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Predation potential of three flatworm species (Platyhelminthes: Turbellaria) on mosquitoes (Diptera: Culicidae)

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ABSTRACT

We conducted a field survey for flatworms to select species as potential biological control agents against *Aedes aegypti* and *Culex pipiens* (Diptera, Culicidae) breeding in artificial containers. Laboratory experiments were performed to determine the daily predation rate, differential predation on each mosquito larval instar, selective predation on either *A. aegypti* or *C. pipiens*, and predator tolerance to water from artificial containers. *Girardia anceps* (Tricladida, Paludicola, Dugesiidae), *Mesostoma ehrenbergii* and *Bothromesostoma* cf. *evelinae* (Rhabdocoela, Typhloplanoida, Typhloplanidae) were found in temporary puddles and permanent pools. In the laboratory, they killed between 52% and 100% of immature mosquitoes coexisting in the same habitat. No preference of flatworms for mosquito preys was detected. Predation rate was related to predator size and instar of preys. *Girardia anceps* and *B. evelinae* survived after a dry period and when re-flooding occurred, they laid eggs. Tolerance to water from artificial containers was highest in *G. anceps* and this species could be a suitable predator to reduce mosquito populations from artificial containers using an inoculative approach.

1. Introduction

Turbellarians (Platyhelminthes, Tricladida, Paludicola), often referred to as planarians, are free-living flatworms that live in fresh water. They are acoelomate bilateria with simple life cycles. Flatworms are hermaphrodites with cross fertilization following copulation. They lay cocoons from which several (2–10) young emerge, grow and differentiate without metamorphosis to the adult. Cocoons are assembles of fertilized eggs, yolk cells and cocoon-shell globules. Together with the secretion of the shell glands these cocoon-shell globules harden to form the shell of the cocoon.

Flatworms are known predators of some invertebrates. Prey capture is achieved by a trapping tactic using mucus secreted when they detect a disturbance in the water caused by prey, through mechanoreceptors in the body surface; thus, they are tactile predators with external digestion (Wrona and Koopowitz, 1998; Trochine et al., 2005). Free-living flatworms are known to feed on several animals including oligochaetes, arthropods, and mollusks in the field (Hyman, 1951; Jennings, 1957; Reynoldson and Young, 1963; Mitchell, 1974). In the laboratory, *Daphnia* spp. and the nauplii stage of the brine shrimp, *Artemia salina* (L.) are commonly used as food for flatworms (McConnell, 1967).

The Rhabdocoela, another closely related group of flatworms (Turbellaria), have been less studied as predators of aquatic organisms (Motoyoshi, 2007). However, they can impact populations of other invertebrates. *Mesostoma ehrenbergii* (Focke) Örsted has been cited as a consumer of zooplankton in habitats not occupied by fishes (Maly et al., 1980; Brendonck et al., 2002; Eitam et al., 2004; Trochine et al., 2005).

Predation of mosquito larvae by free-living flatworms was first recorded by Lischetti (1919), and since then some researchers have tried to use these animals to control culicids. Experimental field populations of *Culex* larvae were reduced by over 90% in 26 days by *Dugesia dorotocephala* (Woodsworth) in California (Legner and Yu, 1975). Another species, *Dugesia tigrina* (Girard), significantly reduced *Culex* spp. larvae in catch basins in Ontario, Canada (George et al., 1983) but failed to control mosquitoes in temporary pools in North Dakota (Meyer and Learned, 1981). Suprakash and Aditya (2003) used *Anopheles* and *Culex* mosquitoes as prey for flatworms and verified that mosquito larvae are more palatable for them than eggs and pupae. They also noticed that flatworms generally avoid very small mosquito larvae (1st instar) because of their fast movement. All these observations suggested that flatworms could be applied as biological control agents but, as Legner (1995) pointed out, further research on free-living flatworms as natural enemies of mosquitoes declined as the use of *Bacillus thuringiensis* Berliner subsp. *israelensis* as an efficient microbial insecticide increased. The above mentioned references also showed that

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the use of flatworms for mosquito control depends on a series of constraints that restrict their successful use to specific environments. For this reason, it is necessary to study the local predator-prey system and the applicability as biological control agents before its integration into vector control programs can be established. Thus, contributions to the knowledge of the local flatworm fauna are useful in order to select potential predator species.

