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B. Byttebier
Departamento de Ecología, Genética y Evolución
Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires
Ciudad Universitaria, Pabellón 2, 4to piso, Laboratorio 54
Nuñez C1428EHA, Ciudad Autónoma de Buenos Aires, Argentina
Phone: 54 11 5285-7400 (int. 58381)
E-mail: bbyttebier@gmail.com

Predation on eggs of *Aedes aegypti* (Diptera: Culicidae): temporal dynamics and identification of potential predators during the winter season in a temperate region

Barbara Byttebier and Sylvia Fischer

Departamento de Ecología, Genética y Evolución, and IEGEBA (UBA-CONICET), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina.

Abstract

In temperate regions, the populations of *Aedes aegypti* (L.) (Diptera: Culicidae) remain in the egg stage during the cold season. The ability of these eggs to survive until the next favorable season is affected by several mortality factors, including the action of predators. In the present study, we analyzed the temporal dynamics of the loss of eggs and identified predators of *Ae. aegypti* eggs along the unfavorable season in a temperate region of Argentina. To this end, eggs were exposed in field conditions in pitfall traps, where walking arthropod taxa were captured during one-week periods from early June to early September (Austral winter). The association of arthropod taxa with the loss of eggs was analyzed to identify potential predators. Based on the results obtained, two taxa were chosen to confirm their capacity to consume eggs in a laboratory study. The proportion of lost eggs and the abundance of predators were significantly higher in the winter-spring transition, although results were heterogeneous among traps in all exposure periods. Ants of the genus *Strumigenys*, isopods of the species *Armadillidium vulgare*, and dermapterans of the species *Euborellia annulipes* were positively associated with a high proportion of lost eggs. In laboratory conditions, *A. vulgare* and *E. annulipes* consumed the offered eggs, thus confirming their predator capacity. This study represents the first record of predation of *Ae. aegypti* eggs in temperate South America and the first evidence of dermapterans consuming mosquito eggs.

Key words: mosquito eggs, predation, arthropods, winter season

Introduction

Aedes aegypti (Diptera: Culicidae) has increasing importance for public health because it is a competent vector of several viruses. In South America, this species is the main vector of dengue and urban yellow fever, as well as of chikungunya fever and Zika virus disease, which are emergent diseases in this region (Brisola Marcondes et al. 2017).

During the last decades, *Ae. aegypti* has expanded its distribution, currently covering tropical, subtropical and also temperate regions in all continents except Antarctica (Kraemer et al. 2015). In America, the temperate regions where this species occurs include part of the United States in the northern hemisphere, high elevation areas in Mexico and western South America, and central Argentina, including Buenos Aires

province. In these regions, the development and reproduction of *Ae. aegypti* are limited by low temperatures, and the larval development and adult activity are restricted to the warm months (Eisen et al. 2014). However, during the warm period, adults reach very high abundances and contribute to the transmission of viral diseases (Fischer et al. 2017). In the Metropolitan Area of Buenos Aires alone, 4739 cases of local transmission of dengue were confirmed during the epidemics in 2016 (Ministry of Health of Argentina 2016).

In temperate areas, the winter season represents the period of highest vulnerability for this species, since most of the population remains in the form of dormant eggs. Thus, knowledge about mortality factors acting on that stage of development is needed to understand the potential limitations for this species. Most studies on mortality factors affecting *Ae. aegypti* have been performed on the aquatic stages of development (larvae and pupae), and, to a lesser extent, on the adult stage (especially of females, which have been studied extensively because of their epidemiological implications). In contrast, very few studies have explored the mortality factors affecting eggs, especially in field conditions (Mogi 2007).

The eggs of *Ae. aegypti* are laid individually on the internal side of artificial containers, above the line of water. These eggs may either hatch immediately after the completion of embryonic development or remain dormant for months and hatch when favorable conditions return. Although eggs are known to survive for long periods, in some cases up to 15 months in laboratory conditions, survival decreases after longer storage times (Christophers 1960). In natural conditions, egg mortality after three months was found to be of 86% in Queensland, Australia (Russell et al. 2001), 65% in Tanzania, East Africa (Trpis 1972), and 49% in the city of Resistencia, Argentina (Giménez et al. 2015). The most relevant factors affecting mortality include the climate conditions, the amount of sun exposure, the container type and the action of natural enemies, all of which present geographic variability (Trpis 1972, Russell et al. 2001). The natural enemies affecting the mortality of eggs include fungi, which have been associated with a decrease in egg viability (Russell et al. 2001, Giménez et al. 2015), and arthropods, which may prey on eggs (Christophers 1960, James 1966, Pérez Insueta et al. 2004, Yang 2006). Since, under natural conditions, the proportion of lost eggs associated with predation by arthropods in Resistencia (Chaco, Argentina) and Queensland (Australia) during the

unfavorable season has been found to vary around 10-12% (Giménez et al. 2015, Russell et al. 2001), this factor could represent a significant source of mortality.

