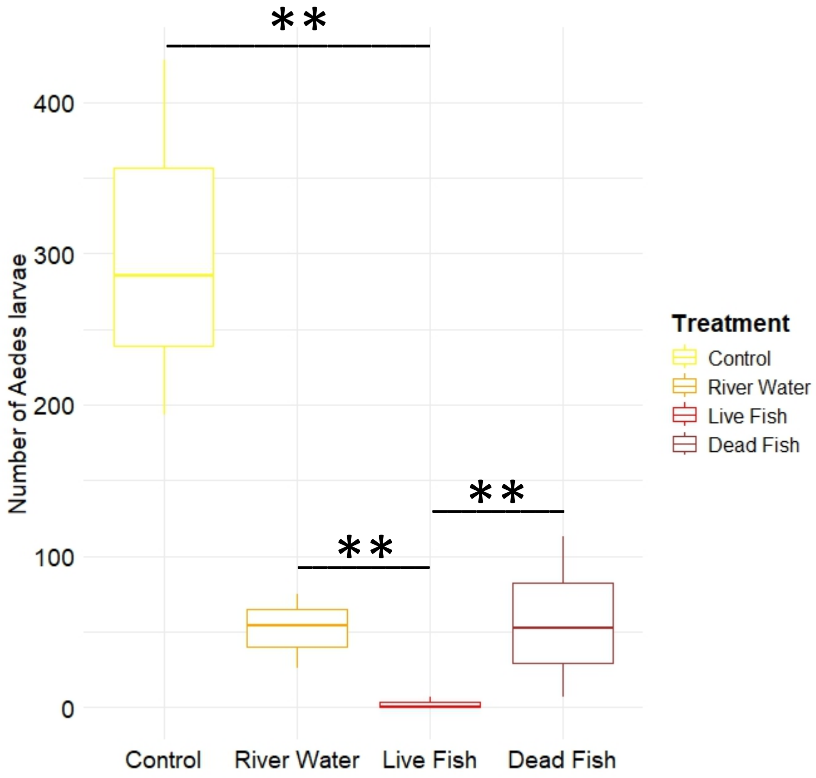

Figure 16. Total abundance of *Aedes* larvae in the four treatments.

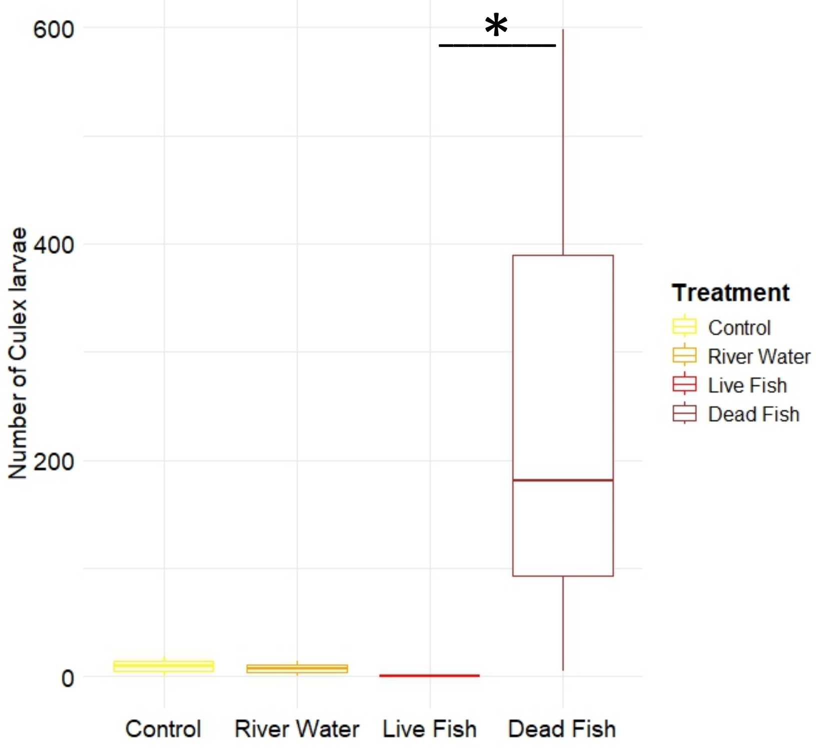

Figure 17. Total abundance of *Culex* larvae in the four treatments.

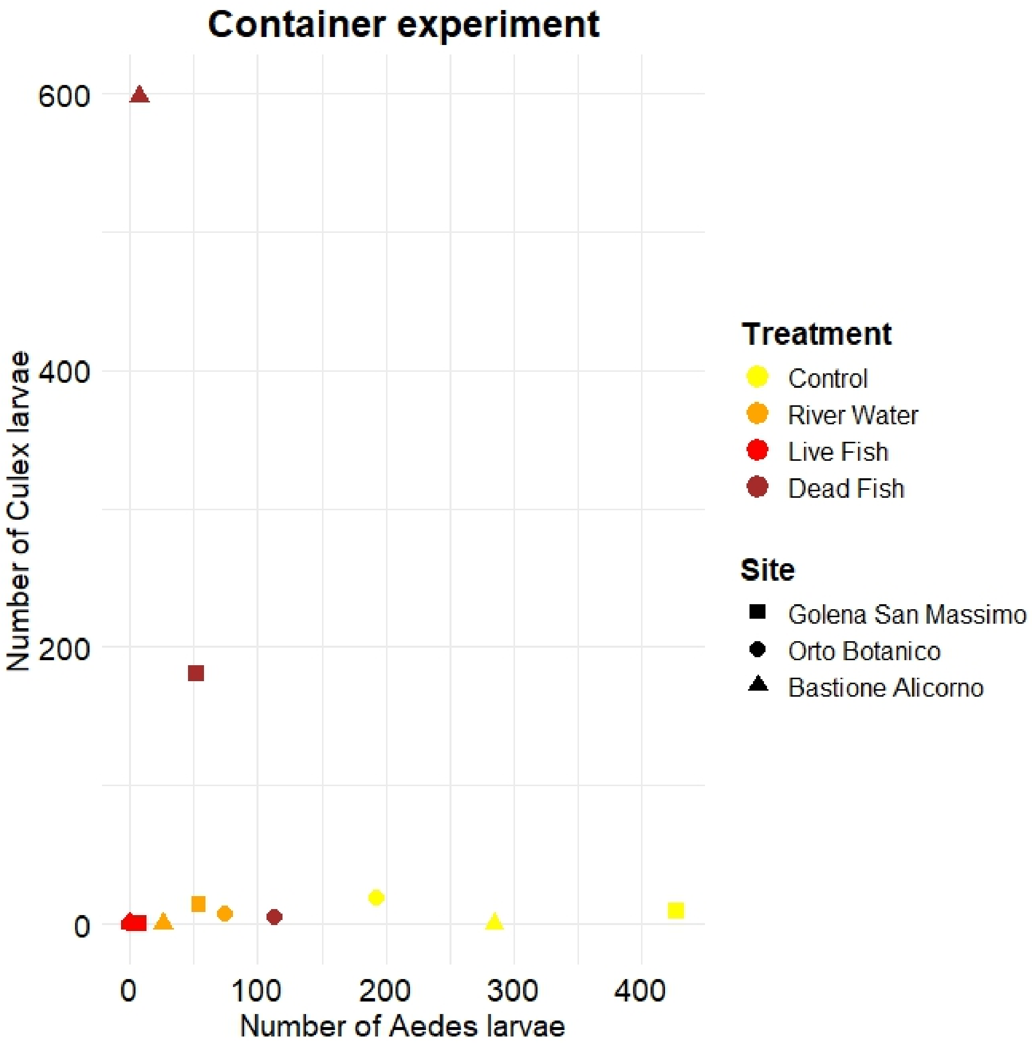

Figure 18. Plot with the total abundance of *Aedes* larvae on the x axis and the total abundance of *Culex* larvae on the y axis, for all the sites and all the treatments.

4.2.3 Temporal trends

Looking at the temporal trend of the mosquito assemblage history in the containers what can be observed is that the trajectories are very dependent both on the dominant species in the container and the treatment.

Culex mosquitoes quickly colonized the “Dead Fish” containers out of the city center, with the first larvae present between day 5 and 6, but then after the first outbreak these containers became almost devoid of mosquitoes by day 13 until the end of the experimentations (Figure 19). *Culex* larvae appeared also in the “Control” and “River Water” containers, both in the “Golena San Massimo” and “Orto Botanico” sites between days 17 and 18 since the beginning. On the other hand, in the “Bastione Alicorno” site this taxon was exclusive to the “Dead Fish” treatment (Figure 21).