In Buenos Aires province, Argentina, two mosquito species, *Aedes aegypti* (L.) and *Culex pipiens* L., are common in urban areas. Both species have major medical relevance as vectors of important human diseases, such as dengue and yellow fever, and certain encephalitis and filarial worms, respectively. They usually breed in water accumulated in tanks, cemetery vases, discarded receptacles, and automobile tires as well as unattended swimming pools. Both mosquito species may co-occur in the same container.

Our research on flatworms in freshwater habitats of La Plata city, Buenos Aires province, had three goals: (1) to identify the communities of free-living flatworms that are more abundant in places where immature mosquitoes breed in different sites in the vicinity of La Plata; (2) to establish whether flatworms and mosquitoes co-occur in those sites, and (3) to evaluate the performance of native flatworms as predators of mosquitoes that breed in artificial containers under laboratory conditions.

2. Materials and method

2.1. Field survey

Collections were made from several mosquito breeding sites in and near La Plata city, (34°51′07″S, 58°57′30″W). Samples were collected from September to April during 2005 and 2006 when large populations of flatworms and mosquito larvae were known to occur. The freshwater bodies surveyed included three transient ponds, two permanent pools, a rice field, 12 drainage ditches, 50 artificial containers and 10 water-filled leaf axils of plants. The artificial containers and water-filled leaf axils differed on the different sample dates. Unless otherwise stated, 100 samples were taken weekly with a 300-ml ladle and concentrated with a $15 \times 10 \times 15$ cm 100-μm mesh net in a single sample. The water samples were placed into a 3-liter plastic bottle and taken to the laboratory for processing.

The three transient ponds were shallow depressions in the ground, ranging from 20 to 100 m^2, with a maximum depth of 0.35 m, filled by rainfall and persisting from several days up to 5 weeks. These were localized in a meadow about 6 km from the urban area of La Plata. The two permanent pools, located near Río de la Plata river (9 km away from La Plata) and in a field close to La Plata, respectively, had an approximate size of $25 \times 25 \times 1$ m (wide × length × depth). The large rice field had several irrigation canals, devoted to rice which was artificially flooded in December and maintained with water until April. Forty internal irrigation canals ($20 \times 0.25 \times 0.15$ m) within the field were sampled. The 12 drainage ditches were trenches dug in the ground ($25 \times 0.5 \times 0.4$ m) in a suburban area of La Plata city which were used to drain excess rainwater and household wastewater containing detergent, soap, bleach, and grease from nearby houses, with aquatic vegetation and frequently included some domestic wastes such as, glass and plastic bottles, papers and cans. In this case, five samples were taken per week from each ditch using the ladle and net technique.

The small artificial containers and water-filled leaf axils of plants required different sampling techniques. The artificial containers were 50 flower vases made of zinc or ceramic, approximately 1 l capacity, from a cemetery in La Plata city. To sample for the flatworms and mosquito larvae, the contents of the vases were individually poured into a pan in the field, and samples were concentrated and combined into one using the mesh net technique. The water-filled leaf axils of the plant, *Eryngium cabrerae* Pontiroli (Umbelliferae), which are common in non-cropped fields surrounding the city were selected at random and sampled. Water samples were extracted with a plastic pipette from 10 plants weekly, and placed into a 3-liter plastic container.

In the laboratory, flatworms were extracted from the water samples with a plastic pipette. Specimens were kept alive to start breeding cultures. In order to identify flatworms (planarians and rhabdocoelans), some individuals from the cultures were mounted in Bouin's fixative, processed into paraffin, then cut in serial sagittal sections (4-μm thick), and finally stained with the hematoxylin-eosin method. Taxonomy identification followed Noreña-Janssen and Faubel (1992) and Sluys et al. (2005). As all species were readily distinguished by naked eye on the basis of external morphology and color, subsequent separation of species was based on external features of flatworms.

Mosquito species were identified following Darsie's (1985) identification key.

2.2. Flatworm culture

Field-collected individuals were sorted by species and placed in plastic containers with 500 ml of dechlorinated tap water. Early instar mosquito larvae were added as food. As cocoons and young flatworms appeared, they were transferred to new containers to increase the number of individuals in cultures.

2.3. Source of mosquito larvae

Mosquito larvae were obtained from separate colonies of *A. aegypti* and *C. pipiens* maintained at Centro de Estudios Parasitológicos y de Vectores (CEPAVE). Adults were kept in cages and had free access to raisins. Females were fed with blood from a restrained chicken. *A. aegypti* females were offered a 750 ml black jar half filled with dechlorinated water and lined with paper towel. Eggs laid on the paper were air-dried and stored in plastic bags. Artificial hatching was achieved by submerging them in water. *C. pipiens* egg rafts were collected from a water-filled plastic bowl placed inside the cage. Larvae of both species were raised under a L:D 16:8 photoperiod, 80% relative humidity and 26 ± 2 °C in 3-liter pans and fed with powdered rabbit chow.