Some recognized predators of *Ae. aegypti* eggs are *Tapinoma melanocephalum* ants (Pérez Insueta et al. 2004), pillbugs (Focks et al. 1993), and cockroaches (Christophers 1960), in particular *Periplaneta americana* (Dyctioptera: Blattidae) (Russell et al. 2001).

In Buenos Aires city, where the population of *Ae. aegypti* persists mainly in the egg stage during the cold season (Vezzani et al. 2004), the mortality of winter eggs after exposure to field conditions over three months has been estimated at 30.6 % (Fischer et al. 2011). In addition, between 12% and 30 % of the eggs exposed to natural conditions in different studies during the winter were lost, probably as a consequence of egg predation (Fischer et al. 2011, Byttebier 2017).

Besides anecdotic information, few studies about predation on mosquito eggs have evaluated the predation capacity of a particular species, and most of them have been performed under controlled conditions. The scarcity of works on this subject provides very incomplete information about the potential predators of *Ae. aegypti* eggs, and none of them has been performed in the temperate region of South America. Furthermore, no ecological studies have been performed to recognize the main predators or assess relevant aspects in the predator-prey relationship such as the degree of temporal and spatial coincidence, an essential factor of the dynamics of this interaction. Such studies are necessary to collect information to choose effective predators and select potential agents for biological control as part of an integrated vector control program.

Based on the above, the aims of the present study were: a) to analyze the temporal dynamics of *Ae. aegypti* egg loss during the winter season, b) to identify potential predators on these eggs, and c) to test the ability of two potential predators to consume eggs.

Materials and Methods

Study area

Buenos Aires city (34° 36' S, 58° 26' W) is characterized by a temperate climate with a temperature that varies seasonally. The mean temperature in fall and spring varies around 17 °C, with cool mornings and nights. In winter, the mean temperature is 11.5

°C, and the weather is moderately cold during the day and cold during the night. In summer, solar radiation is strong and the mean temperature is around 23.6 °C. The annual cumulative rainfall is 1,140 mm on average, and rainfall events are recorded regularly throughout the year, with a decrease in the winter months (National Meteorological Service 2016).

Source of *Aedes aegypti* eggs for the experiments

The eggs used in the experiments were obtained from a colony of *Ae. aegypti* initiated from eggs collected with ovitraps placed in different quarters of Buenos Aires city, during the warm season priorto the experiment. The colony was maintained for 5 months at ambient temperature (≈ 27 °C) and a photoperiod of 14:10 h (L:D) to simulate the summer conditions of Buenos Aires. The field-collected eggs were submerged in a solution of water and powdered baker´s yeast to stimulate hatching. During their development, larvae were fed with a solution of yeast *ad libitum*. Once pupated, individuals were transferred to new containers (8 cm in diameter and 5.5 cm in height), which were placed into an acrylic box (60 x 60 x 60 cm), where the adults were maintained after emergence. For the blood feeding procedure, one or two days old mice were placed into the adult box for two hours. After blood feeding, oviposition substrates (wooden paddles) were placed into the box, attached to the pupal container (Gerberg et al. 1994). The eggs used in the experiments were those laid by F2 females reared in the laboratory, to reduce differences due to maternal age, life history, geographic origin, or oviposition period in the field. The blood feeding and oviposition processes were repeated through four weeks. A total of 21,872 eggs were collected during those four weeks.

Experimental design

Two experiments were carried out. The first one was performed in field conditions to study the temporal dynamics of egg loss and the associated potential predators. The second was carried out in laboratory conditions to assess the ability of two potential predators to consume eggs.

Field study

The experiment was performed at the campus of the Faculty of Exact and Natural Science, University of Buenos Aires, at the north-eastern edge of Buenos Aires city. The

experimental site consisted of a fenced green area of 2,130 m² surface, with patches of tall grass, trees and bushes, and part of the ground covered with dead leaves.