Aedes larvae appeared on average some days after, around day 9, in all of the containers, but only the population in the “Control” containers remained very productive until the end of the experiment (Figure 20). The treatments “Dead Fish” and “River Water” remained both almost devoid of mosquitoes at the end of the second week, with some larvae present at very low densities (Figure 21).

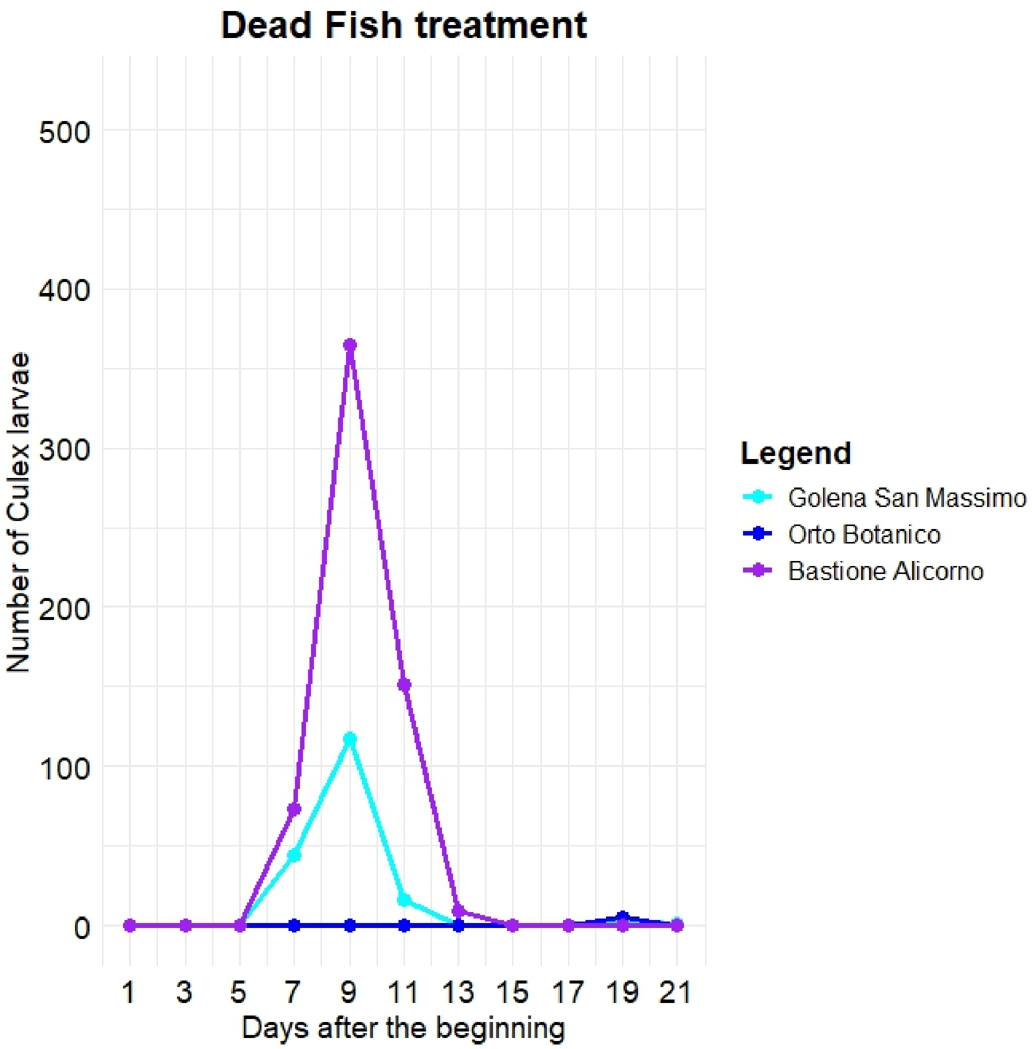

Figure 19. Temporal trend of *Culex* larvae abundance in the "Dead Fish" treatment in the three sites. The abundance is reported every two days, with the value being the sum of the larvae collected in that time frame. This was done because otherwise a lot of null values would have appeared, given the fact the sampling was not performed every day.

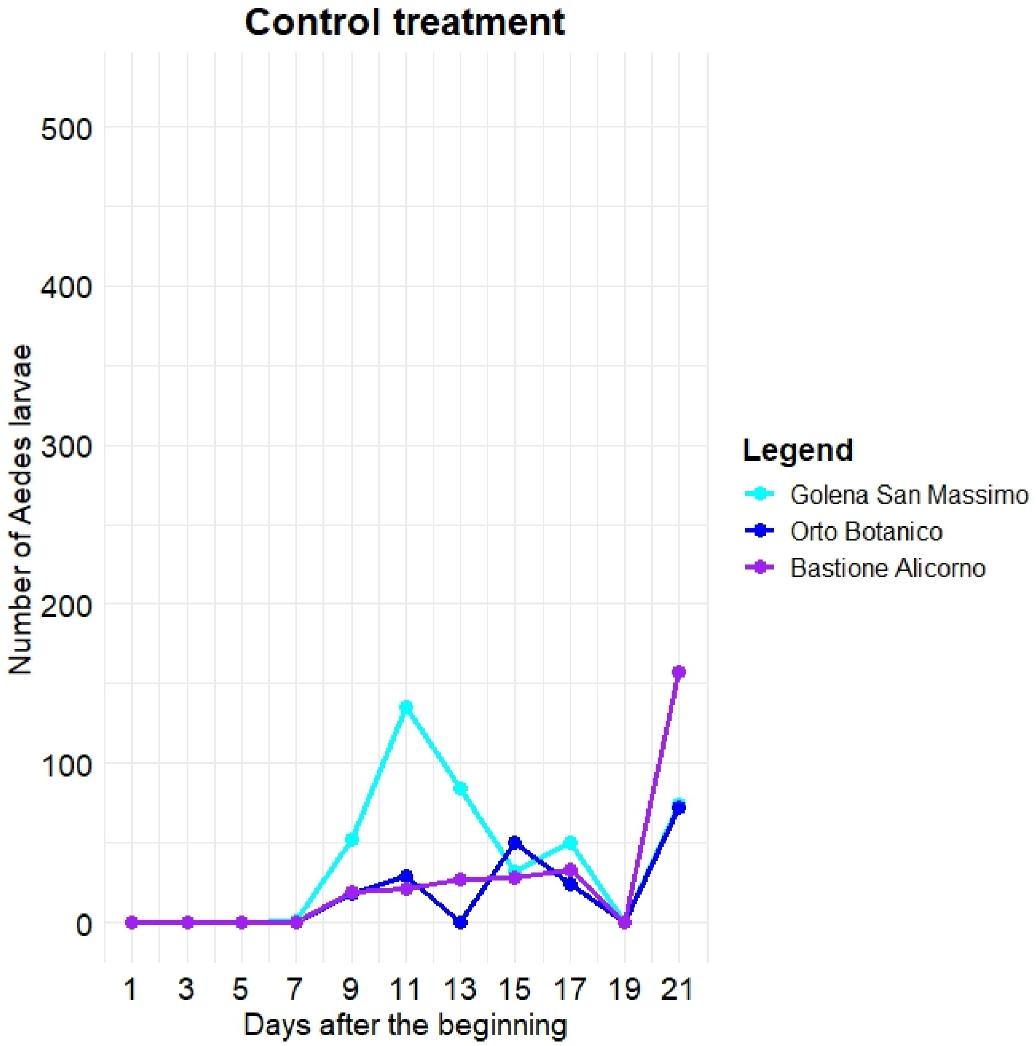

Figure 20. Temporal trend of *Aedes* larvae abundance in the “Control” treatment in the three sites, reported every two days.

