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Cold Season Mortality Under Natural Conditions and Subsequent Hatching Response of *Aedes* (*Stegomyia*) *aegypti* (Diptera: Culicidae) Eggs in a Subtropical City of Argentina

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ABSTRACT In temperate and subtropical regions, populations of *Aedes* (*Stegomyia*) *aegypti* (L.) survive unfavorable winter conditions in the egg stage. Knowing their survival rates can be of great interest for the health authorities in charge of control activities. In this study, we analyzed the mortality of *Ae. aegypti* eggs exposed to the cold season as well as their hatching patterns under laboratory conditions in the city of Resistencia, Chaco, Argentina. The mortality rate was 48.6%. No statistically significant differences were observed in the mortality of eggs exposed at different sites. Hatching response differed significantly among the successive postexposure immersions, with the highest proportion of hatched eggs during the first immersion. These results show that the mortality rate of *Ae. aegypti* eggs exposed to the cold season in a subtropical city of Argentina was higher than those from temperate climate region. The additional mortality of eggs in our study might be related to fungal development (an unexpected event), which was not observed in research in temperate climate. The hatching pattern observed in this study ensures a rapid increase of the population at the beginning of the favorable breeding season, but it also maintains a batch with delayed hatching eggs, posing a risk for the community.

KEY WORDS dengue vector, survival, egg, Chaco, Argentina

In 2009, Argentina suffered the largest outbreak of dengue fever by serotype 1, with >26,000 confirmed cases, three dengue hemorrhagic cases, five deaths, and autochthonous cases reported in 13 provinces, including the province of Chaco with ∼10,000 cases on a total of 1 million inhabitants (Ministerio de Salud de Argentina 2009, Estallo et al. 2014). Since its emergence, three serotypes circulated in our country up to 2009, and only in 2003 they circulated simultaneously in Salta (Ministerio de Salud de Argentina 2009), but in 2013 the four serotypes circulated in the country, which constitutes a serious risk to the health of the population (Ministerio de Salud de Argentina 2013). An interesting fact is that two cities of Argentina registered the simultaneous circulation of two serotypes during 2013: Presidencia Roque Saenz Peña (Chaco) and Córdoba (Córdoba) (Ministerio de Salud de Argentina 2013). To date, *Aedes aegypti* (L.) is the only vector responsible for the dengue fever outbreaks occurring in our country (Vezzani and Carbajo 2008).

Ae. aegypti is a mosquito species associated with urban areas, where larvae and pupae develop in a wide variety of artificial habitats (Forattini 2002). The eggs of this species are laid individually on the internal side of artificial containers, above the water. After embryonic development is completed, the desiccation-resistant eggs remain quiescent for months (Clements 1992, Service 1993, Estrada-Franco and Craig 1995, Organización Panamericana de la Salud (OPS) 1995), and hatch when favorable conditions return (Clements 1992, Crans 2004).

For *Ae. aegypti*, unfavorable periods are mainly determined by low winter temperatures in temperate regions (Christophers 1960, Eisen and Moore 2013) and by suboptimal temperatures combined with low rainfall and low humidity in subtropical (Micieli and Campos 2003), and tropical regions (Russell et al. 2001). In Resistencia (a city located in the subtropical region of Argentina), the populations of the vector reach their highest levels in spring, and attain lower abundance in summer and fall (Stein et al. 2005). Since unfavorable thermal and rainfall conditions restrict mosquito activity during the winter, populations remain in the egg stage and no adults or larvae are detected; the new breeding season starts in October with the eggs remaining from the previous warm season (Stein et al. 2005).

Therefore, winter survival of the eggs until the next favorable period is a key factor for the persistence of *Ae. aegypti* populations. Mortality of *Ae. aegypti* eggs may be caused by intrinsic factors such as senescence, but also by environmental conditions such as temperature extremes or humidity deficit (Christophers 1960,

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Trpis 1972, Juliano et al. 2002). Furthermore, the predation by insects such as cockroaches, and the action of fungal mycotoxins have been also documented as mortality sources of *Ae. aegypti* eggs under natural conditions (Russell et al. 2001).

After surviving the unfavorable season, *Ae. aegypti* eggs do not necessarily hatch at the first stimulus, and certain variability in the hatching response over successive immersions can be observed even among eggs from the same batch, subjected to similar environmental conditions (Gillett 1955, Vinogradova 2007).