The assay consisted in exposing *Ae. aegypti* eggs placed in pitfall traps arranged to collect walking arthropods. Each trap consisted of a plastic container (8 cm in diameter and 5.5 cm in height) buried at ground level. To prevent the flooding of the containers by rain, a plastic protection was attached 2 cm above each trap, fixed to the ground with wire (Fig. 1). Every three weeks throughout the winter, a group of eggs were exposed for one week (Table 1). In each opportunity, a new substrate with a known number of *Ae. aegypti* eggs (at least 20) was placed into each trap.
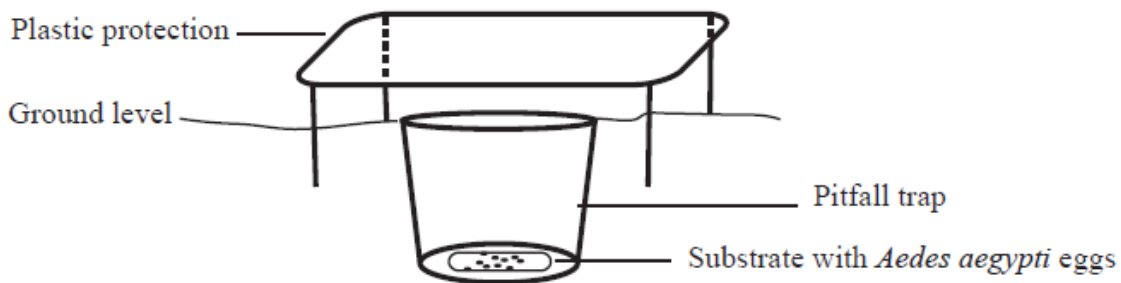
Fig. 1. Pitfall trap diagram containing a substrate with *Aedes aegypti* eggs, covered by a plastic protection, placed in the field.

Table 1. Exposure periods of pitfall traps with *Aedes aegypti* eggs inside, placed in the field, and atmospheric conditions of each period.

exposure period	mean temperature (ºC)	min-max temperature (ºC)	rainfall (mm)
early June	12.7	6.5-21.5	0
end of June	12.3	4.9-18.6	0
early July	12.2	4.5-20.4	9.8
end of July	10.3	3.5-18	0.6
mid-August	11.1	4.2-18	6.5
early September	15.4	10-24	35

After each exposure period, pitfall traps were transferred to the laboratory. The eggs recovered from each substrate were counted under a stereoscopic microscope, discriminating intact, dead (collapsed and semicollapsed), and hatched eggs. Captured arthropods were fixed in 80 % ethanol for later identification by using specialized taxonomic keys (Morrone and Coscaron 1998). The level of taxonomic identification of each group of arthropods depended on their potential capacity to prey upon *Ae. aegypti*

eggs according to previous information, and on the availability of adequate taxonomic keys. Ants were identified to genus level (Fernández et al. 2003), whereas isopods and earwigs were identified to species level (Araujo 1999, Klostermeyer 1942). Heteroptera, Homoptera, Orthoptera, Coleoptera, Amphipoda and Opilionida were identified to family or superfamily level. Ticks and spiders were separated in morphospecies. Individuals of Chilopoda and Diplopoda were identified to order or subclass level. All individuals of Blattodea captured were juveniles, which allowed identification only to suborder level because wings (absent in the early stages) present the key taxonomic characters. Collembolans were not counted because they are not considered consumers of mosquito eggs due to their size and foraging habits.

Data analyses

The proportion of lost eggs from each trap was calculated by subtracting the number of recovered eggs from the number of initial eggs, divided by the number of initial eggs. The proportions of lost eggs were compared among periods with the Kruskal-Wallis test, because data did not meet the assumptions of normality and homogeneity of variances. Post-hoc comparisons between periods were performed with the mean rank test.

The relationship between egg loss and the different arthropod taxa was analyzed with a Generalized Linear Mixed Model (GLMM) by using the R software. The proportion of lost eggs in each trap was included as the dependent variable, whereas the number of individuals from each arthropod taxon captured that did not have herbivore habits were included as independent variables. The exposure period was added as a random factor. The binomial family with the logit link function was selected. An observation-level random intercept variable was included in the model to account for overdispersion (Zuur et al. 2013).

The abundances of the predatory taxa identified by the model were summed for each trap, and the differences in predator abundances per trap between periods were compared with the Kruskal-Wallis test. *A-posteriori* paired comparisons were made with the mean rank test.

