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Published online: 27 Jan 2017.

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Predation efficiency of copepods against the new invasive mosquito species *Aedes koreicus* (Diptera: Culicidae) in Italy

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Abstract

Aedes koreicus (Edwards) (Diptera: Culicidae) is an invasive mosquito species and potential vector of pathogens which has recently colonised a large part of northeastern Italy and other European countries. Several species of cyclopoid copepods are natural predators of mosquito larvae and can be useful biological control agents in artificial containers used as breeding sites by *Aedes* mosquitoes. However, to ensure behavioural efficiency of these agents, and to avoid the introduction of non-native species, predatory copepods should be selected from the local fauna. In this study, we evaluated the predation efficiency under laboratory conditions of two locally bred populations of cyclopoid copepod species, *Macrocyclops albidus* (Jurine, 1820) and *Mesocyclops leuckarti* (Claus, 1857) (Copepoda: Cyclopoida), which are two of the most common European species of cyclopoid copepods, against *Ae. koreicus* larvae. Predation experiments were also conducted with *Aedes (Stegomyia) albopictus* (Skuse) as a "reference". In each predation test, one adult female copepod was placed with 50 first instar larvae of a single mosquito species in a Petri dish filled with 10 mL of water. After 24 hours, the mean number (± standard error) of first instar larvae killed by one *M. albidus* female was 18.6 ± 1.3 *Ae. koreicus* and 20.9 ± 1.3 *Ae. albopictus*, and the mean number killed by one *M. leuckarti* female was 25.8 ± 2.8 *Ae. koreicus* and 36.1 ± 4.2 *Ae. albopictus*. In addition, *M. leuckarti* was slighly less effective against *Ae. koreicus* than against *Ae. albopictus* after 48 hours, probably because first instar larvae of *Ae. koreicus* were larger than first instar larvae of *Ae. albopictus*. Our findings indicate for the first time that copepods are effective predators of first instar larvae of *Ae. koreicus*.

Keywords: *Vector control, biocontrol, larval predator, container-inhabiting mosquito, predatory copepod*

Introduction

In recent decades, non-native species of *Aedes* mosquitoes (Diptera: Culicidae) such as *Aedes (Stegomyia) albopictus* (Skuse) have colonised several areas of Europe. Among these, *Aedes (Hulecoeteomyia) koreicus* (Edwards) is a species native to Korea, Japan, northeastern China and eastern Russia (Tanaka et al. 1979). It was first detected in Belgium in 2008 and in Italy in 2011 (Capelli et al. 2011; Versteirt et al. 2012). Having colonised a large part of northeastern Italy, *Ae. koreicus* was also observed in Switzerland and western Russia in 2013, in southern Germany in 2015, and in Hungary in 2016 (Bezzhonova et al. 2014; Montarsi et al. 2015b; Suter et al. 2015; Werner et al. 2015; Kurucz et al. 2016). It has been suggested that *Ae. koreicus* transmits the Japanese encephalitis virus (Miles 1964) and *Dirofilaria immitis*, the agent of the dog heartworm (Montarsi et al. 2015a). However, despite its rapid expansion and potential role as vector of pathogens, information about the ecology and the control of this species is lacking. *Aedes koreicus* is a container-breeding mosquito, and larvae are mainly found in medium- to large-sized artificial containers permanently filled

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with organic-rich water, such as discarded tires, drums, concrete containers and construction equipment (Baldacchino et al. 2017). In addition, field observations show that this species is cold tolerant since it has been collected up to 1000 m above sea level (asl) (Baldacchino et al. 2017).