Studies related to the mortality of *Ae. aegypti* eggs under unfavorable environmental conditions are necessary for selecting the most vulnerable periods for a population to take control measures in a city. Specifically in Argentina, we only know the study by Fischer et al. (2011) on the mortality of *Ae. aegypti* eggs in winter under natural conditions in temperate regions. This research aimed the following objectives: To assess the mortality of *Ae. aegypti* eggs during the winter season in the city of Resistencia, and to observe the hatching behavior of *Ae. aegypti* eggs after being exposed to natural environmental conditions during the cold period, under the assumption that environmental conditions during winter in the subtropical region of Argentina are not so severe, and therefore would not affect the survival of *Ae. aegypti* eggs during quiescence, ensuring future populations of the vector at the beginning of the new warm season.

Materials and Methods

Study Area. The city of Resistencia is located at 27° 30' S and 59° W, toward the south-east end of the province of Chaco (Fig. 1), and is part of the Atlantic humid subtropical climate area, which belongs to the Parana-Paraguay river system. The average annual temperature and rainfall of the region are 20.9°C and 1.280 mm, respectively. The highest concentration of rainfall is registered between the months of November and April with two peaks, one in spring (November) and another one during late summer and early fall (March). In the summer, the average temperature is 27°C, with an absolute maximum of over 43°C. Between the months of June and September, rainfall reaches 300 mm and the average temperature is 15°C, with an average minimum of 10°C. In July, there is a period of frosts that can last 20 d (Bruniard 1978). The study was performed in four sites within the city of Resistencia, located in different directions starting from the center of the city toward the periphery: East site (E), North-East site (NE), West site (W), and South-West site (SW; Fig. 1). The sites selected were single-family dwellings with tree-shaded gardens and backyards, in residential neighborhoods with a similar construction type, and with a previous history of *Ae. aegypti* presence. The backyards of houses located to SW and NE were larger (12 by 20 m) than the others (E and W houses; 10 by 3 m) and were completely covered with abundant grass, shrubs, and arboreal plants, whereas E and W backyards had 50% of their surfaces covered with concrete.

Obtention of *Ae. aegypti* Eggs. The eggs used in this study were obtained from the *Ae. aegypti* larvae collected in the cemetery of the city of Resistencia, which were reared to reach adult stage, in the insectarium of the Instituto de Medicina Regional (IMR) of the Universidad Nacional del Nordeste (UNNE). After the emergence, adults were placed in rearing cages and fed with sugar water. Every 4 or 5 d, a blood source (*Coturnix coturnix*) was offered to the females.

Once the females (F1 generation) were blood fed, oviposition substrates (10- by 2-cm wooden paddles) were attached to the inside wall of plastic containers filled with 2 cm of water, and placed inside the rearing cages. Substrates with 20 or more eggs were removed from the rearing cage, and maintained in wet conditions until the beginning of the experiment to ensure the complete development of embryos. Before starting the experiment, each paddle was inspected under a stereoscopic microscope, intact (turgid) eggs were counted and damaged or collapsed eggs were removed with histological needles. All paddles with at least 10 eggs were used in the experiment (average 45, range 10–198). All eggs were collected between March and May 2013.

Experimental Design. The experiment consisted in the exposure of a known number of eggs to natural conditions during the cold season. To this end, the paddles with eggs were placed in earthen pots (10 cm high by 10 cm diameter pots) with a plastic cover (plate) attached 0.8 cm over the top, completely covering the pot to prevent rain from falling into it and eggs from hatching (Fischer et al. 2011). Each pot was located in one of the four backyards, on the ground and under shade. An average of 267 eggs (range 220–308) distributed on 5–6 paddles were placed in each pot. Paddles were attached in a vertical position and fixed to the wall with an office clip. Each pot had a label indicating site and type of treatment. Two different treatments were performed: natural condition treatment (NC), as described above, and protected condition treatment (PC), where each pot was covered completely from the top with a fine nylon mesh to prevent access of macroscopic organisms (potential predators; Fischer et al. 2011). Three replicates were placed for each treatment at each site, resulting in six devices (pots) per site.

The devices remained in the field for 90 d (between 1 June and 1 September 2013). The relative temperature and humidity of the period of study were recorded by the weather station of the UNNE (Fig. 1). After 90 d and coinciding with the rising temperatures, the devices were removed and the paddles transferred to the insectarium of the IMR to count and classify the eggs into collapsed, missing, or intact by means of a stereoscopic microscope. All collapsed eggs were discarded and removed from the paddles, and recorded as dead, while intact eggs were maintained on the paddles.