The relationships of average temperature during exposure with the proportion of lost eggs (calculated for each period), and with the abundance of potential predators

were analyzed with correlation analyses. In addition, the correlation between the proportion of lost eggs and the abundance of potential predators was analyzed.

Laboratory study

Based on the results of the field study, two potential predatory taxa were selected for the laboratory study: *Armadillidium vulgare* (Isopoda: Armadilloidea) and *Euborellia annulipes* (Dermaptera: Anisolabidae). Predators were collected with pitfall traps located in the site where the field study was performed. Each predator was placed individually in a plastic container of 8 cm in diameter and 5.5 cm in height, and starved for 48 hours previous to the experiment. The experiment consisted of exposing a substrate with a known number of *Ae. aegypti* eggs (at least 15 eggs for each replicate) and one individual of the corresponding predator (Fig. 2).

Fig. 2. Experimental container with a substrate with *Aedes aegypti* eggs and a potential predator.

Two treatments with five replicates each were performed for each arthropod taxon. In the first treatment, a rectangular dish towel of 3 x 1 cm soaked in water as humidity source was added. In the second treatment, 1 cm of humid soil as alternative food source or refuge for predators was added. Soil was collected in the same area where the field study was carried out, and then frozen to kill macroscopic organisms that might affect or consume the eggs. Five control replicates without a predator were included for each treatment.

After 72 h, the substrata with eggs were recovered and the number of remaining eggs was counted under a stereoscopic microscope.

Data analyses

The proportion of consumed eggs was calculated as the initial number of eggs minus the remaining eggs, divided by the initial number of eggs. On some substrata, spontaneous hatching of some eggs was observed (larvae emerging from eggs); these were discounted from the initial number of eggs and not considered in the analysis.

Because data did not meet the assumptions of normality and homogeneity of variances, the Sheirer-Ray-Hare test (Sokal and Rohlf 1995) was used to assess the effects of predator identity (*Armadillidium vulgare*, *Euborellia annulipes*, control) and treatment (with or without soil) on the proportion of consumed eggs. *A-posteriori* paired comparisons were made with the mean rank test.

Results

Field study

A total of 3,534 (17 %) of the 20,661 initial eggs placed in the field were lost during the experiment. Of those recovered, 94 % were intact, 5.5 % collapsed and 0.7 % hatched, mostly from early September (4.5 % of the recovered eggs of that period). In early June and early September, the proportion of lost eggs was significantly higher (H = 94.46, df = 5; p < 0.001), although results were heterogeneous between traps in all exposure periods (Fig. 3). In 255 of the traps, all eggs were recovered, distributed in all exposure periods, whereas in 19 of the traps, all the eggs were lost in early and late June, early July, and September (Fig. 3). At the end of July and mid-August, the highest losses were observed in one trap, with 90 % and 80% of eggs lost respectively. In 125 of the remaining traps, less than 25 % of the eggs were lost, whereas in 78 traps, between 25 and 99 % of eggs were lost.

Regarding the exposure periods with higher loss of eggs, in early June, a total of 996 eggs (21 % of the initial eggs) were lost, and in early September a total of 1296 of the eggs (50 % of the initial eggs) were lost,. The number of eggs lost was significantly higher in early June than at the end of July and mid-August, and no differences were detected for the end of June and early July. At the beginning of September, the