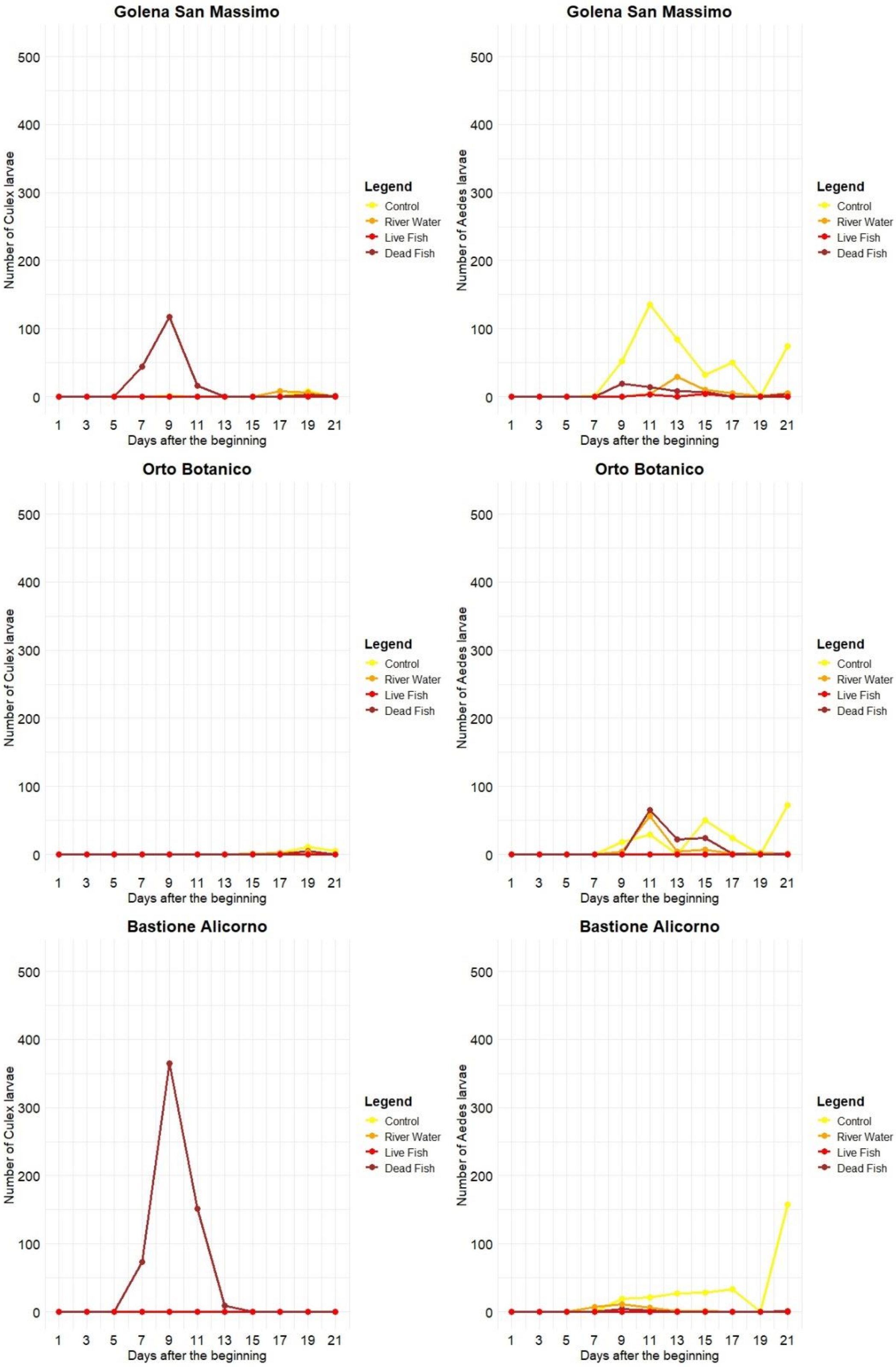

Figure 21. Temporal trend of *Culex* larvae (left column) and *Aedes* larvae (right column) abundance in the four treatments, for each of the three sites (rows), reported every two days.

5. DISCUSSION

Our field experiments do not allow us to detect an influence by either the presence or activity of fish or the passage of time on the animal community diversity in the slow flowing urban canals in Padova. On the other hand, communities associated with vegetated patches show great spatial heterogeneity, given the fact that the difference between sites resulted highly significant, raising questions about the appropriateness of our sample size to detect effects. In particular the area closer to the water enter point in the urban canals shows reduced diversity and richness, this could be due to the higher water speed in that area, making the persistence of organisms attached to underwater plants more difficult; moreover that area is partly drained every time the gates protecting Padova from floods are closed. This finding could also lead to a reduced plant diversity in this site with respect to the other two (a pure *Ludwigia* patch, compared to the other two where some *Vallisneria* or *Potamogeton* were present). This environment with reduced complexity could lead to an increased predation, thus being less suitable for small organisms (Diehl, 1992). It is possible that a combination of these causes leads to the reduced diversity and low abundances in the "Torrione Ghirlanda" site compared to the others. Other possible causes are the imperfect exclusion of the fish predators by the nets or the short time left to the system to evolve, although in other experiments noticeable top-down effects of fish on invertebrates were detected after a couple of weeks (Meissner & Muotka, 2006).

The complexity of effects shaping the overall community in these environments is also confirmed looking at the main taxa (shrimps, mayflies and damselflies) that do not show a common trend through time, sites and treatments. Both shrimp and mayfly populations seem to be less abundant in the "Torrione Ghirlanda site", and the latter also decreased in abundance between the beginning and the end of the experiment, but this could be due to the fact that many larvae could have emerged as flying adults during that time. The only taxon that seems to be influenced by the treatment are the damselflies, with greater abundances the more the environment was closed. No mosquito larvae were detected in the manipulative experiment, probably because the adults usually do not choose flowing water sources to oviposit in (Yadav et al., 2012) and because the nets, even if closed, allowed the passage of water with fish chemical cues, another negative driver of mosquito oviposition (Angelon & Petranka, 2002; Louca et al., 2009; Kraus & Vonesh, 2010). In 2024, indeed, water flow in the Bacchiglione river and Padova canals was much higher than in 2022, the year of an important summer drought.

Looking more specifically at the influence of fish cues in mosquito oviposition behaviour in the container experiment, the effect of the random "site" variable was never significant, meaning that the main driver of the observed differences was the treatment, which resulted significant both for the two-species composition, for total mosquito larvae and for *Aedes* larvae only, and a p-value near the significance threshold for *Culex* larvae. The distribution of the two genera was almost as expected, with *Aedes* mosquitoes found in all of the sites in the urban environment, while the *Culex* mosquitoes were present mostly outside the center, where their favourite breeding grounds are more common (barrels and ditches) (Becker et al., 2010), but in any case we did not expect that many *Culex* mosquitoes in such proximity to the ancient walls of Padova. Interestingly, the fact that some female *Culex* mosquitoes reached the "Orto Botanico" to oviposit means that it is not uncommon for them to spread to the city center which, by the way, is well within their flight capabilities (Cui et al., 2013). The fact that the two species show different preferences for the four treatments can be explained by the fact that, in their natural habitats, they are adapted to two different ecological niches. *Aedes albopictus* showed a marked avoidance for the "Live Fish" treatment, with the most larvae found in the "Control" containers with as few fish cues as possible. These mosquitoes usually oviposit in tree holes or bamboo stumps, namely very little and ephemeral environments that are very rarely colonized by predators, moreover the larvae are bottom-feeders and show very little anti-predator response, so are more exposed to predation because most of macroinvertebrate predators are benthic organisms (Sih, 1986; Becker et al., 2010). Conversely, *Culex pipiens* usually oviposit in larger, semi-permanent water bodies, where their chances to encounter predators are greater. The larvae feed in the water column or hanging down the surface and show a stronger anti-predatory response (Sih, 1986; Becker et al., 2010), so in theory they should have been found also in the containers with fish cues, but actually the only clear effect was a strong difference between "Dead Fish" and "Live Fish" treatments. A possible explanation to this observation has already been suggested by Becker et al. (2010), with *Culex pipiens* females being able to detect volatile compounds derived from organic material degradation, which provides a more suitable environment for larval development; this explanation makes sense also because the *Culex* eggs are not resistant to dessiccation, so the choice of an environment that can ensure a larval development as fast as possible is favoured. Another possibility is that the mosquitoes can detect the specific cues of dead fish, indicators of a predator-free environment, but this hypothesis needs to be tested. Interestingly the strong preference of *Culex* mosquitoes for dead fish could explain the high number of West Nile cases in Padova in the summer of 2022, because, due to an extended drought period, the canals were dry and the level, especially near the "Ponte dei Cavai" gate, lowered significantly, causing the oxygen levels to drop leading to a mass fish death (Faccin et al., 2023). These could have been favourable conditions for a *Culex* mosquito outbreak, with an increased risk of trasmission of West Nile fever in a densely populated area.

Also the abundance through time of the two species was pretty different. *Aedes* larvae started to appear after *Culex* ones and they continued to develop for a broader time window than their counterparts. On the other hand *Culex* females, where present, responded swiftly to the presence of dead fish, reaching the maximum abundance very soon and seemingly abandoning the site afterwards. Interestingly all the containers, except the control, show an extinction pattern regarding the mosquito larvae abundance, contrary to what was expected, because if the fish cues in containers filled with river water were the main drivers that discourage mosquito oviposition, the female preference should have increased through time, with the degradation of the fish-derived chemical compounds. A possible explanation is the excessive organic enrichment of the containers filled with water from the river, that contains more organic material than the tap water. In some of these containers, between the second and third week of the experiment, a thin surface film developed, probably due to the degradation of organic material. This film also happened to develop in some plastic glasses during the rearing phase in the laboratory, especially when too much food was added, and ended up causing the death of many larvae and pupae, probably because it interferes with the hydrophobic properties of the respiratory siphon in the pre-imago stages.