Permanent water containers used by *Aedes* mosquitoes can be treated by larvicidal products (e.g. *Bacillus thuringiensis* var. *israelensis* and insect growth regulators) as well as natural predators of mosquito larvae (Baldacchino et al. 2015). Cyclopoid copepods (Copepoda: Cyclopoida; hereafter referred to as copepods) are the most effective invertebrate mosquito predators (Veronesi et al. 2015). Because they survive for several months in artificial containers, they provide long-lasting control of mosquito populations (Marten & Reid 2007; Veronesi et al. 2015). Copepods prey mainly on first and second instar mosquito larvae, and the largest copepod species (> 1.4 mm body length) can kill up to 30–40 *Aedes* larvae per day (Marten & Reid 2007). Because copepods are usually numerically abundant in water bodies and mass production is simple and relatively inexpensive, they are considered cost-effective biological control agents (Lazaro et al. 2015). Even if several copepod species can survive droughts by special resting stages and are among the first organisms to recolonise temporary water after rainfalls (Bruno et al. 2001; Frisch & Green 2007; Kroeger et al. 2014), most of them are prone to desiccation and can only be used in permanent water containers.

To date, the predatory efficiency of about 50 copepod species belonging to 15 genera has been tested mostly in the laboratory, but also in the field, mainly against *Aedes* and *Culex* mosquitoes (Marten & Reid 2007; Veronesi et al. 2015). Two taxa of cyclopoids have proved to be particularly effective for controlling *Aedes* species such as *Ae. albopictus*: *Macrocyclops albidus* (Jurine, 1820) and several species of *Mesocyclops* (Marten & Reid 2007). While several laboratory and field studies have already been carried out on the use of *M. albidus* against *Ae. albopictus* in the United States, Brazil and, in one study, Italy (Marten 1990; Santos & Andrade 1997; Rey et al. 2004; Veronesi et al. 2015), no copepod species had been previously tested against *Ae. koreicus* larvae.

In order to avoid the introduction of non-native copepod species as control agents, two of the most common European copepod species, *M. albidus* and *Mesocyclops leuckarti* (Claus, 1857), were selected from the local fauna. Since different populations of the same copepod species can differ in predation behaviour (Marten & Reid 2007), these copepod species were mass-reared under laboratory conditions to assess their predation efficiency. *Macrocyclops albidus* and *M. leuckarti* live in temperate climatic regions: in Italy, *M. leuckarti* is most common below 500 m asl, whereas *M. albidus* has been found at altitudes of up to 1000 m asl (Stoch 2004). Both these copepods can survive in a wide range of water temperatures (0–40°C) and pH values (4.5–8), and in hypoxic conditions (Tinson & Laybourn-Parry 1985; Williams-Howze 1997; Nilssen & Waervagen 2000; Marten & Reid 2007). These ecological features make these two species suitable to use for biological control.

In the present study, we aimed to evaluate for the first time the predation efficiency of copepods against *Ae. koreicus* under laboratory conditions. Predation experiments were also conducted with *Ae. albopictus* larvae as a "reference" since copepods have already proven to be effective predators against this species. Thus, we assessed the predation efficiency of the two selected copepod populations of *M. albidus* and *M. leuckarti*, and compared their efficiency against *Ae. koreicus* and *Ae. albopictus*. We hypothesised that (i) both copepod species would be effective predators on the first instar larvae of *Ae. koreicus*, and (ii) their predation efficiency would be different according to the *Aedes* species larvae because of the size difference (i.e. *Ae. koreicus* larvae are larger than *Ae. albopictus* larvae).