proportion of lost eggs was significantly higher than in the remaining exposure periods. From the end of June to mid-August, only 9.5 % of the sum of initial eggs was lost (Fig. 3).
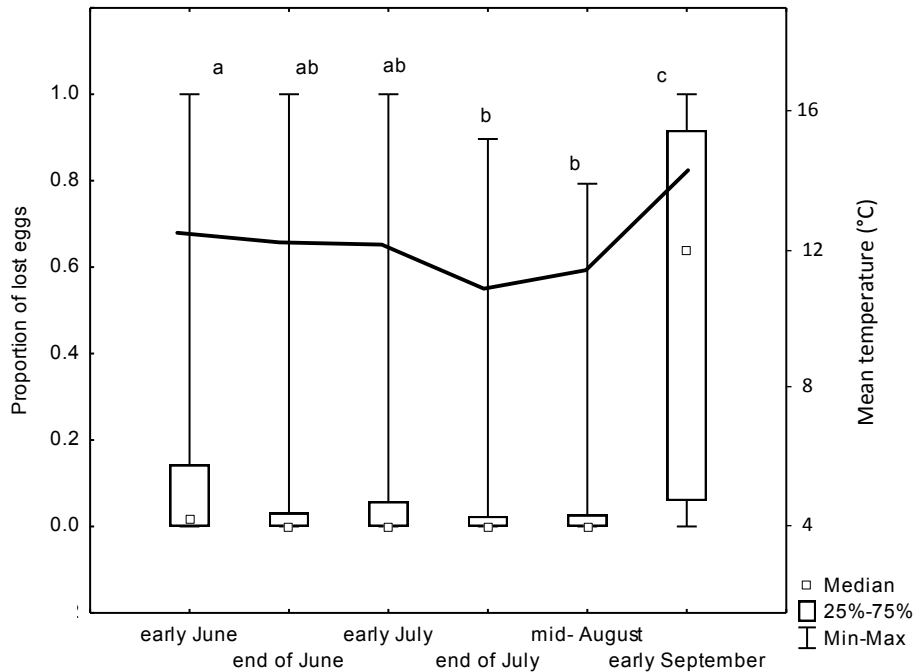
Fig. 3. Proportion of lost eggs per trap for each exposure period (median and quartile) and mean temperature (bold line). Periods with similar letters indicate no significant differences in the proportion of lost eggs.

During the experiment, a total of 4,421 arthropods were captured. Among them, 3,091 were insects belonging to 12 genera of Formicidae, 14 families, larvae and morphospecies of Coleoptera, two families of Heteroptera, two of Homoptera, and one of Orthoptera, Dyctioptera, Thysanoptera and Dermaptera respectively. The 519 arachnids captured belonged to four morphospecies of Acari, four morphospecies of Araneae and one family of Opilionida. Among Myriapoda, individuals of two classes were captured: Chilopoda and Diplopoda. Three of them belonged to the subclass Chilognatha and five to the order Lithobiomorpha. A total of 803 crustaceans were captured. These belonged to three species of the order Isopoda and one family of the order Amphipoda (Supp. Table 1).

The results of the GLMM identified ants of the genus *Strumigenys*, isopods of the species *Armadillidium vulgare*, and dermapterans of the species *Euborellia annulipes*, positively associated with a high proportion of lost eggs (Table 2).

Table 2. Coefficients of the generalized linear model analysis significantly associated with the loss of eggs.

Variable	Coefficient	z value	P value
Armadillidium vulgare	0.398	5.917	< 0.0001
Euborellia annulipes	10.4	3.380	< 0.001
Strumigenys sp.	3.295	2.220	< 0.05

The abundance of potential predators showed differences between exposure periods (H = 29.917; df = 5; p < 0.001). The highest abundance was found in early September (343 individuals), and was significantly different from that at the end of July and August, when the lowest abundance of potential predators was found (45 individuals in each exposure period). From early June to early July, intermediate values of potential predators were found (Fig. 4).
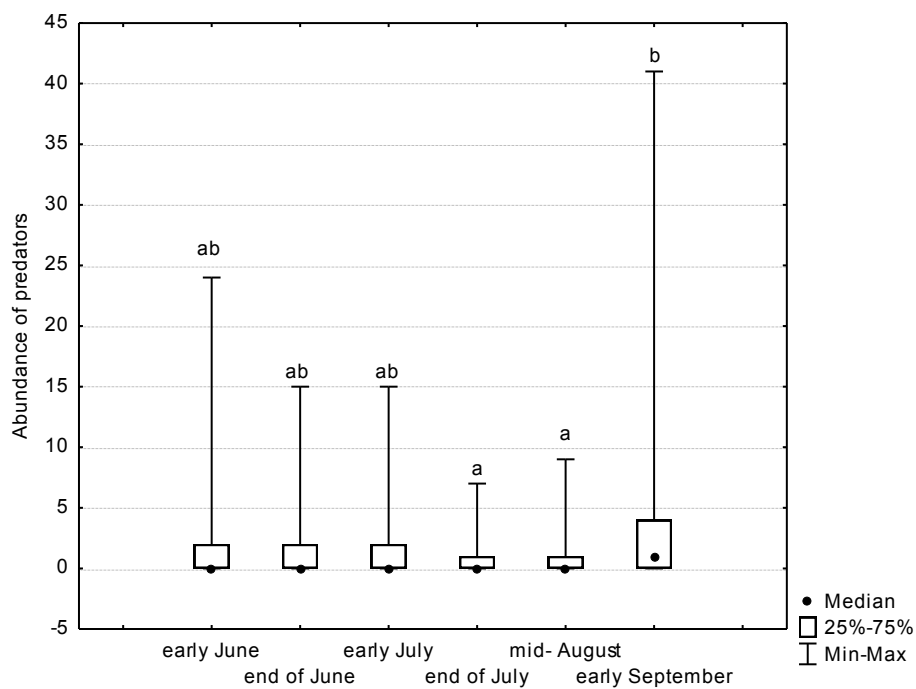
Fig. 4. Number of potential predators per trap for each exposure period (median and quartile). Periods with similar letters indicate no significant differences in the number of predators per trap.