6. CONCLUSIONS

The top-down effect of fish on both species of mosquitoes found in the study area seems to be primarily discourage oviposition, both in the wild (no mosquito larvae found during the net manipulative experiment) and in semi-natural mesocosms (the “River Water” and “Live Fish” treatments were the two with less mosquito larvae collected). On the other hand the effect of fish in shaping the overall macroinvertebrate community associated with vegetated patches is not clear, probably because it is a complex multifactorial process.

The two investigated mosquito species show different patterns of colonization, looking both at the time and treatment components. The explanation for these differences can be found in the ecological niches of the two species in their natural environment, with *Aedes* having the predator avoidance as main evolutionary pressure and *Culex* females looking swiftly for an environment that can provide better conditions for a faster larval development.

The oviposition preferences of *Culex* females in our study can provide a tentative explanation for the big outbreak of West Nile cases in the area of Padova in the summer of 2022 when a mass fish death occurred, linking directly canal management to threats to public health. This hypothesis deserves further investigation, for example based on further experiments and an analysis of epidemiological data over time and space.

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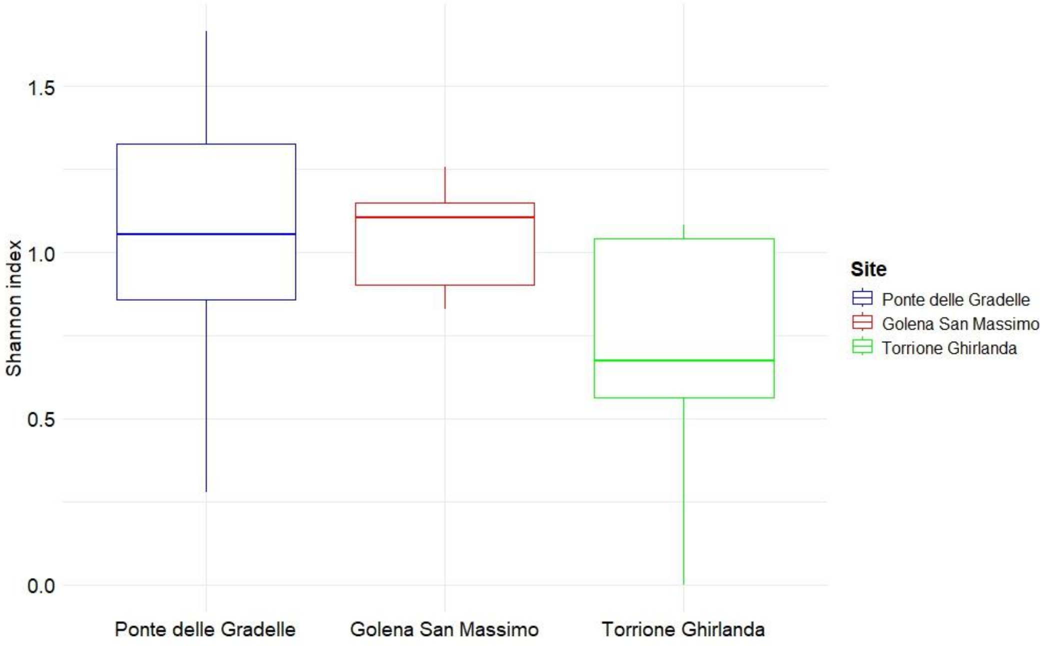
Finally thanks to my girlfriend Aurora, who (fortunately) shares my passion for nature and animals and for being the best friend and partner I could hope for.

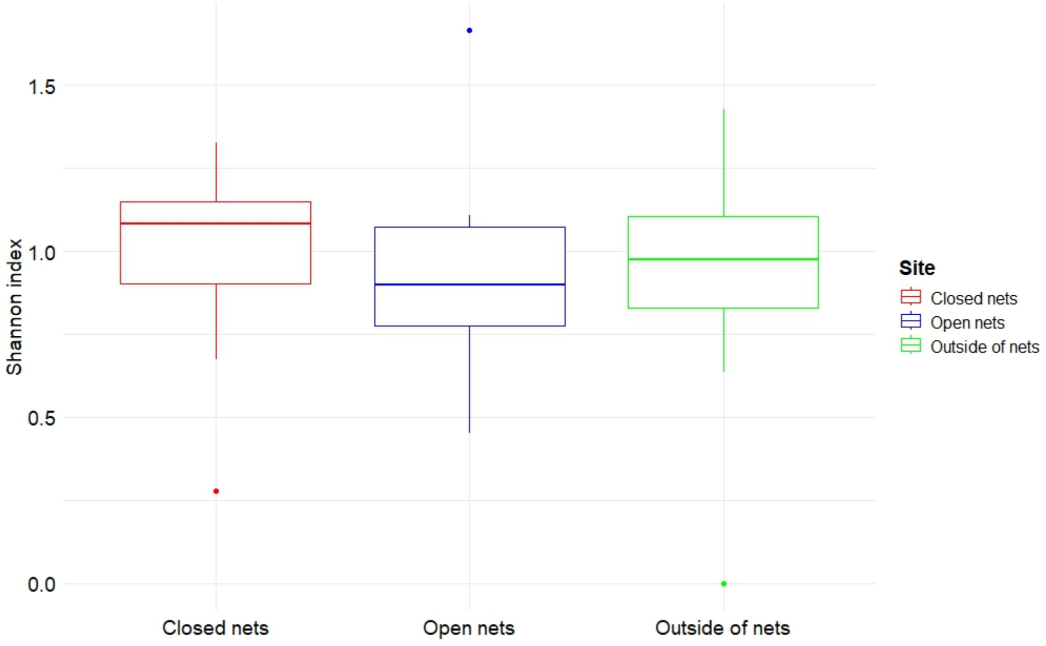
9. APPENDIX

Table 1. Taxa found by scooping the net environments and abundances relative to each experimental site.

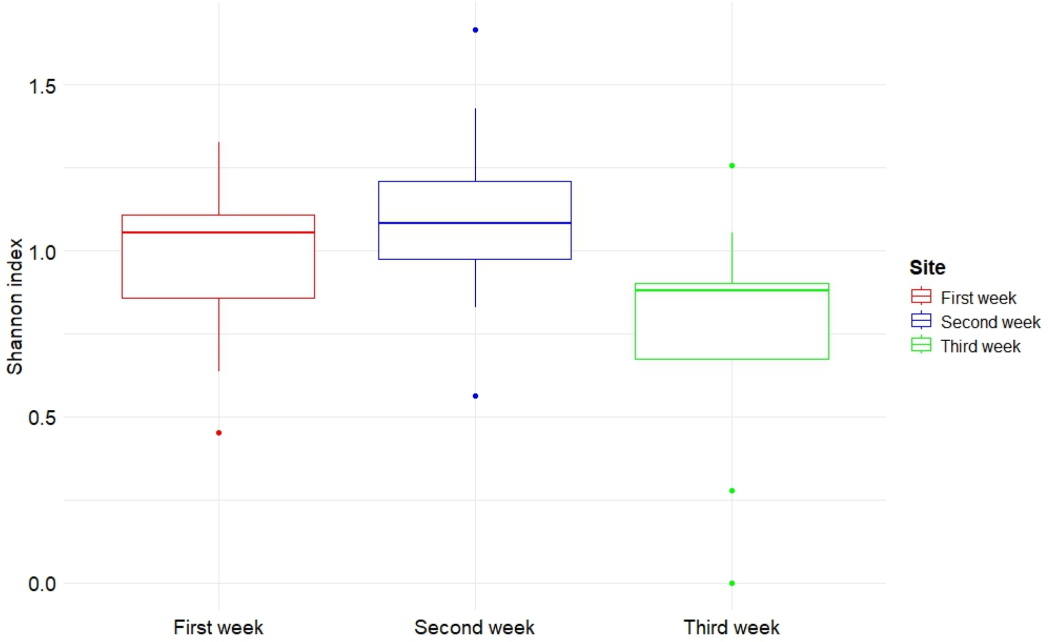
Taxa	Ponte delle Gradelle	Golena San Massimo	Torrione Ghirlanda	Total
Phylum Anellida				
Class Clitellata				
Subclass Hirudinea	10	3	2	15
Phylum Arthropoda				
Class Insecta				
Order Coleoptera	5	0	0	5
Order Diptera				
Family Chironomidae	3	0	0	3
Order Ephemeroptera	41	74	10	125
Order Odonata				
Suborder Zygoptera	57	40	7	104
Suborder Anisoptera	0	1	0	1
Order Rhynchota				
Infraorder Nepomorpha	0	1	0	1
Class Malacostraca				
Order Amphipoda	1	0	0	1
Order Decapoda				
Procambarus clarkii	0	2	0	2
Infraorder Caridea	287	122	32	441
Order Isopoda	65	0	2	67
Phylum Chordata				
Class Actinopterygii				
Order Cyprinodontiformes				
Gambusia holbrooki	5	9	1	15
Order Perciformes				
Padogobius bonelli	2	0	0	2
Phylum Platyhelminthes				
Class Turbellaria				
Order Tricladida	9	0	0	9