To stimulate hatching, the paddles with intact eggs were placed in plastic trays and immersed in 250 ml of dechlorinated water. Laboratory conditions were more or less constant, with temperatures average 26 ± 2°C and relative humidity average 70% ± 10. After 48 h, the paddles were removed from the water, they were left

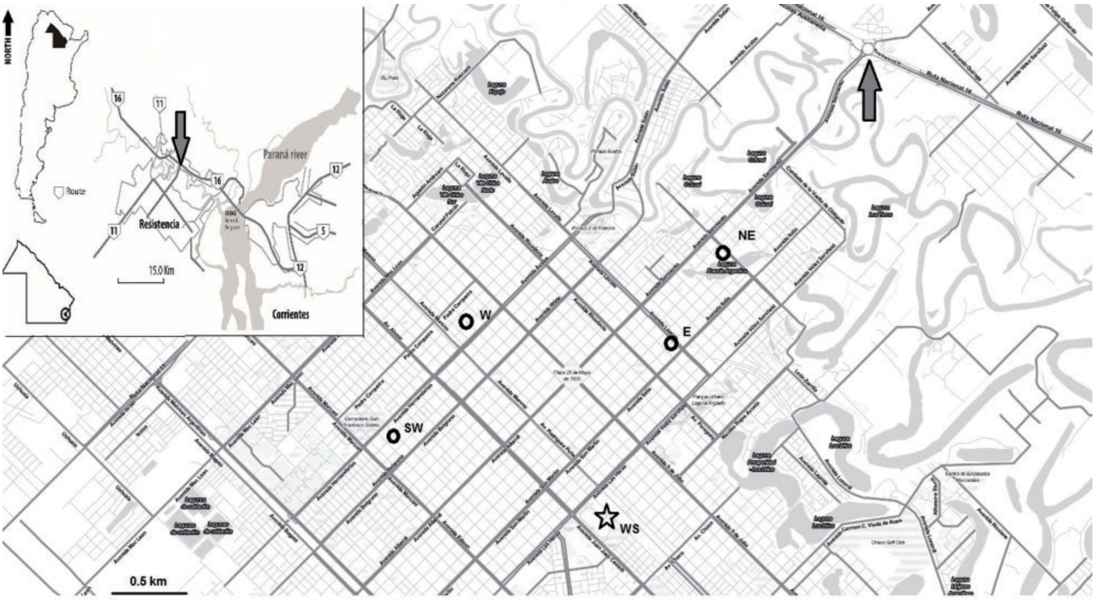

Fig. 1. Location of the study sites and weather station (WS) in Resistencia, Chaco, Argentina. Modified from Stein et al. (2013).

to dry, and the number of hatched eggs or emerged larvae was counted. Four or five days after being removed from the trays, the paddles were immersed once again. This procedure was repeated a total of eight times.

After carrying out the eight immersions, chorions of unhatched eggs were bleached with a commercial 50% sodium hypochlorite solution to allow direct observation of the embryos. Creamy white embryos with eye spots, a hatching spine, and abdominal segmentation were considered viable (alive). Embryos with characteristics different from the ones mentioned were considered nonviable (dead; McHaffey and Harwood 1970).

Data Analysis. The proportion of missing eggs (eggs that could not be recovered after field exposure) in each pot was calculated as the difference between the number of eggs initially exposed and the number of eggs recovered after the 3-mo exposure, divided by the number of eggs initially exposed in that pot. Effects of site and treatment on the proportion of missing eggs were analyzed with a nonparametric two-way ANOVA using the Sheirer–Ray–Hare method (Sokal and Rohlf 1995), because different transformations did not meet the necessary assumptions.

Egg mortality in each pot was calculated by dividing the number of dead eggs by the total number of eggs recovered after the field exposure. Two categories of dead eggs were distinguished: collapsed eggs, and eggs appearing intact but with nonviable embryos. Effects of site and treatment on the proportion collapsed eggs and of nonviable eggs were analyzed by means of a two-way ANOVA on arcsine square root transformed data.

