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Delayed mortality effects of cold fronts during the winter season on *Aedes aegypti* in a temperate region

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Abstract

The expansion of the invasive mosquito *Aedes aegypti* L. (Diptera: Culicidae) towards temperate regions in the Americas is causing concern because of its public health implications. As for other insects, the distribution limits of *Ae. aegypti* have been suggested to be related to minimum temperatures and to be controlled mainly by cold tolerance. The aim of this study was to assess the daily mortality of immature stages of *Ae. aegypti* under natural winter conditions in Buenos Aires, Argentina, in relation to preceding thermal conditions. The experiment was performed outdoors, and one cohort of larvae was started each week for 16 weeks, and reared up to the emergence of the adults. Three times a week, larvae, pupae and emerged adults were counted, and these data were used to calculate the daily mortality of larvae, pupae and adults and to analyze their relationship with thermal conditions. The results showed that mortality was generally low, with a few peaks of high mortality after cold front events. The mortality of pupae and larvae showed a higher correlation with the cooling degree hours of previous days than with the minimum, maximum or mean temperatures. Pupae and adults showed to be more vulnerable to low temperatures than larvae. A delay in mortality was observed in relation to the low temperature events, with a proportion of individuals dying in a later stage after the end of the cold front. These results suggest that thermal conditions during cold fronts in Buenos Aires are close to the tolerance limit of the local *Ae. aegypti* population. The wide range of responses of different individuals suggests that low winter temperatures may constitute a selective force, leading the population to a higher tolerance to low temperatures, which might favor the further expansion of this species towards colder regions.

Key words: winter mortality, low temperature, yellow fever mosquito, Diptera, Culicidae

Introduction

Insects and most of their biological processes are largely affected by low temperatures (Kingsolver, 2009). Their possibility of adaptation to local conditions depends on the impact of extreme rather than average temperatures on the population because of the larger impacts of the former on fitness (Williams *et al*., 2016). This affects the species distribution range, which can be determined and predicted by their tolerance to low temperatures, especially during the cold season (Sinclair *et al*., 2015).

Studies on the cold hardiness of insects generally assess the mortality that occurs few hours after few hours after exposing them to cold temperatures (Nedved *et al*., 1998), probably overestimating their tolerance to the conditions analyzed (Kostal *et al*., 2019). However, the mortality after cold exposure may have a certain delay, related to the effects of cold injury that could not be appropriately repaired (Nedved *et al*., 1998, Kostal *et al*., 2019), which should be considered for an accurate estimation of the effects of cold on a certain population.

The thermal biology of the mosquito *Aedes aegypti* (Diptera: Culicidae) has been extensively studied. Most studies have focused on populations from tropical and subtropical regions, with low emphasis on the lower temperature limits for development, and only few studies have been performed for temperate climate populations. The survival of *Ae. aegypti* immature stages is known to vary with temperatures, decreasing as temperatures descend below 20ºC (Bar Zeev, 1958, Rueda *et al*., 1990, Tun Lin *et al*., 2000, Richardson *et al*., 2011, De Majo *et al*., 2019). Regarding this issue, the lowest temperature at which *Ae. aegypti* completed development was 14ºC, with a survival of 24% for a population of Israel (Bar Zeev, 1958). However, for the population of Buenos Aires, Argentina (which is located in a temperate region), we have previously observed that 21% of individuals completed development at 12ºC, both under constant and fluctuating temperature regimes, demonstrating a higher tolerance to low temperatures of this population in comparison to others previously studied (De Majo *et al*., 2019).

Ae. aegypti, original from Africa, has shown to be highly invasive, and has currently colonized almost all continents except for Antarctica (Kraemer *et al*., 2015). This species is expanding towards temperate regions in the Americas (Eisen *et al*., 2014), and in Argentina it is currently present in temperate climate provinces like Buenos Aires (Zanotti *et al*., 2015), La Pampa (Rossi *et al*., 2006; Diez *et al*., 2014), Mendoza (Domínguez & Lagos, 2001), Neuquén (Grech *et al*., 2013), Río Negro (Rubio *et al*., 2020), San Luis (Visintin *et al*., 2009), and San Juan (Carrizo Páez *et al*., 2016).

As known, *Ae. aegypti* is the main vector of different arboviruses including dengue (Simmons *et al*., 2012), chikungunya (Leparc-Goffart *et al*., 2014) and Zika (Tilak *et al*., 2016). Thus, the recent expansion in the geographic range of this species is causing concern, especially under scenarios of climate change, because of its public health implications. The models of the distribution limits have pointed out that one of the main constraints for the persistence of populations and the transmission of diseases is temperature (Brady *et al*., 2014). In particular, minimum temperatures have been identified as one of the strongest predictors of the presence of *Ae. aegypti*, and cold tolerance has been suggested to be one of the main biological parameters controlling the distribution limits (Dickens *et al*., 2018).