2.4. Predation on field-collected mosquitoes

Flatworms collected from the different breeding places were sorted into two size categories (small: <0.5 cm in length, and large: >0.5 cm in length) and exposed to larvae of the mosquito species found at each site. Each replicate consisted of a plastic beaker (8 cm diameter) with one individual of each size category per flatworm species and early instar mosquito larvae in 50 ml of dechlorinated water. The daily predation rate was recorded on the basis of missing larvae (considered as eaten by the flatworm) as well as uneaten dead larvae. A beaker containing only the test mosquito species served as the control. No other source of food source was added. Three replicates plus one control were performed for each combination of mosquito species per flatworm species (total number of experimental beakers = 48).

2.5. Experimental flatworm predation on A. aegypti and C. pipiens

To evaluate the predation capacity of three flatworm species on larvae of *A. aegypti* and *C. pipiens*, a series of experiments were carried out in the laboratory. Unless specified, all experiments were conducted in incubators at 26 ± 1 °C and a 12:12 h (L:D) photoperiod. Nine replicates plus three controls were set for each combina-

tion of predator size and species. No food source was added, but individuals were not previously starved. Flatworms were sorted into two size categories (small and large). When all individuals of one species were less than 0.5 cm, they were not classified by size.

2.5.1. Experiment 1

To assess the possible dependence of predatory capacity on the size of both prey and predator, 25 mosquito larvae of each instar were exposed to one small or large flatworm in plastic containers (8 cm diameter) containing 150 ml of dechlorinated tap water. Prey was sorted by instar (1st to 4th) in different containers. Data were analyzed by two-way ANOVA (raw data after checking for homogeneity of variances and normality) to test the effects of larval instar of prey and flatworm size, on the larval mortalities of both *A. aegypti* and *C. pipiens*. Separate ANOVAs were performed for each combination of prey and predator species.

2.5.2. Experiment 2

To assess possible differential predation of flatworm on either mosquito species, mosquito larvae, comprising 25 2nd instar of *A. aegypti* and 25 2nd instar of *C. pipiens* (50 larvae/container) were exposed to one flatworm. The same containers were used as before but with 250 ml of water and with only one large flatworm. Raw data (after checking for homogeneity of variances and normality) were analyzed by parametric one-way ANOVA, followed by a Duncan's Multiple Range Test at $P < 0.05$.

In Experiments 1 and 2, the numbers of missing and dead larvae were recorded 24 h after the start of the experiment.

2.5.3. Experiment 3

To assess the daily predation rate during 5 consecutive days for each flatworm species, 25 2nd instar larvae of either *A. aegypti* and *C. pipiens* were exposed to one flatworm (small or large) in a container with 150 ml of water. Every 24 h, dead larvae were counted, and the remaining larvae were replaced by a new batch of 25 larvae. Unless otherwise specified, data were analyzed by repeated measures ANOVA with days as the within-subject factor after log-transformation of number of missing larvae in order to achieve homoscedasticity and normality, followed by a Duncan's test.

2.5.4. Experiment 4

This experiment was designed to determine if any of the flatworm species has higher tolerance to dry periods relative to the other. Resistance eggs (cocoons) left in containers would enable the flatworms to survive after desiccation, which frequently occurs in *A. aegypti* breeding containers. Fifteen individuals of each species were placed in petri dishes (10 cm diameter) with 50 ml dechlorinated tap water. Dishes were kept at room temperature until the water evaporated, which was achieved about 7 days later. Dishes were divided into two groups. One of them was flooded 10 days after the water evaporated, and the other after 20 days. Three containers for each flatworm species were flooded each time, and another was used as control maintaining 50 ml of water throughout the experiment. After the dry periods, the number of cocoons left by flatworms in each replicate was counted. Collapsed cocoons were considered as non viable and were not counted. Following each flood event, newly hatched flatworms were scored as present or absent.