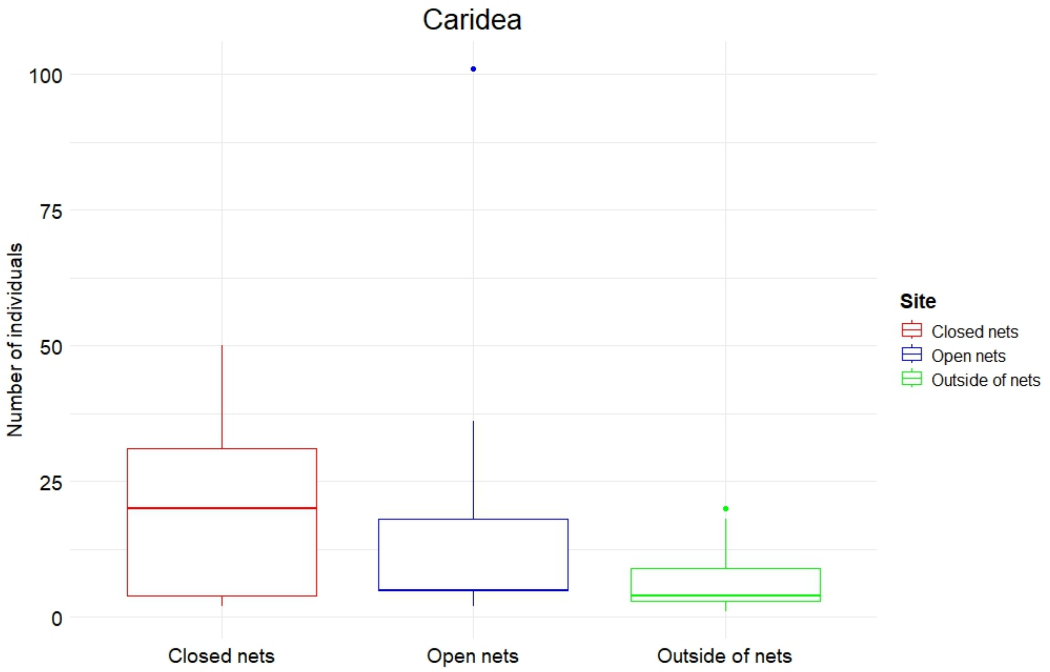
Appendix 1. Shannon diversity index in the three sites.



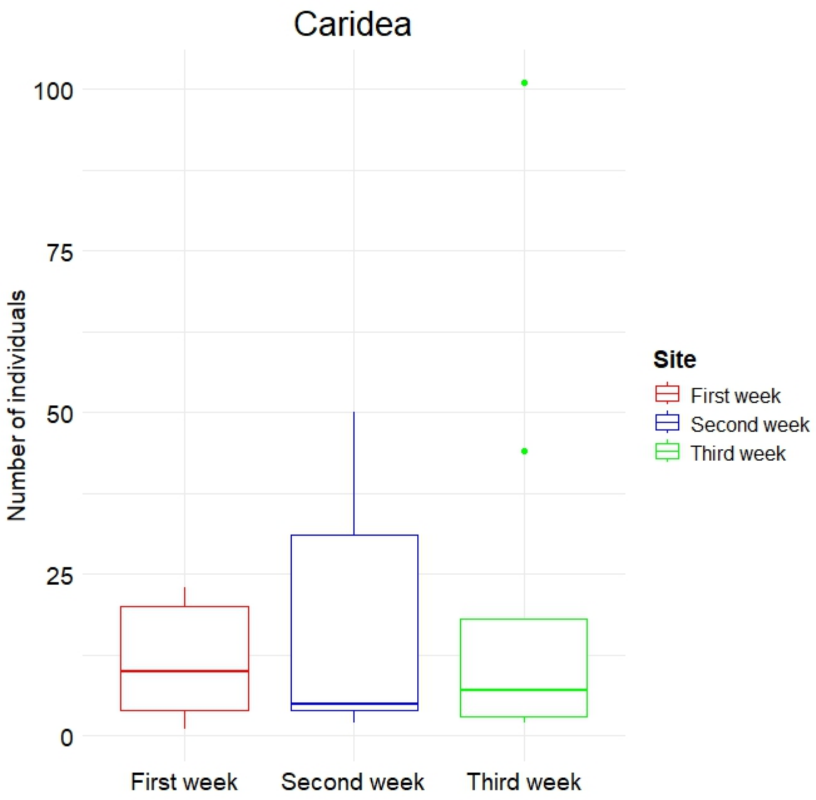
Appendix 2. Shannon diversity index in the three treatments.



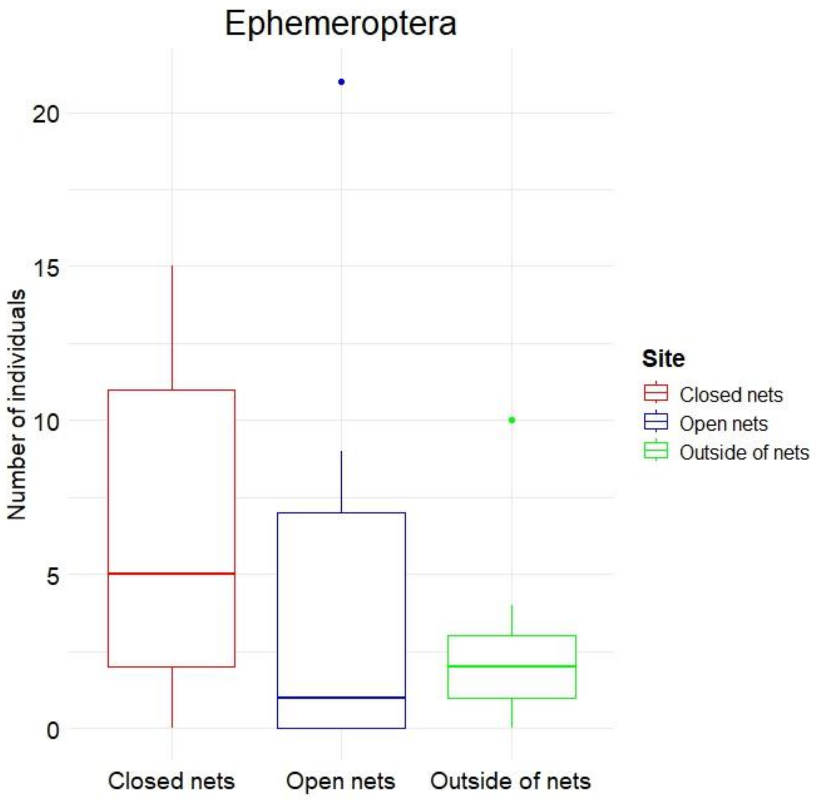
Appendix 3. Shannon diversity index through the three weeks of sampling.