Materials and methods

Copepod rearing

Macrocyclops albidus was collected from the littoral zone of Levico Lake in the province of Trento, Italy (46°00′52.72″N, 11°16′40.89″E) using a 100-µm-mesh plankton net (Figure 1A). In the laboratory, ovigerous females were isolated using a stereomicroscope and reared individually in 200-mL plastic cups filled with 50 mL well water, 50 mL protozoan culture (see below) and 1 g/L ground cat food (Friskies® adult, Nestlé Italiana S.p.A., Assago, Italy). The cups were kept in natural light conditions and placed in a thermostatic bath with a progressively increasing temperature (from 12 to 20°C) for 2 weeks to acclimatise copepods to room temperature. After the nauplius larvae hatched, the females were killed, dissected and mounted on permanent slides for identification to the species level following Dussart and Defaye (2001). Nauplii from *M. albidus* were kept aside to start the cultures. *Mesocyclops leuckarti* from Crevalcore in the province of Bologna, Italy (44°43′00″N, 11°09′00″E) was commercially available and purchased online (http://www.eugea.it/). Subsequently, cultures of *M.*
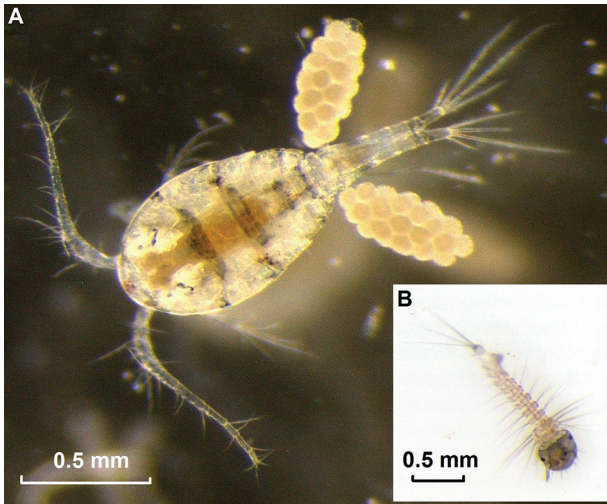
Figure 1. **A**, Ovigerous female of *M. albidus*, and **B**, first instar larva of *Ae. koreicus* (Photo: F. Baldacchino.)

albidus and *M. leuckarti* were established to provide the large number of individuals needed for the experiments.

The copepods were mass-reared under laboratory conditions according to the available literature (Suarez et al. 1992; Marten & Reid 2007). The cultures were maintained in 40-L tanks filled with 20 L of water pumped from a deep (40 m) well, and kept at room temperature and in natural light conditions. The copepods were fed weekly with pure protozoan cultures of commercially available *Chilomonas paramecium* and *Paramecium caudatum* (http://www.sciento.co.uk/). The former protozoan is the preferred food for naupliar and first copepod stages, whereas the latter protozoan is the preferred food for the last copepod stages and mature copepods. The protozoan cultures were maintained in semi-darkness at room temperature in 1-L glass jars and fed weekly with 1 g/L of ground cat food.

Mosquito rearing

Ae. albopictus and *Ae. koreicus* eggs were obtained from laboratory colonies established from mosquitoes collected in the field. *Aedes albopictus* and *Ae. koreicus* were originally collected in Trento, Italy (46° 04′N, 11°07′E) and Villa-Agnedo, Italy (46°02′N, 11°32′E), respectively. The colonies were kept in a climatic chamber at 23 ± 1°C and 75 ± 5% relative humidity with a 16:8 (light:dark) photoperiod with crepuscular periods (1 h). *Aedes albopictus* adults were maintained in a 22 × 22 × 22 cm cage whereas *Ae. koreicus* adults were maintained in a 45 × 45 × 45 cm cage (Bugdorm, MegaView

Science Co., Ltd., Taiwan). The larvae were reared in 500-mL plastic cups filled with dechlorinated water and were fed daily with ground cat food. Adults were supplied with cotton soaked in 10% sucrose solution, *ad libitum*. Adult females were fed twice a week with fresh whole cow blood using a Hemotek® blood-feeding system (Hemotek Ltd., Accrington, England) and were provided with filter paper in water-filled ovitraps for egg deposition. After 1–2 months, the eggs were immersed in plastic cups filled with 250 mL of dechlorinated water without adding food, and the resulting first instar larvae were used in the predation tests.