2.6. Tolerance to water from artificial containers

This experiment was designed to determine if flatworms can tolerate unnatural environment conditions such as water-holding artificial containers in which *C. pipiens* and *A. aegypti* commonly breed. To assess if high mortality occurs in flatworms developing in water from artificial containers, we collected the contents of 25 flower vases from La Plata Cemetery and mixed them in a 3-liter bucket. In the laboratory, the liquid from vases was filtered through Whatman® No 5 paper filter (particle retention: 2.5 μm) on the same day of collection. Ten flatworms of each species were placed into 250 ml plastic beakers with 150 ml of filtered water from flower vases. No food was added, so that the only food items available were the microorganisms able to pass through the paper filter. Three replicates plus a control using dechlorinated tap water per flatworm species were performed. Percentage of mortality was recorded at 10 and 20 days.

3. Results

3.1. Field survey

The flatworms were identified to the species level using the serial sagittal sections and histologic techniques following Noreña-Janssen and Faubel (1992) and Sluys et al. (2005) which revealed distinctive features of the reproductive system anatomy. Three species were recognized: *Bothromesostoma* cf. *evelinae* Marcus and *M. ehrenbergii* (Rhabdocoela, Typhloplanoida, Typhloplanidae), and *Girardia anceps* (Kenk) Ball (Tricladida, Paludicola, Dugesiidae), were found in the field survey (Table 1). In the transient freshwater ponds, immature stages of *Ochlerotatus albifasciatus* (Macquart) and *Culex dolosus* Lynch Arribalzaga were found along with the three flatworm species. In the permanent pools, *M. ehrenbergii* and *B.* cf. *evelianae* were found with floating vegetation colonized by *C. dolosus* and *Culex eduardoi* Casal & García.

Flatworms were not found in the rice field, drainage dishes, artificial containers or water-filled leaf axils of *E. cabrerae*. However, mosquito species were isolated from these habitats. *Anopheles albitarsis* Lynch Arribalzaga and *C. dolosus* were found in the rice field, *C. pipiens* in drainage ditches, *Culex renatoi* (Lane & Ramallo) in the axils of *E. cabrerae*, and *A. aegypti* and *C. pipiens* in artificial containers.

3.2. Predation on field-collected mosquitoes

In this experiment, the flatworms eliminated most of *C. dolosus* and *O. albifasciatus* field-collected larvae. The three flatworm species fed on early instar mosquito larvae and the percentage of larvae killed ranged from 52% to 100% (Table 1). In flatworm species separated by size, both large and small individuals consumed mosquito larvae at a similar rate. Larvae of *C. eduardoi* were not exposed to the flatworms because they were found in low numbers in the field.

Table 1
Mosquito and flatworm species found in natural breeding places from La Plata and mosquito larval mortality in laboratory tests.

Habitat	Flatworm species	Flatworm size (cm)	Mosquito species (number exposed)	Dead larvae (%)
Transient pools	*M. ehrembergii*	>0.5	*O. albifasciatus* (15)	100
		<0.5		87
	G. anceps	>0.5		100
		<0.5		100
	B. cf. *evelinae*	<0.5		80
	M. ehrembergii	>0.5	*Culex dolosus* (25)	100
		<0.5		92
	G. anceps	>0.5		100
		<0.5		96
	B. cf. *evelinae*	<0.5		60
Permanent pools	*M. ehrembergii*	>0.5	*Culex dolosus* (25)	100
		<0.5		88
	B. cf. *evelinae*	<0.5		52

3.3. Experimental flatworm predation on A. aegypti and C. pipiens

3.3.1. Experiment 1

Girardia anceps vs. *A. aegypti*: In this and other experiments, no mortality was recorded in the controls, and these data were excluded from the analysis. Our results showed that the large *G. anceps* killed more 1st and 2nd instar than 3rd and 4th instar larvae. Predation by small *G. anceps* varies significantly only among 1st and 3rd instars (Table 2, Fig. 1A).

Table 2
Two-way ANOVA results from Experiment 1 to evaluate predation of small and large *Girardia anceps* and *Mesostoma ehrenbergii* on 1st to 4th larval instars of *Aedes aegypti* and *Culex pipiens*. For *Bothromesostoma* cf. *evelinae*, a one-way ANOVA was performed because size was excluded as a factor.