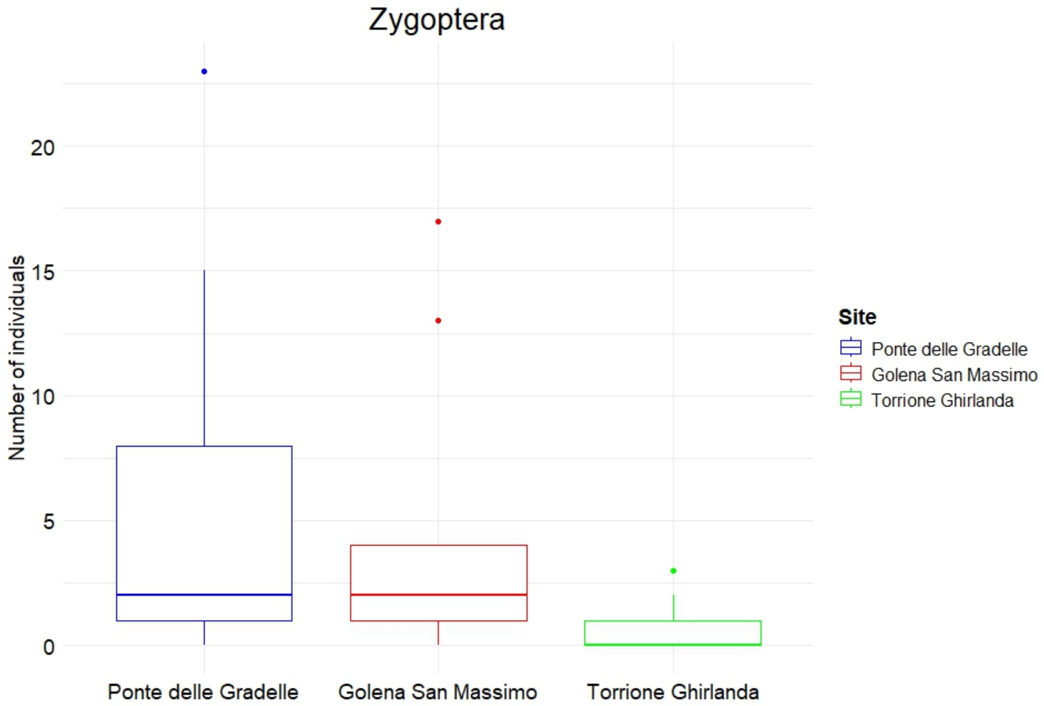
Appendix 4. Total abundance of Caridea in the three treatments.



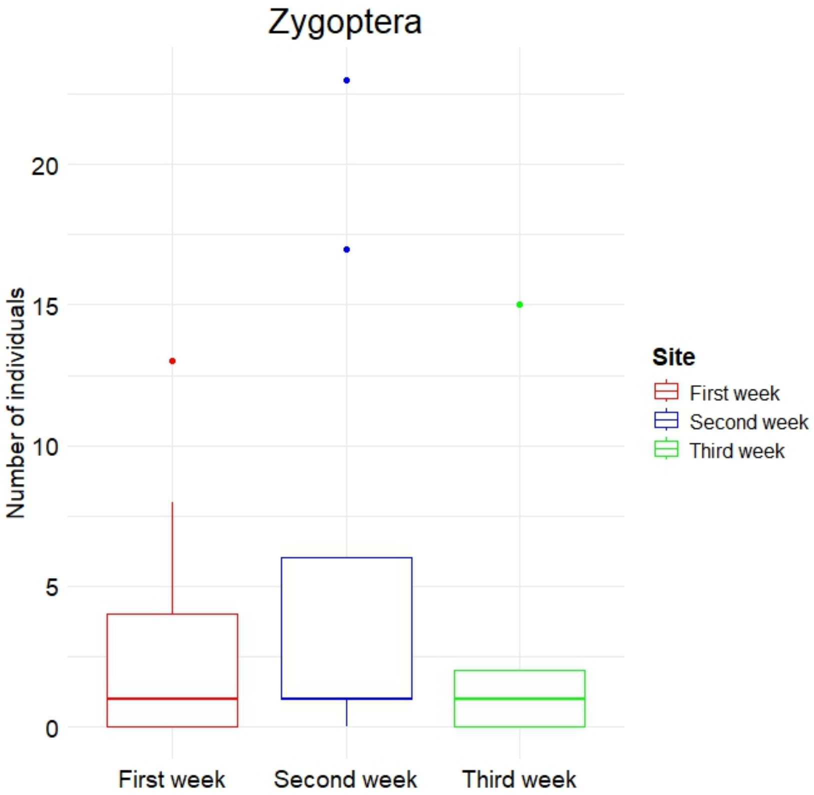
Appendix 5. Total abundance of Caridea through the three weeks of sampling.



Appendix 6. Total abundance of Ephemeroptera in the three treatments.

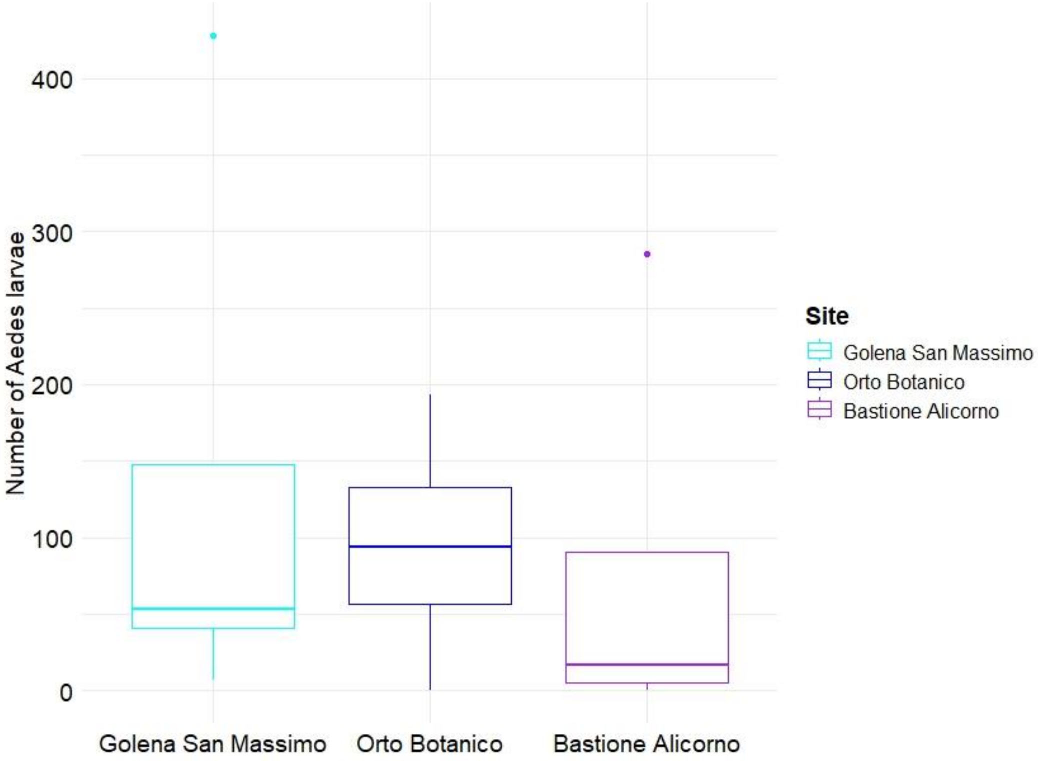


Appendix 7. Total abundance of Zygoptera in the three sites.

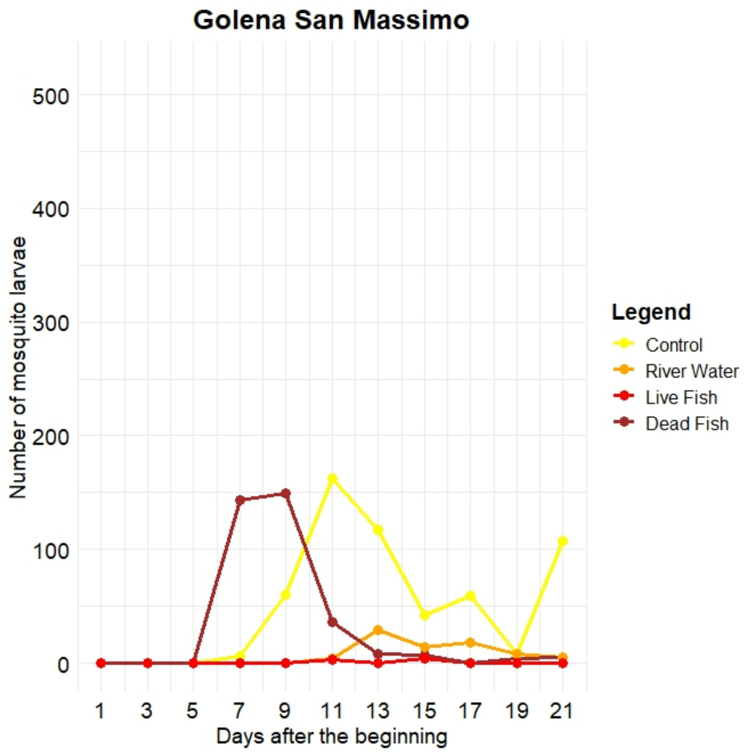


Appendix 8. Total abundance of Zygoptera through the three weeks of sampling.