Hatching response of eggs was determined by analyzing the intensity of the response after each immersion and the cumulative response to the successive immersions. The intensity of the hatching response for each immersion was assessed by calculating the number of larvae observed on each occasion divided by the number of living eggs before the respective immersion (eggs were considered alive either if they hatched after any stimulation or if they contained viable embryos after bleaching). Here nonviable eggs were not considered. For each immersion the proportions of hatched eggs were compared between the sites by means of a chi square test for independent proportions. This test is comparable to computing the Pearson chi square statistic for contingency tables. Differences between pairs of sites were examined by subdividing the contingency tables and computing the chi square value on the partial tables (Fleiss et al. 2003). The cumulative hatching response for each site was calculated as the sum of hatched eggs in previous immersions and the current immersion, divided by the total number of living eggs.

Results

During the study period, the average temperature of the warmest month (July) was 16.12°C (SD = 5.95°C), and that of the coldest month (August) was 15.87°C (SD = 5.92°C); the average relative humidity was 76.35% (SD = 17.51).

Out of a total of 6,411 eggs exposed, 638 (9.95%) were missing at the end of the study. Egg loss was recorded for all sites and treatments, and the proportion of missing eggs did not show statistically significant differences among treatments ($H = 0.013$, $df = 1$, $P = 0.91$), sites ($H = 3.033$, $df = 3$, $P = 0.38$), or the interaction of treatment × site ($H = 1.3$, $df = 3$, $P = 0.73$). The percentage of paddles with missing eggs under protected conditions was 56.33%, and under natural conditions, 54.41%. In general a large variability among paddles within the same pot was observed, with almost total loss in some paddles and close to zero loss in others, with exception of one pot (NC at site NE) where a great number of missing eggs was observed in all paddles.

Among 5,773 recovered eggs, 2,119 (36.71%) were collapsed at the end of the period of the exposure, and 683 (11.83%) appeared intact but contained nonviable embryos, so the mortality of *Ae. aegypti* eggs found in this study after 3 mo of exposure to the cold-dry season resulted 48.53%. The proportion of collapsed eggs did not show statistically significant differences among treatments ($F = 1.18$, $df = 1$, $P = 0.29$), sites ($F = 0.08$, $df = 3$, $P = 0.97$), or the interaction of treatment × site ($F = 0.74$, $df = 3$, $P = 0.54$).

During the successive immersions, 32.35% of the paddles from NC pots developed fungi, while 40.84% of the paddles exposed to treatment in PC developed fungi. Among the 683 eggs with nonviable embryos, 571 were located on paddles with fungi, and 469 were completely covered by fungi during the successive immersions. These fungi were identified as *Choetonium globosum* (Kunze ex Fries), *Pericocia byssoides* (Persoon), and *Torula herborum* (Pers.), and belong to the soil microbiota (Ellis 1976, Von Arx et al. 1986). The proportion of nonviable eggs did not show statistically significant differences among treatments ($F = 0.23$, $df = 1$, $P = 0.63$), sites ($F = 0.27$, $df = 3$, $P = 0.85$), or the interaction of treatment × site ($F = 0.39$, $df = 3$, $P = 0.76$).

Out of 2,968 viable eggs, 2,957 (99.63%) hatched during the eight immersions. Significant differences in hatching intensity among treatments were detected for the first, second, and fifth immersion (Fig. 2a), and among sites for the first, third, fourth, and sixth immersion (Fig. 2b). The intensity of hatching (all sites and treatments pooled) showed a U shape, with highest proportions at the first (0.674) and last (0.667) immersions, and lowest proportions at the fifth (0.32) and sixth (0.395) immersions.

The cumulative hatched response showed a 95% of the eggs hatched after the fourth immersion.