Flatworm species	Mosquito species	Effect	df	*F*	*P*
G. anceps	*A. aegypti*	*A. aegypti* instar	3,64	62.40	<0.001
		G. anceps size	1,64	85.55	<0.001
		Instar × size	3,64	18.58	<0.001
	C. pipiens	*C. pipiens* instar	3,64	17.28	<0.001
		G. anceps size	1,64	16.11	<0.001
		Instar × size	3,64	4.98	0.004
M. ehrenbergii	*A. aegypti*	*A. aegypti* instar	3,64	19.91	<0.001
		M. ehrenbergii size	1,64	25.40	<0.001
		Instar × size	3,64	10.70	<0.001
	C. pipiens	*C. pipiens* instar	3,64	21.72	<0.001
		M. ehrenbergii size	1,64	48.34	<0.001
		Instar × size	3,64	1.41	0.24
B. cf *evelinae*	*A. aegypti*	*A. aegypti* instar	3,32	10.95	<0.001
	C. pipiens	*C. pipiens* instar	3,32	9.50	<0.001

Girardia anceps vs. *C. pipiens*: Small and large *G. anceps* preyed differentially on *Culex* 1st and 2nd instars. In the case of large-sized prey, predator size did not change predation capacity (Table 2, Fig. 1B).

Mesostoma ehrenbergii vs. *A. aegypti*: The predation capacity of small *M. ehrenbergii* decreased as larvae increased in size. No differences were detected in predation by large *M. ehrenbergii* on different instar larvae (Table 2, Fig. 1C).

Mesostoma ehrenbergii vs. *C. pipiens*: For small *M. ehrenbergii*, predation capacity was significantly lower when exposed to 3rd and 4th instar larvae; no differences were found among predation rates of large *M. ehrenbergii* (Table 2, Fig. 1D).

Bothromesostoma cf. *evelinae* vs. *A. aegypti* and *C. pipiens*: Both mosquitoes were preyed upon, but significant differences were only found between 1st–3rd and 4th instars (Table 2, Fig. 1E and 1F).

3.3.2. Experiment 2

None of the flatworm species exhibited a preference for particular prey species (*G. anceps*: $F_{1,\ 16} = 1.51$, $P = 0.23$; *M. ehrenbergii*: $F_{1,\ 16} = 1.02$, $P = 0.32$; *B.* cf. *evelinae*: $F_{1,\ 16} = 0.36$, $P = 0.85$). *B.* cf. *evelinae* preyed upon fewer larvae (mean ± SE number of killed larvae, *C. pipiens*: 5.0 ± 1.1, *A. aegypti*: 4.3 ± 0.8) than *M. ehrenbergii* (*C. pipiens*: 14.5 ± 1.3, *A. aegypti*: 13.8 ± 0.8) and *G. anceps* (*C. pipiens*: 20.5 ± 1.3, *A. aegypti*: 22.3 ± 0.5).

3.3.3. Experiment 3

Girardia anceps was able to maintain its predation rate over the 5 days on both mosquito species (Table 3, Fig. 2A). *M. ehrenbergii* fed on fewer larvae on the 5th day, so that its predation rate on both *C. pipiens* and *A. aegypti* was not sustained evenly (Table 3,