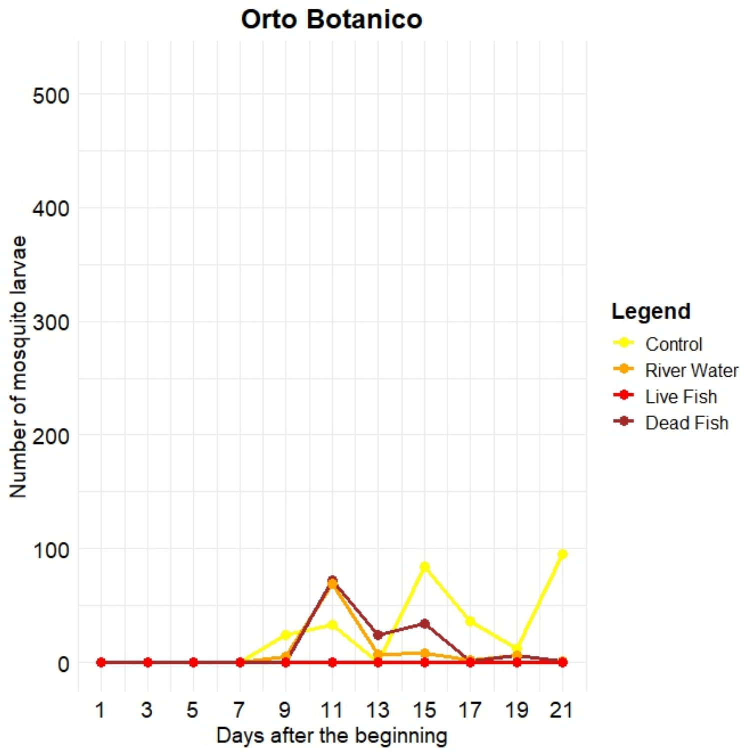
Appendix 9. Total abundance of *Culex* larvae in the three sites.



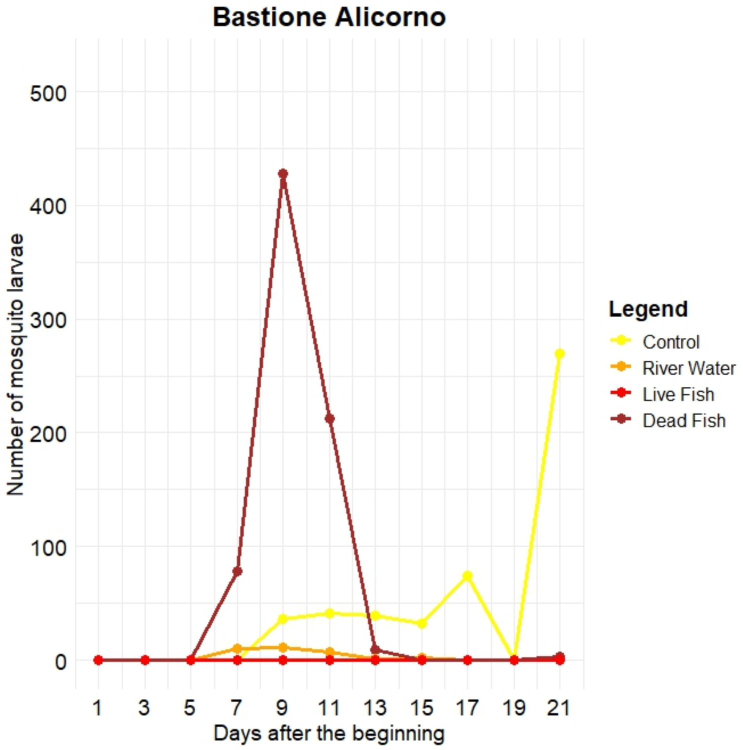
Appendix 10. Total abundance of *Aedes* larvae in the three sites.



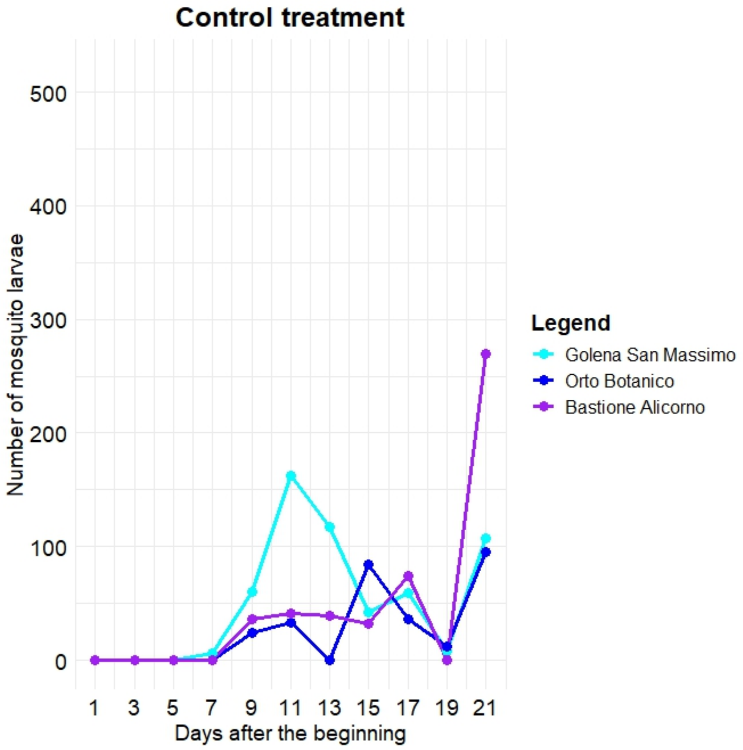
Appendix 11. Temporal trend of mosquito larvae abundance in the "Golena San Massimo" site in the four treatments, reported every two days.



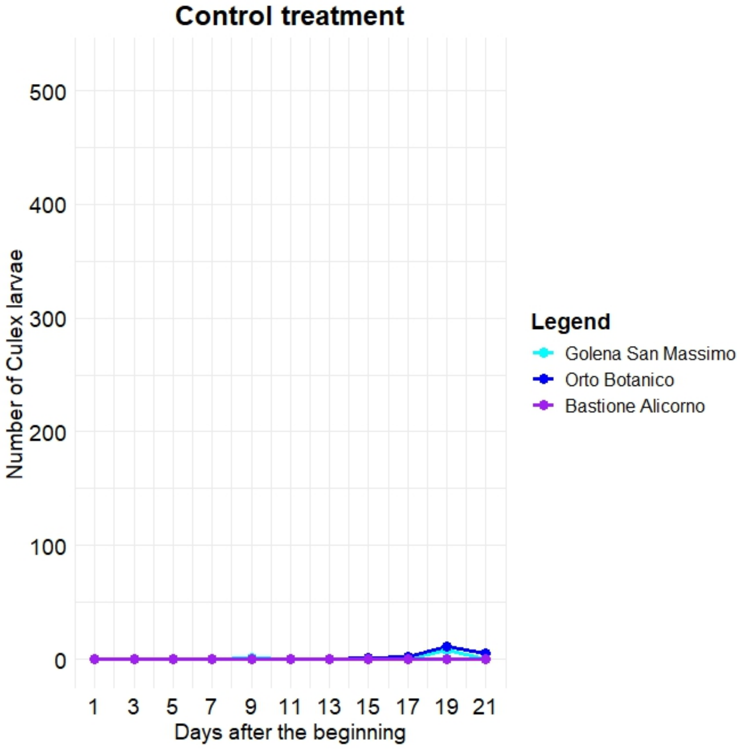
Appendix 12. Temporal trend of mosquito larvae abundance in the "Orto Botanico" site in the four treatments, reported every two days.



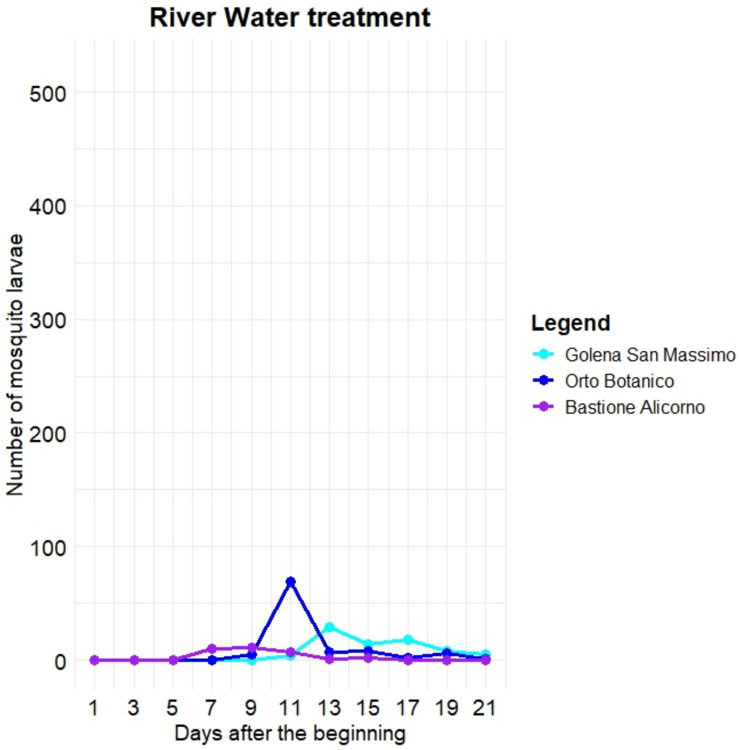
Appendix 13. Temporal trend of mosquito larvae abundance in the "Bastione Alicorno" site in the four treatments, reported every two days.