Discussion

The mortality of *Ae. aegypti* eggs found in this study after 3 mo of exposure to the cold-dry season was lower than the mortality registered in the dry season of Tanzania (East Africa; up to 90%; Trpis 1972), and in Queensland (Australia; 86%; Russell et al. 2001), which are two regions with tropical climate. The main explanation for the lower mortality of eggs obtained in this study, in relation to the results found in the dry season of tropical climates, would be the more favorable climatic and environmental conditions occurring in Resistencia. The temperature conditions experienced in Resistencia during the winter (around 16°C) are nearly optimal for the survival of *Ae. aegypti* eggs (Meola 1964). Furthermore, the high relative humidity recorded during our experiment (>70%) is also favorable for the survival of eggs (Juliano et al. 2002). These conditions are in contrast with those experienced in artificial and natural habitats in tropical regions where heat and low relative humidity (50–60%) during the dry-cool season are responsible for the limited survival of the eggs of this species in Tanzania (Trpis 1972) and low rainfall (mean monthly minimum 10 mm), temperature (mean monthly minimum 11°C), and relative humidity (mean monthly minimum 30%), are conditions less than ideal for the survival of *Ae. aegypti* in Queensland (Russell et al. 2001). In our study, the mortality recorded was higher than that found in the city of Buenos Aires (30.6%), with similar relative humidity to Resistencia, located in a temperate climate region of Argentina (Fischer et al. 2011), for the same period of exposure and season. The differences might be mainly related with the additional mortality in our study related to fungal development (an unexpected event), which occurred in a percentage of the paddles, and might have caused the mortality of eggs present in those paddles. In Queensland, *Penicilium* fungi (Link) represented a significant cause of egg mortality (Russell et al. 2001), but in Argentina this is the first record of fungi affecting *Ae. aegypti* eggs survival. Because these fungi are found in the soil, eggs remaining in the field that do not hatch immediately after the beginning of the favorable, warm and rainy season, could be affected and their survival significantly reduced. Further studies on the interactions of fungi with field-exposed eggs are necessary to accurately assess the importance of this cause of mortality. The proportion of missing eggs was slightly lower in our study (9.95%) as compared to that observed in Buenos Aires city (13%; Fischer et al. 2011). The similar egg loss in protected and natural conditions might be caused by the random detachment of eggs from most of the substrates. One exception is the almost total loss of eggs in one of the pots at site NE (NC), which could suggest the possible action of a predator. Unfortunately, we were not able to observe the predator identity; thus, this subject would require further investigation. Predators such as *Periplaneta americana* (L.) would be candidates for further research, as consumption by this species is one of the main causes of the mortality of *Ae. aegypti* eggs in Queensland (Russell et al. 2001).

Regarding spatial variability, the similarity in egg mortality (both for collapsed eggs and for nonviable eggs) among the studied sites distant 2 or 3 km, suggests that the values obtained in our study can be considered as representative for Resistencia city. On the other hand, the large number of missing eggs in only one replica indicates that egg predation was not a generalized phenomenon, but when it occurs, impacts are potentially important. It is not possible from the results of the present study to conclude about the spatial pattern of egg predation.

The intensity of hatching in successive immersions showed a relatively homogeneous pattern in our study. Based on previous results obtained for the temperate region in Argentina (Fischer et al. 2011) higher hatching percentages at the first immersion would be expected.