A positive correlation of the mean temperatures during the exposure period with the proportion of lost eggs (r = 0.977; p < 0.001) and with predator abundance (r = 0.969; p < 0.005) was observed. The correlation of the proportion of lost eggs with predator abundance was also positive and significant (r = 0.993; p < 0.001) (Fig. 5).
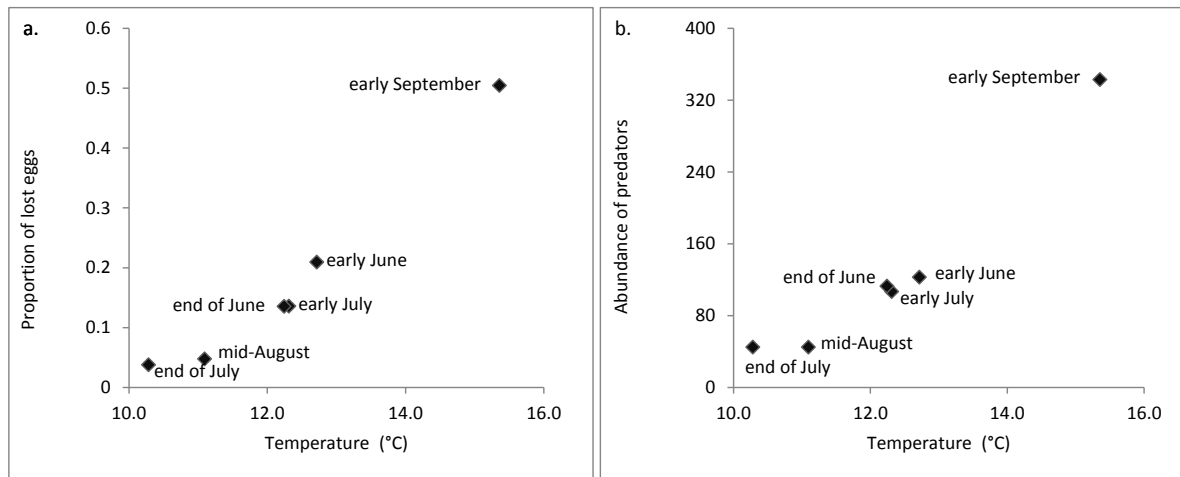
Fig. 5. Relationship of: a) mean temperature of each exposure period with proportion of lost eggs, b) mean temperature with abundance of potential predators and c) abundance of potential predators with proportion of lost eggs.