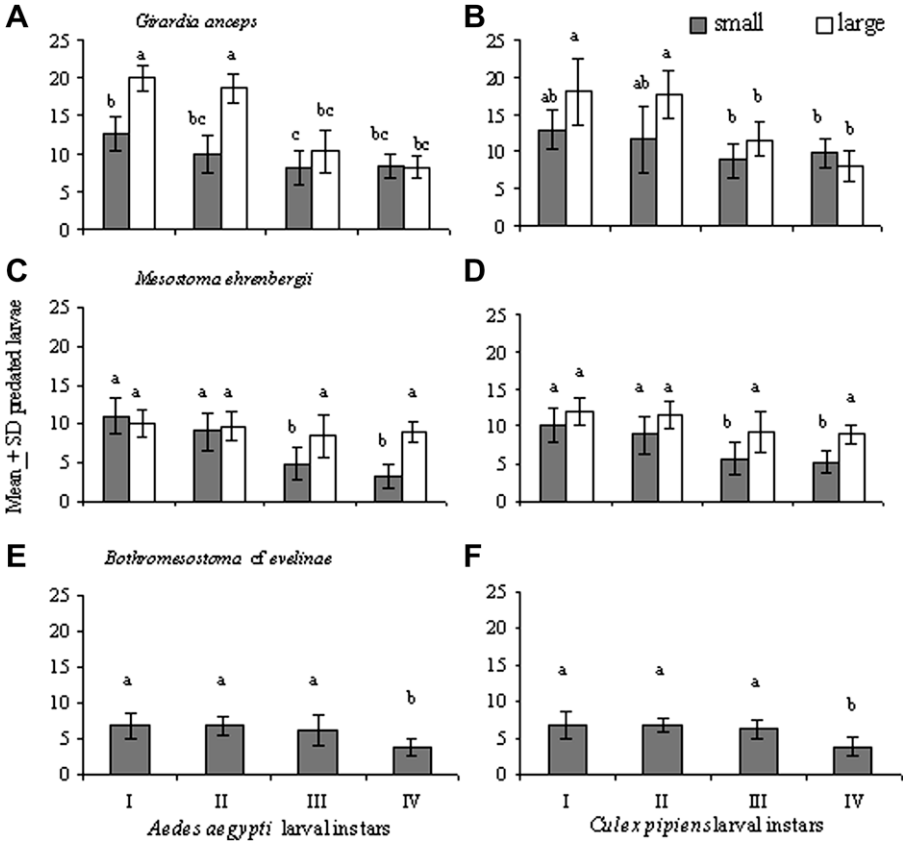

Fig. 1. Average number of 1st, 2nd, 3rd and 4th instars larvae (designated as I–IV) of *Aedes aegypti* and *Culex pipiens* killed by small and large *Girardia anceps* (A and B), *Mesostoma ehrenbergii* (C and D) and by *Bothromesostoma* cf. *evelinae* (not segregated by size) (E and F). Vertical bars show ±1 SD. Different letters above the bars show significant differences ($P < 0.05$) after Duncan's test.

Table 3
Repeated measures ANOVA results from experiment to evaluate predation capacity of *Bothromesostoma* cf. *evelinae* and small and large *Girardia anceps* and *Mesostoma ehrenbergii* against larvae of *Aedes aegypti* and *Culex pipiens*. For *B.* cf. *evelinae*, a one-way ANOVA was performed because factor size was excluded, as all individuals were the same size. Data were transformed as $Y = \log$ (number of killed larvae) to meet ANOVA's assumptions.

Flatworm species	Mosquito species	Effect	df	*F*	*P*
G. anceps	*A. aegypti*	Day	4,64	1.98	0.11
		Size	1,16	348.92	<0.001
		Day × size	4,64	1.02	0.40
	C. pipiens	Day	4,64	0.28	0.88
		Size	1,16	525.43	<0.001
		Day × size	4,64	0.29	0.87
M. ehrenbergii	*A. aegypti*	Day	4,64	41.51	<0.001
		Size	1,16	255.21	<0.001
		Day × size	4,64	5.77	<0.001
	C. pipiens	Day	4,64	77.98	<0.001
		Size	1,16	162.89	<0.001
		Day × size	4,64	9.12	<0.001
B. cf. *evelinae*	*A. aegypti*	Days	4,40	2.7	0.04
	C. pipiens	Days	4,40	1.37	0.26

Fig. 2B). Predation of *B.* cf. *evelinae* on *C. pipiens* was constant over 5 days but the predation capacity of this species on *A. aegypti* was not constant, with a maximum peak occurring on the 3rd day (Table 3, Fig. 2C).

3.3.4. Experiment 4

Girardia anceps deposited 9 ± 1.5 (mean ± SE) viable eggs/replicate of 15 individuals after 10 days of desiccation, and 6 ± 3.6 eggs after 20 days. *M. ehrenbergii* laid 3 ± 1.5 after 10 days of desiccation, and 1 ± 1 after 20 days. *B.* cf. *evelinae* was not able to survive under desiccation conditions, and no eggs were recorded at any period of time.

3.4. Tolerance to water from artificial containers

In water from flower vases, no mortality was recorded for *G. anceps* after 10 days of exposure, and 4% of mortality was recorded after 20 days. *M. ehrenbergii* showed 0% and 14% of mortality after 10 and 20 days, respectively. Mortality in *B.* cf. *evelinae* reached 4% after 10 days and 44% after 20 days. In addition, *M. ehrenbergii* laid 3 eggs and *B.* cf *evelinae*, 5 eggs after 20 days.