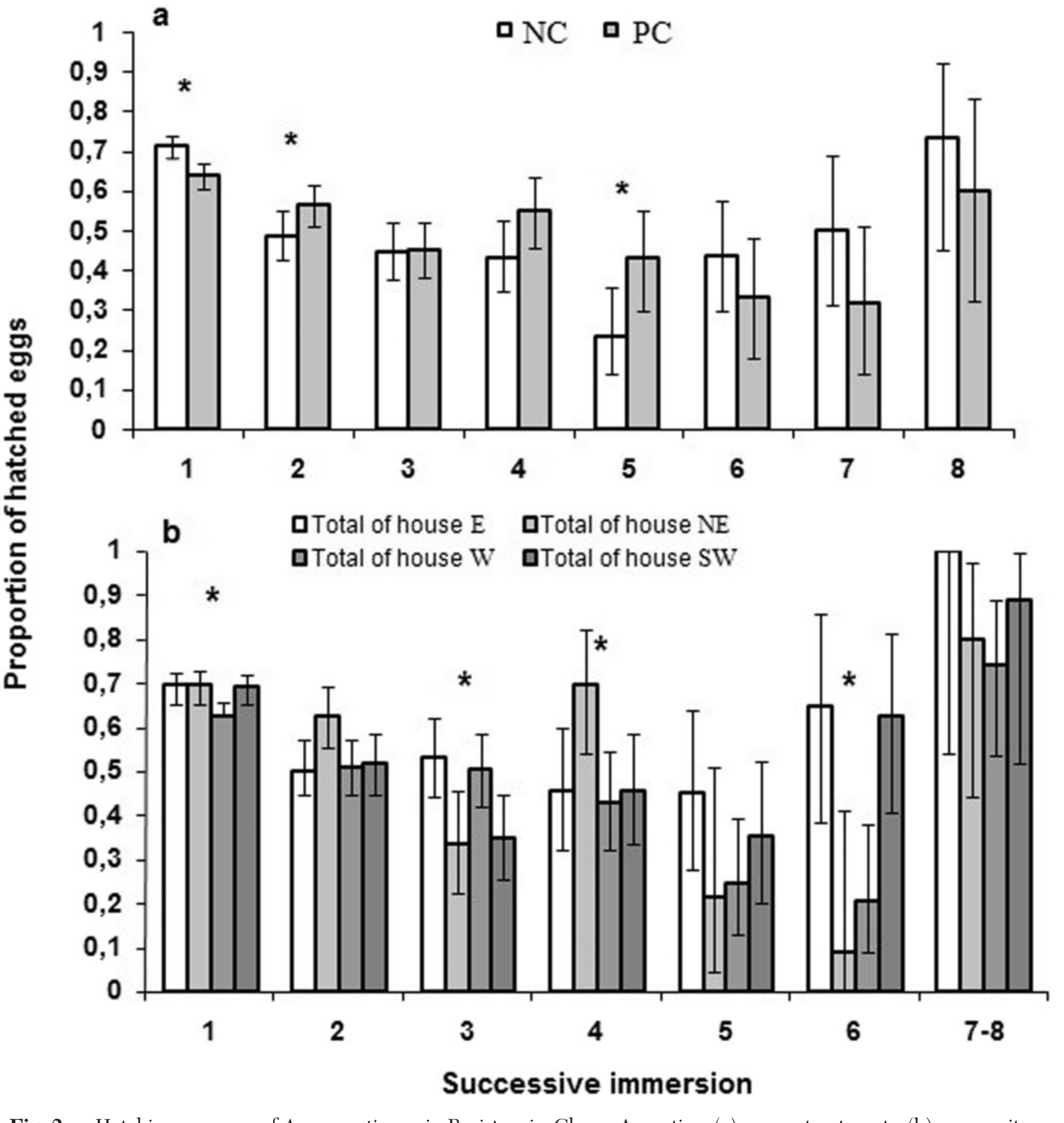

Fig. 2. Hatching response of *Ae. aegypti* eggs in Resistencia, Chaco, Argentina: (a) among treatments, (b) among sites.

In mosquitoes, the pattern of delayed hatching can be explained by different factors, including plasticity in response to environmental factors and genetic variation as a consequence of the adaptation to local climatic conditions (Evans and Dennehy 2005). Differences in the hatching behavior have been observed for *Ae. aegypti* populations from different geographic origins, which have been related to an adaptive selection in response to local climatic conditions (Gillett 1955), being this the most plausible explanation for the lower hatching response obtained in our study, in contrast to findings at Buenos Aires city.

In general, when the risk of survival of a vulnerable stage (immature development for mosquito populations) is high, a trend toward staggering the entrance to that stage is expected, in order to avoid catastrophic reproductive failures. This prediction has been tested with the treehole mosquito *Aedes triseriatus*, which exhibited a higher hatching delay in regions with low or unpredictable precipitation pattern (Khatchikian et al. 2008). In the case of Resistencia, rainfall is scarce during early spring, thus developmental success is rather unpredictable for individuals hatched during this period because insufficient water supply and high insolation cause the rapid drying of larval habitats. Therefore, a delay in hatching, together with a relatively low mortality risk, would favor a "bet hedging" strategy, ensuring that a proportion of eggs survive until the next opportunity.

The results of this study provide new insight about the effect of season winter in eggs mortality and spring hatching pattern of *Ae. aegypti* populations in the study area. The mortality rate of *Ae. aegypti* eggs in the winter season in Resistencia was higher than in Buenos Aires city, even so, a percentage of the eggs of the species that survive from the previous warm season, start the new one with a large batch of eggs. On the other hand, the hatching pattern observed ensures a rapid increase of the remaining population at the beginning of the favorable breeding season, but it also maintains a batch with delayed hatching eggs, due to the possible occurrence of unfavorable events, such as the drying of the habitat, which would affect the larvae present in them.

Health authorities should consider these findings (high winter survival of eggs across the city), and implement measures such as the elimination during the cold and dry season, when the population remains mainly in the eggs stage of containers abandoned or stored in backyards and other outdoor locations. This would not only reduce the size of the egg bank available at the beginning of the warm and rainy season, but also reduce the availability of potential larval habitats at the beginning of the warm and rainy season, thus slowing the increase of abundances of the adult population during the favorable season.

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