Laboratory study

Predators consumed 324 (60 %) of the 567 *Ae. aegypti* eggs offered. On average, *A. vulgare* individuals consumed 16 eggs (64 %), whereas *E. annulipes* individuals consumed 17 eggs (62 %), and variability among individuals was observed within each predator species (Fig. 6). A significant effect of predator on the proportion of consumed eggs was detected (H = 1089.32; d.f = 2; p < 0.001). Post-hoc comparisons detected significant differences between the absence of predator (control treatment in which no eggs were lost) and the presence of predator (p < 0.001 and p < 0.005 for *A. vulgare* and *E. annulipes* respectively), but no differences between predators. No significant effect of treatment was detected (p > 0.25), although a slightly higher consumption was observed in the treatment without soil in the container. The predator by treatment interaction was not significant (p > 0.5).
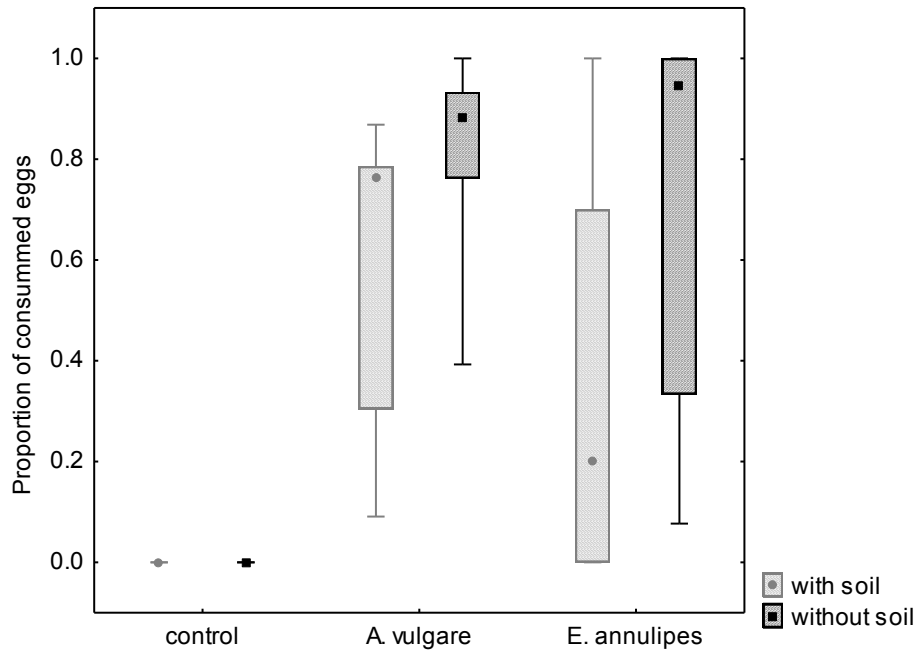
Fig. 6. Proportion of consumed eggs (median, quartile and range) for two predators (*Armadillidium vulgare* and *Euborellia annulipes*) and a control in two environments. Similar letters indicate no significant differences among predator treatments.

Discussion

The loss of eggs was not continuous through the winter, with two marked events of higher loss in early June and September. This was an expected result, which was in agreement with the fact that temperatures at the end of autumn (June) and the winter-spring transition (early September) are usually higher than those recorded during the rest of the winter season, in July and August (National Meteorological Service 2016). Predators were observed during the whole winter, although the higher abundance in the winter-spring transition supports the idea of higher temperatures favoring the activity of arthropods, an observation also reported by other authors (Danks 1987, Porter and Tschinkel 1987). Regarding the numbers of eggs lost during the experiment, it should be considered that the traps were buried at the soil level, which led walking arthropods to fall in the traps, favoring the direct contact with the mosquito eggs. This methodology could have led to an overestimation of the loss because the predators falling into the traps might not have had the possibility to escape and might have consumed mosquito eggs in the absence of alternative food. On the other hand, this overestimation might

have been compensated, at least in part, by the short exposure period of the eggs each time, which lasted for only one week.

The winter-spring transition period coincides approximately with the first time of hatching of the eggs that survived the winter season (Byttebier 2017). Since the hatching of the first cohorts of eggs depends not only on favorable temperatures but also on the rainfall, the impact of early spring predatory activity on the population will be more important during a dry winter-spring transition. In dry years, egg hatching is delayed, and the whole egg bank might be exposed to the predatory activity during early spring. In contrast, in wet years a fraction of the individuals may escape from egg predation by hatching and initiating larval development triggered by early spring rains.

Regarding the potential predators of *Ae. aegypti* eggs, both the association of individuals of *A. vulgare* (Isopoda) with a higher loss of eggs and the confirmation of their capacity to consume eggs in the laboratory assay suggest that this species might be an effective predator of mosquito eggs in natural conditions. Previous studies have also mentioned isopods consuming *Ae. aegypti* eggs in the field, although the published information was only anecdotal (Focks et al. 1993). *A. vulgare* individuals coexist with the eggs of *Ae. aegypti*, since these arthropods are usually associated with human environments, and are even considered a garden pest (Robinson 2005). Furthermore, the capture of this species in high abundance during the whole unfavorable season suggests its capacity to reduce the mosquito egg bank of *Ae. aegypti* during the period of highest vulnerability, when the mosquito population remains in the egg stage.

The identification of *E. annulipes* (Dermaptera) as a predator of mosquito eggs both in the field and in the laboratory assays is a novel result, which, to our knowledge, has not been previously reported. As *A. vulgare*, this species has a cosmopolitan, urban distribution, frequently associated with houses and gardens (Rankin and Palmer 2009), where it shares the environment with *Ae. aegypti*. This is why the low abundance of *E. annulipes* captured in our field study was unexpected, considering that they are very frequent insects in residential areas in general (Robinson 2005), and in Buenos Aires city in particular (Byttebier, personal observation). A possible explanation for this might be a low efficiency of the traps to capture a representative number of this taxon or the ability of the captured individuals to escape from the traps, which should be assessed in future studies.