4. Discussion

Mesostoma ehrenbergii occurs worldwide (Noreña-Janssen and Faubel, 1992), whereas *G. anceps* was recorded in Argentina and Paraguay (Sluys et al., 2005), and *B.* cf. *evelinae* is distributed in Brazil, Uruguay, Argentina and Peru (Noreña-Janssen and Faubel, 1992; Noreña et al., 2006). The three species mentioned in our study are common in permanent and temporary freshwater sites in Argentina. Free-living Platyhelminthes inhabit freshwater environments characterized by temporarily fluctuating abiotic factors and are able to resist these fluctuations in the environment (Noreña et al., 2004). Given these habitat characteristics, our findings on the capabilities of *G. anceps*, *B.* cf. *evelinae* and *M. ehrenbergii* to survive adverse conditions, including tolerance to desiccation, are not surprising. However, we did not find flatworms in human-made water bodies such as ditches and artificial containers, suggesting that they would not be able to easily colonize such places from their natural breeding sites. Perhaps, the lack of vegetation would make it difficult for them to reach these human-made habitats (Noreña et al., 2004). Yet, as demonstrated by the experiment of tolerance to water from cemetery vases, these flatworm species have the capacity to live in artificial containers such as flower vases. This highlights the possibility of introducing them in habitats which are naturally colonized by the target mosquito species, but not by *G. anceps*, *B.* cf. *evelinae* and *M. ehrenbergii*.

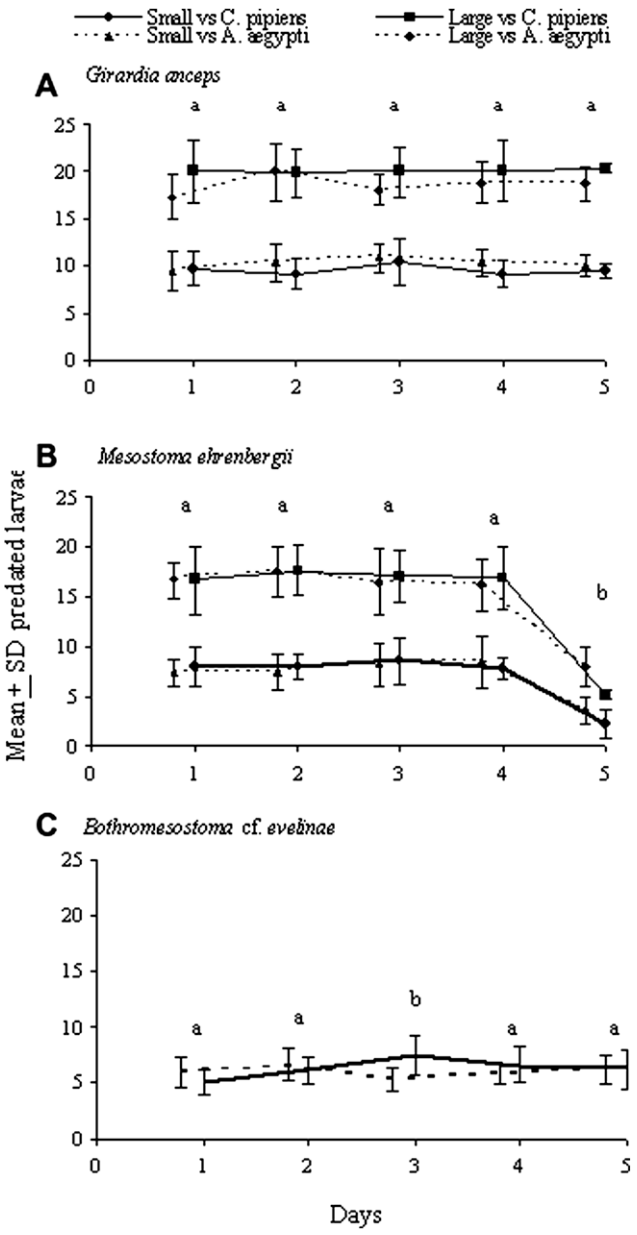

Fig. 2. Average daily predation rates of small and large *Girardia anceps* (A) and *Mesostoma ehrenbergii* (B), and *Bothromesostoma* cf. *evelinae* (not segregated by size) (C) on *Culex pipiens* and *Aedes aegypti* larvae. Days across different treatments were not compared. Vertical bars show ±1 SD. Different letters above the data points show significant differences between days within the same treatment ($P < 0.05$) after Duncan's test.

We have shown that the three species of flatworms do not feed differentially on the two mosquito species offered as a prey. Experimentally, each species of flatworms consumed similar numbers of both prey species in 24 h. Thus, the idea that depletion of one prey species is followed by consumption of the other can be dismissed. In the case of *M. ehrenbergii* and *G. anceps* which consumed more than half of the individuals of each mosquito species, any preference could have been masked by the intense consumption. The lack of preference can be explained because free-living flatworms