Predation tests

The comparative predation efficiency of *M. albidus* and *M. leuckarti* was assessed against *Ae. koreicus* and *Ae. albopictus* larvae under laboratory conditions as described by Marten (1990). Copepod females and mosquito larvae came from laboratory colonies that were less than 6 months old. This predation experiment was conducted in the same climatic chamber used for breeding the mosquitoes. In each test, one female copepod, starved for 24–48 hours, was placed in a Petri dish (5 cm in diameter) filled with 10 mL of boiled well water. Subsequently, 50 first instar larvae (less than 24 hours old) of one of the two mosquito species were introduced to each Petri dish (Figure 1B). Petri dishes containing 50 first instar mosquito larvae but no copepod were used as a control. The number of living larvae was counted after 24 and 48 hours. At the end of each test, the copepod was dissected, mounted on a permanent slide and observed under a light microscope to confirm the species identification following Dussart and Defaye (2001). Each test was replicated 15 times for each mosquito species and each copepod population, for a total of 60 tests.

Data analysis

Differences in the number of killed larvae were analysed at two different times, i.e. 24 hours and 48 hours, to compare: (i) the predation efficiency of the two copepod species on the same mosquito species, and (ii) the predation efficiency of the same copepod species on the two different mosquito species. As the data were not normally distributed, we used the non-parametric Mann–Whitney test to compare the median values of killed larvae. All statistical analyses were performed using PAST version 3.08 (Hammer et al. 2001). The mean number of larvae killed by a female copepod was also corrected using Abbott's formula as follows: Corrected mean $= 50 \times (\text{Mean}_{test} - \text{Mean}_{control})/(50 -$

Table I. Number of *Aedes koreicus* or *Ae. albopictus* killed by one female of *Macrocyclops albidus* or *Mesocyclops leuckarti* in 24 and 48 hours.

Copepod species	Ae. koreicus			Ae. albopictus		
	Mean ± SE	Corrected[†]	Range	Mean ± SE	Corrected[†]	Range
After 24 hours:						
M. albidus	18.6 ± 1.3[aA]	17.8	11–28	20.9 ± 1.3[aA]	20.1	12–33
M. leuckarti	25.8 ± 2.8[aA]	25.2	17–47	36.1 ± 4.2[bA]	35.7	5–50
After 48 hours:						
M. albidus	31.6 ± 2.2[aA]	27.0	16–45	36.6 ± 1.3[aA]	35.8	26–46
M. leuckarti	34.3 ± 2.8[aA]	30.4	15–49	41.6 ± 3.2[bB]	41.1	14–50

*N: the number of replicates; SE: standard error.

[†]Abbott's corrected mean = 50 × (Mean $_{test}$ − Mean $_{control}$)/(50 − Mean $_{control}$)

Fifty larvae were available to each copepod in small and large Petri dishes. Median values after 24 and 48 hours were tested separately (Mann–Whitney test, $P \leq 0.05$). Significant differences between copepod species (values in the same column) are represented by different lower case letters (a, b), while significant differences between mosquito species (values in the same row) are represented by different capital letters (A, B).

Mean$_{control}$). Abbott's formula is a standard in bioassay evaluation to correct data for control response.

Results

In 24 hours under laboratory conditions, and regardless of mosquito species, *M. albidus* females killed and/or consumed almost half of the 50 first instar larvae (a mean of 17.8 and 20.1 *Ae. koreicus* and *Ae. albopictus* larvae, respectively), while *M. leuckarti* females killed more: well over half of the larvae (25.2 and 35.7 respectively; Table I). After 48 hours, the mean mortality rates increased in all the test combinations. Mortality rates in control tests were about 2.5% for both *Aedes* species after 24 hours, and 5.7% for *Ae. albopictus* and 20% for *Ae. koreicus* after 48 hours. Differences in mortality rates between treatments and controls were all significant after 24 and 48 hours. *Mesocyclops leuckarti* showed a significantly higher predation efficiency than *M. albidus* against *Ae. albopictus* ($U = 55.5$ and $p = 0.02$ after 24 h; $U = 53.5$ and $p = 0.01$ after 48 h), whereas the predation rates for the two copepod species against *Ae. koreicus* did not differ significantly (Table I). In addition, the predation efficiency of *M. albidus* against both *Aedes* species did not differ significantly, whereas *M. leuckarti* was significantly more effective against *Ae. albopictus* than against *Ae. koreicus* after 48 hours ($U = 65.5$ and $p = 0.05$).