On the other hand, contrary to our expectations, we found no evidence of predatory activity by some other taxa such as Formicidae, in spite of their reported ability to consume eggs of mosquitoes. For example, previous studies have demonstrated the capacity of different species of Formicidae to consume mosquito eggs (Lee et al. 1994, Burnham et al. 1994, Duhrkopf et al. 2011), including those of *Ae. aegypti* (Pérez Insueta et al. 2004). However, among the eleven genera captured in our study, only the genus *Strumigenys* was associated with a significant loss of eggs. All known species of this genus are predatory, and are known to prey upon a wide range of small arthropods, such as collembolans (Bolton 1999). Although not detected in the present field study, it could be possible that other local species of the family Formicidae are able to predate on mosquito eggs, considering both the abundance and the diversity observed for this family, and the previous information for ant species in other regions. This could be the case of the genus *Wasmannia* (presumably *W. auropunctata*), which is considered a valuable pest control species in some regions (Way and Khoo 1992). This species, captured in high abundance in our study, has been observed associated with important losses of mosquito eggs in a garden near the study site (Fischer, unpublished observation). It is possible that the high representation of individuals of *Wasmannia* in all traps and along the whole study might have hidden the predatory activity, which should be assessed in future studies.

Regarding coleopterans and cockroaches, Yang (2006) confirmed the ability of larvae of *Curinus coeruleus* (Coleoptera: Coccinellidae) to consume eggs of *Ae. albopictus* in laboratory conditions. In contrast, in our experiment, none of the 14 groups of Coleoptera captured (both larvae and adults) showed associations with egg loss, despite their different feeding habits, diversity and abundance. Similarly to that discussed for ants, this might be related to the presence of some families of this group, for example individuals of Carabidae and Staphylinidae, in a large number of study units along the whole winter season, which could have hidden the predatory activity.

Regarding the individuals of the order Blattodea found in the present study, they were not associated with the loss of eggs either. This result was particularly unexpected because other studies have shown that some species of this order, such as *Periplaneta americana*, are effective predators of *Ae. aegypti* eggs, both in controlled conditions and in the field (Christophers 1960, Russell et al. 2001). It is possible that the cockroaches captured in our study belong to species that do not consume *Ae. aegypti* eggs. Thus,

future experiments are necessary to assess the abilities of different candidate taxa of ants, coleopterans and cockroaches to consume eggs both in the laboratory and in field conditions.

The high heterogeneity in the loss of eggs between experimental units could indicate that the effect of predators is not spatially homogeneous, even over short distances. This is important when considering predators as potential control agents, since the impact generated could be very local, and not extrapolable to the whole egg bank. Furthermore, it is generally accepted that biological control agents on mosquitoes would be most effective if they attack late larval or pupal stages (but see exception in Southwood et al. 1972). In contrast, preying on eggs or early instar larvae would be less effective, because the individuals killed at this stage might have been controlled by density-dependent effects in the absence of the predator (Juliano 2007). However, since, in temperate regions, the size of the egg bank is related to the persistence of the *Ae. aegypti* population through the winter until the next warm season, a significant loss of eggs by predation might jeopardize this persistence at a local scale.

Furthermore, the aquatic stages of *Ae. aegypti* do not have many natural enemies, since larval development is, to a large extent, restricted to small, domestic man-made containers that are relatively free from predatory organisms (Christophers 1960, Sunahara et al. 2002). The egg stage is one of the two terrestrial stages of the mosquito life cycle that shares the environment with arthropod predators, since females lay their eggs on the wall of containers above the water surface. Because of the lack of movement, the egg stage is an easy prey for arthropods, thus representing the stage most susceptible to predation. In fact, some studies have shown that the behavior of laying eggs on the water surface is related to a strategy to avoid egg predation by arthropods (Madeira et al. 2002, Abreu et al. 2015).

Finally, if we consider that the activity of arthropods in the warmer period of our study led to a significant loss of eggs compared to the rest of the winter, we might expect an even higher loss in spring and summer time. Field experiments are necessary to further assess the effects of predators on the egg population during the warm period, when the *Ae. aegypti* population is in the reproductive activity period, and to evaluate the importance of including predation on eggs in the planning of vector control strategies.

Acknowledgments

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