are not specific (Yu et al., 1996) and are aggressive predators of invertebrates in shallow aquatic habitats (Blaustein and Dumont, 1990). Alternatively, our results could be related to the spatial simplicity of the experiments. Because increasing structural complexity of the environment plays an important role in predation dynamics by providing shelter for prey species (Dutilleul, 1993), predation success could be higher in structurally simple habitats, where prey may be more easily detectable and susceptible to attack by predators (Gilinsky, 1984). The probability and frequency of encounters between prey and predator are influenced by the presence of refuges and their behaviors (Trochine et al., 2005). For example, Melo and Andrade (2001) found that the larval mobility of *Culex quinquefasciatus* L., made them more susceptible than *Aedes albopictus* (Skuse) to predation by *Girardia tigrina* (Girard) in automobile tires. In our experiments, a faster reaction of *C. pipiens* larvae could be cancelled by the short distance between predators and prey, due to the small size of our experimental containers. Finally, the presence of non-mosquito prey in the containers could also affect the predation rates of the flatworms. The efficiency of flatworms as mosquito predators would be affected if they prefer other invertebrates (arthropods, nematodes, oligochaetes, etc.) as prey. On the other hand, the consumption of other organisms such as copepods by flatworms would promote the survival of flatworms at times when mosquitoes are absent, i.e. outside the mosquito breeding season. *M. ehrenbergii* will feed on copepods (Trochine et al., 2005) and can also consume larvae of Chironomidae (M.C. Tranchida, personal observations). Further research on the interactions between predatory copepods and flatworms would be interesting in order to know their influence on the control of mosquito populations. In any case, no predatory copepod species were found in artificial containers from La Plata (Tranchida et al., in press) where flatworms can be artificially introduced.

The predatory capacity of each species of flatworms can be related to body size. Adults of *B.* cf. *evelinae* are smaller than the other species (Noreña-Janssen and Faubel, 1992), so pharynx size can be a constraining factor for predation rate. *M. ehrenbergii* and *G. anceps* were capable of preying on all larval instars because of their larger size. Clearly, this capacity was more intense on 1st and 2nd than on 3rd and 4th instars, but Suprakash and Aditya (2003) detected a lower predation on 1st instar due to their faster movements. In addition, although *G. anceps* and *M. ehrenbergii* showed good predatory capacity on all *A. aegypti* and *C. pipiens* instars, *M. ehrenbergii* was not able to maintain its daily predation rate. Our data showed that on the fifth day of the experiment, the number of larvae preyed upon decreased significantly after 4 days, and therefore, *G. anceps* is a better candidate for mosquito control than *M. ehrenbergii*.

Members of the genus *Girardia* lay a single egg with a stalk used to stick to the substrate, and *Mesostoma* holds a large number of eggs inside its uterus. Eggs are released after death of the individual, and are resistant to desiccation. *Bothromesostoma* lays only one egg without retaining it in the uterus for a long time. Our results from Experiment 4 were not performed to record the number of eggs laid by each individual, but at least in the cases of *Bothromesostoma* and *Girardia* egg-laying habits are consistent with the above. In *Mesostoma*, the number of eggs obtained was very low, but the previous history of the individuals was unknown, and there may be an underlying effect of age and/or maturity of flatworms. Besides this, *M. ehrenbergii* can sexually reproduce by either dormant or subitaneous eggs. The latter hatch without going through a period of dormancy and promote a fast numerical response (Fiore, 1971) resulting in rapid population increase. This aspect was not explored in our work but is worth pursuing in the future.

Although several culture techniques for flatworms are available (Legner and Tsai, 1978), the fact that members of genus *Girardia* can multiply both by tissue regeneration through artificial methods (Callahan and Morris, 1989) and egg production, makes *G. anceps* more suitable for mass production than the other predators studied herein.

In conclusion, we propose that *G. anceps* is, among the species of flatworms under study, the most appropriate organism to be used as a biological control agent for *A. aegypti* and *C. pipiens* in artificial containers by means of an inoculation strategy. *G. anceps* can prey on all instars of both mosquito species and maintain a steady predation rate over time. This species can also be artificially cultured by mass production methods and is able to resist environmental variations within human-made water-containing habitats. Although these types of organisms are not broadly applicable against mosquitoes, they may be successful in specific situations. An integrated control approach could be applied consisting of the release of flatworms along with low volumes of low-risk larvicides. This practice could contribute to decrease the use of pesticides in situations where the reduction of mosquito breeding sites is difficult, as in cemeteries. A combination of flatworms with other means of control such as *B. thuringiensis* subsp. *israelensis* (Perich et al., 1990), and insect growth regulators (Nelson et al., 1994) could be achieved. From this perspective further research on the integration of flatworms into biological control programs is recommended.

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