The population dynamics of *Ae. aegypti* in temperate climate regions are modulated by seasonal variations in temperature, and low temperatures during the winter season are considered unfavorable for its development and reproduction (Eisen *et al*., 2014). In the Metropolitan Area of Buenos Aires, which is near the *Ae. aegypti* distribution limit in South America, high abundances of adults are recorded during the warm season, exceeding the limits for the transmission of dengue, as has been demonstrated by the 8500 and over 11000 locally transmitted dengue cases during the 2016 and 2020 epidemics respectively (National Health Ministry, 2016, 2020). In contrast, during the winter season, no activity of adults or presence of larvae are usually recorded (Vezzani *et al*., 2004), and the reproductive season begins in the spring (October onwards), together with increasing temperatures (Fischer *et al*., 2017). This background is consistent with the idea that the population passes the winter season in the egg stage.

This permanence in the egg stage during the winter season has the advantage of a reduced mortality caused by low temperatures, since eggs have a higher tolerance to low temperatures than larvae, pupae or adults. For example, it has been shown that 50% of *Ae. aegypti* eggs survive to an exposure of 394 h at 0.5ºC, whereas at the same temperature larval mortality is 50% before 24 h (Bar Zeev, 1957). On the other hand, if temperatures are not too extreme, the continued development of immature stages and the reproduction of adults throughout the cold season may contribute to an increased number of reproduction cycles per year, and the anticipated beginning of the population increase associated with spring temperatures would allow for higher peak abundances during the warm season.

In contrast to the large number of laboratory studies, only few field studies have evaluated the effects of naturally fluctuating low temperatures on mosquito immature stages, despite their importance for the realistic prediction of population dynamics. Tsuda & Takagi

(2001), for example, found that persistent daily mean temperatures below 10ºC seem to cause complete larval mortality, preventing the completion of *Ae. aegypti* development in field conditions in Japan, while Chang *et al*. (2007) determined that mean temperatures around 14-15ºC allowed for completion of development of 30% of the individuals in Taiwan, although they detected a significant effect of cold fronts on survival. In Buenos Aires, we have previously found that *Ae. aegypti* is able to maintain development during the winter season, and that cohorts that develop at average temperatures of 13-14ºC last approximately 50 days to complete development, with 30% survival, while cohorts that develop at average temperatures of 20ºC develop in 18 days, with a survival above 90% (De Majo *et al*., 2017). There are two possible explanations for the lower survival at colder temperatures. The first one is that, under the local thermal conditions, daily mortality remains approximately constant, and the higher mortality responds only to the extended development time, which allows for a higher cumulative mortality. The second explanation is that particular thermal conditions are responsible for high mortality events, and that these events account for the higher mortality of some cohorts.

Based on all the above, the aim of this study was to estimate the daily mortality of *Ae. aegypti* during immature development in the winter and early spring season in Buenos Aires City, to assess its relationship with the preceding thermal conditions, and to identify the most vulnerable stages during immature development.

Methods

Study area

The study was carried out in Buenos Aires City, the capital of Argentina and the largest urban settlement in the country. Buenos Aires is a coastal city, located on the western shore of the Río de la Plata River. The climate is temperate humid, with hot summers and cool winters, and no dry season (Chesini *et al*., 2018). In the period 1991-2019, the mean annual temperature was 18.4ºC, and the annual cumulate rainfall was 1270 mm (Buenos Aires City Government, 2020). The characteristic winter conditions in Buenos Aires begin in early June and last through late August, with average temperatures between 1991 and 2019 of 12.4ºC (mean), 16.6ºC (maximum), and 8.3ºC (minimum). In September, temperatures increase gradually to a mean of 15.2ºC, with average minimum and maximum of 10.6ºC and 19.7ºC respectively. During the cold season, there are eventual cold fronts, defined as periods when temperatures are lower than a defined threshold

(12.7ºC for Buenos Aires City) during at least three consecutive days (National Meteorological Service, 2020).

The experiment was performed in the backyard of a family residence in the urban neighborhood of Mataderos (34°39'22.2"S, 58°31'18.8"W). The backyard (20 m^2) was surrounded by three 9-meter-high walls and one 2.2-meter-high wall with a 2-meter-high window, and had a ceramic floor and no roof. The backyard contained potted plants on the floor and walls, and received sunlight during the afternoon.

Field work

The development of *Ae. aegypti* was studied under natural temperature conditions during the whole winter season. From mid June throughout September (16 weeks), a new cohort was initiated once a week by immersing eggs in water. The eggs were obtained from ovitraps placed in different neighborhoods of Buenos Aires City during late fall (April and May), and maintained at field conditions until immersion. All eggs were assumed to correspond to *Ae. aegypti* because this is the only aedine mosquito that uses containers as larval habitats in this region (Rubio *et al*. 2012). For each cohort, six groups (replicates) of 20 first-instar larvae were separated and placed in 220-ml plastic containers containing 135 ml of a solution of 37.5 mg powdered baker´s yeast/l of water. The amount of yeast was defined according to previous experiments to provide optimal feeding conditions (Romeo Aznar *et al*., 2018).