Discussion

This study represents the first evidence of the predation efficiency of copepods against *Ae. koreicus*. *Mesocyclops leuckarti* and *M. albidus* killed 25.8 ± 2.8 and 18.6 ± 1.3 first instar *Ae. koreicus* larvae, respectively, in 24 hours in a standard test. These predation rates are lower than those

considered "excellent" for mosquito control by Marten and Reid (2007), which is defined as 40 out of 50 larvae killed by one copepod female in a 10-mL dish over 24 hours. Nonetheless, both species did kill more than 50% of the *Ae. koreicus* larvae in 48 hours. Suprisingly, we observed a relatively high mortality (20%) of first instar *Ae. koreicus* larvae in the Petri dishes without copepod females (i.e. control tests) after 48 hours. This might be explained by intra-specific competition between the larvae because of starvation and/or crowding (Armistead et al. 2008).

Mesocyclops leuckarti appears to be a particularly good candidate for controlling *Ae. koreicus* and *Ae. albopictus* in northern Italy, as it killed more than 50% of first instar larvae of both *Aedes* species in 24 hours. *Macrocyclops albidus*, on the other hand, seems less effective than *M. leuckarti*. This is quite surprising as *M. albidus* has proven to be a formidable predator of *Ae. albopictus* worldwide, with predation rates ranging from 28.4 to 45.0 larvae per copepod in 24 hours (Marten 1990; Rey et al. 2004; Veronesi et al. 2015). One explanation may be, as Marten and Reid (2007) described, that different populations of the same copepod species may express differences in predation behaviour. Indeed, we did some preliminary tests on a population of *M. leuckarti* from Levico Lake, and unlike the population of *M. leuckarti* from Crevalcore, the population from Trentino did not prey effectively on the first instar larvae of *Ae. albopictus* (P. Visentin unpub. data). These results support the need for testing different local copepod populations in the laboratory to select the best species to use for mosquito control.

Our predation experiments in the laboratory using *M. albidus* and *M. leuckarti* against first instar larvae of *Ae. koreicus* and *Ae. albopictus* constitute a first step in assessing their eligibility as candidates for biological

control agents in northern Italy. We found that both copepod species were effective predators although the predation efficiency of *M. leuckarti* was slightly lower against *Ae. koreicus* than against *Ae. albopictus*, possibly due to the larger size of *Ae. koreicus* larvae. Copepods might be promising biological control agents against *Ae. koreicus* since permanent water containers with organic-rich water are preferential larval sites for *Ae. koreicus* as well as suitable environments for copepods. However, laboratory tests represent the best-case scenario, and predation rates can be lower in natural conditions, where the systems can be larger or deeper, and where mosquito larvae can occur at much lower densities. Further investigations using large containers (e.g. buckets or plastic drums) in the field are warranted, as the use of copepods in natural conditions can be affected by many factors, such as the size of the container; the temperature; the availability of food sources other than mosquito larvae; and the presence of more than one copepod individual, leading to cannibalism (Veronesi et al. 2015), i.e. all factors which would probably reduce the predation efficiency (Marten & Reid 2007). Moreover, the growth and survival of copepod populations also need to be evaluated in the field. Previous studies have demonstrated that *M. albidus* develops well in tires or drums and is tolerant to a wide range of temperatures (Rey et al. 2004; Veronesi et al. 2015). More specifically, the predation efficiency of *M. albidus* and *M. leuckarti* against *Ae. koreicus* needs to be tested in cold temperate areas, as *Ae. koreicus* has been found at altitudes of up to 1000 m asl, where *M. albidus* is present, but which is higher than the known altitudinal limit for *M. leuckarti*; average temperatures at that altitude might therefore prevent the development of *M. leuckarti*.

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