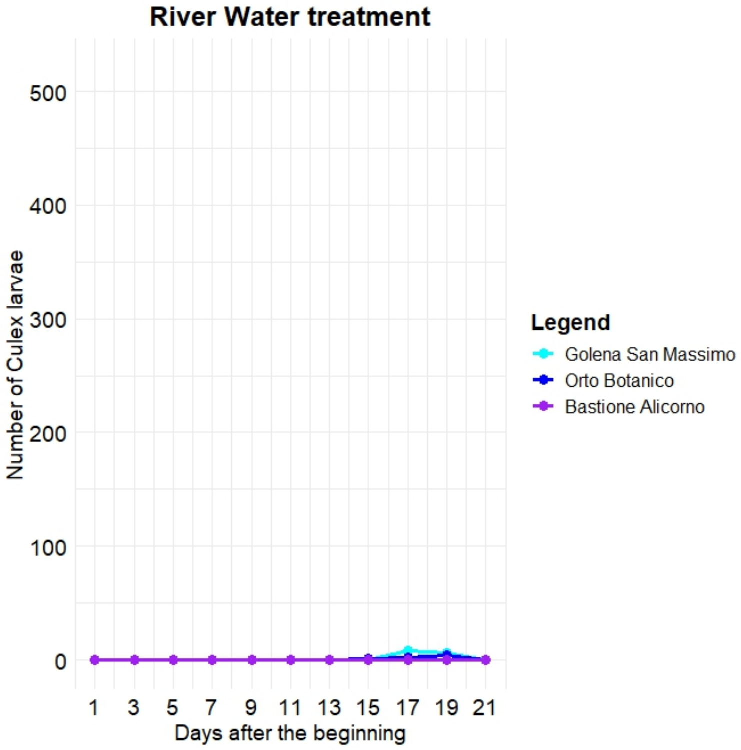
Appendix 14. Temporal trend of mosquito larvae abundance in the "Control" treatment in the three sites, reported every two days.



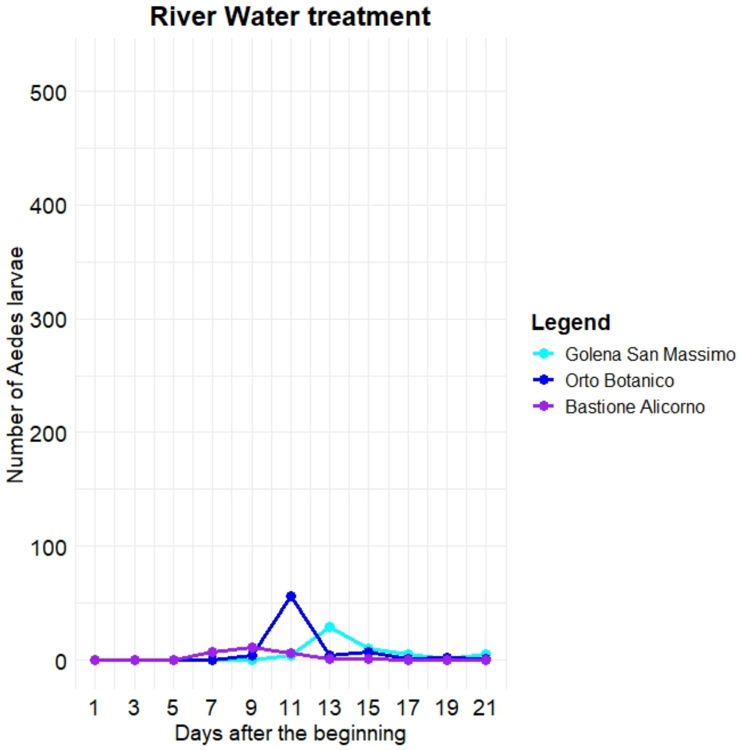
Appendix 15. Temporal trend of *Culex* larvae abundance in the "Control" treatment in the three sites, reported every two days.



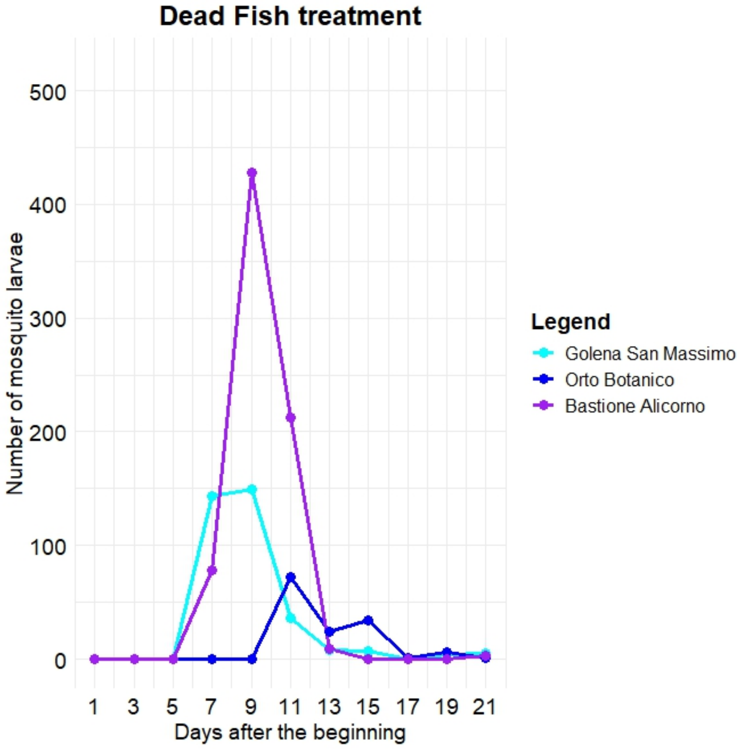
Appendix 16. Temporal trend of mosquito larvae abundance in the "River Water" treatment in the three sites, reported every two days.



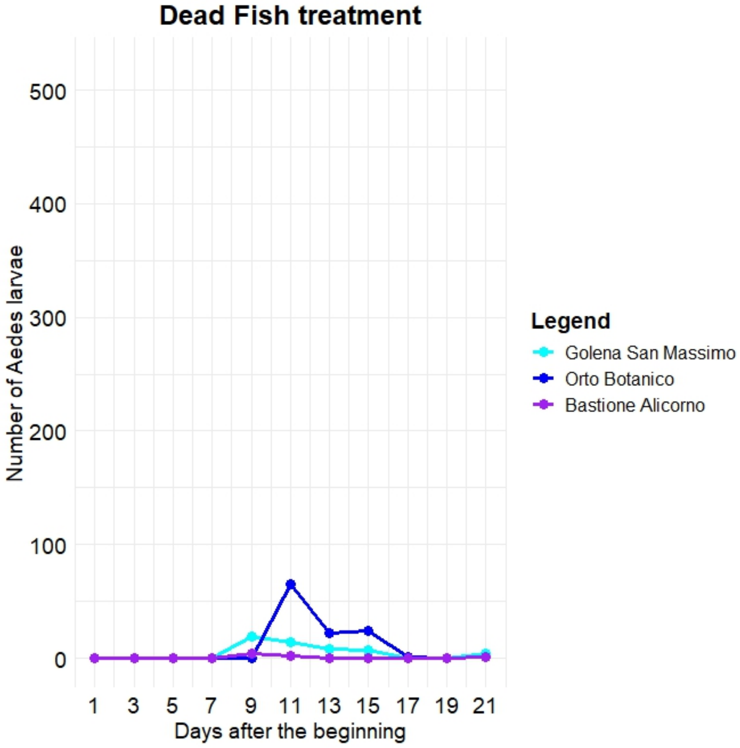
Appendix 17. Temporal trend of *Culex* larvae abundance in the "River Water" treatment in the three sites, reported every two days.



Appendix 18. Temporal trend of *Aedes* larvae abundance in the "River Water" treatment in the three sites, reported every two days.



Appendix 19. Temporal trend of mosquito larvae abundance in the "Dead Fish" treatment in the three sites, reported every two days.



Appendix 20. Temporal trend of *Aedes* larvae abundance in the "Dead Fish" treatment in the three sites, reported every two days.