All cohorts were inspected three times a week up to the adult stage, and the numbers of live and dead individuals of each larval instar and pupal stage were recorded. Live larvae were transferred to a new container with a previously prepared food solution. Pupae were transferred to individual containers conditioned with a resting substrate for the emerged adults, which were killed by freezing.

Data analyses

Temperature data: hourly temperatures were measured *in situ* during the whole experiment, with a meteorological station (Easyweather Model WS3101). These data were used to calculate the mean, maximum and minimum temperatures for periods of 24 hours. Since the counting of mosquitoes started at 9 a.m. and lasted for at least 6 hours each counting day, the time intervals for temperature calculation were considered from 12 p.m. of the previous day to 12 p.m. of the date considered.

Temporal variability in mortality during development: for each counting day, the number of live and dead larvae and pupae was counted separately for each cohort. The mortality in

each time interval (from the previous to the current counting) was calculated as the number of individuals found dead at the end of the interval divided by the number of individuals present at the beginning of the interval. To assess the stage-specific mortality, the analysis was performed separately for larvae and for pupae. To assess the mortality associated with the transition to the adult stage, the proportion of emerged adults found dead was calculated as the number of dead adults divided by the total number of adults emerged during each time interval. Taking into account that the intervals between counting days were not of equal duration (counts were made three times a week, thus intervals lasted 2 or 3 days), the daily mortality within each interval was calculated as the mortality during an interval divided by the duration of the interval (in days).

To assess the relationship of daily mortality with temperature, different indices of temperature were calculated: average minimum, maximum and mean temperatures, and the cumulative cooling degree hours (CCDH). CCDH are defined as the cumulative degree hours that air temperatures fall below a reference or threshold temperature (Gosling *et al*., 2014). The CCDH was calculated as: $\Sigma\ (T_0\text{-}T_{i)}$, for $T_0\text{-}T_i > 0$, where T_0 is the threshold temperature considered, T_i is the one ith-hour interval, and values are summed over a period of interest. CCDH were calculated with threshold temperatures of 5 to 16ºC, accumulated for periods of time ranging from 1 to 8 days. The threshold temperatures were selected to include the minimum temperatures observed in this study (5ºC), and the minimum temperature at which adults are able to fly (15ºC) (Rowley & Graham, 1968). The relationship of the daily mortality of larvae, pupae and adults with these indices was assessed separately with cross-correlation analyses, which allowed analyzing different delay periods between the dependent variable (daily mortality) and the independent variable (the corresponding temperature index). In the case of adults, the period of continued emergence of adults was considered to avoid interruption of the time series, i.e. four early emerged adults were not considered. In the case of CCDH, the threshold temperature with the best correlation was identified for larval, pupal and adult mortality.

For events with high mortality, the temperature conditions during the previous days were analyzed to identify the cold front associated with the increase in mortality. For each of these events, the number of larvae and the number of pupae at the beginning of the cold front were counted, and their survival or death during the 7 days after the end of the cold front recorded, together with the stage at which death occurred. The period analyzed after the end of the cold front was selected to be of seven days since, in some cases, this was the maximum period of time before the beginning of the next cold front. The percentages of larvae and pupae that died during the same stage, the percentages of those that died in a

later stage, and the percentages of those that survived for seven days were calculated and graphically compared. The mortality within 7 days after the end of the cold front of individuals that were at the larval stage at the beginning of the cold front was compared with that of individuals that were at the pupal stage at the beginning of the cold front. Also, among those that died within 7 days after the cold front, the proportion of those that died in a later stage was compared between those that were in the larval stage and those that were in the pupal stage at the beginning of the cold front. These analyses were performed with a Generalized Linear Mixed Model (GLMM) with binomial distribution and the logit link function. The identity of the cold front was included as a random effect to account for the lack of independence of deaths within each cold front.

Results

A total of 1907 larvae were reared along the study period, from which 1229 adults emerged (1151 alive and 78 dead after emergence). Among the 678 individuals that died before emergence, 60% were larvae, whereas the remaining 40% were pupae. The daily mortality showed variations along the study, and differences between the different developmental stages (Figure 1). Larval mortality was low during the whole study period (average 0.0065) except for one event in late July, when it exceeded the average by several times. The daily mortality of pupae was higher (average 0.0375), and several peaks were observed from July to September, which generally occurred after a marked decrease in temperatures, which remained low for at least two consecutive days. A similar pattern was observed for the daily mortality of adults. Excluding the mortality of early emerged adults (N = 4) that died after emergence in July, the daily mortality of adults was similar to that of pupae (average 0.0383) and peaks occurred during August and September (Figure 1).

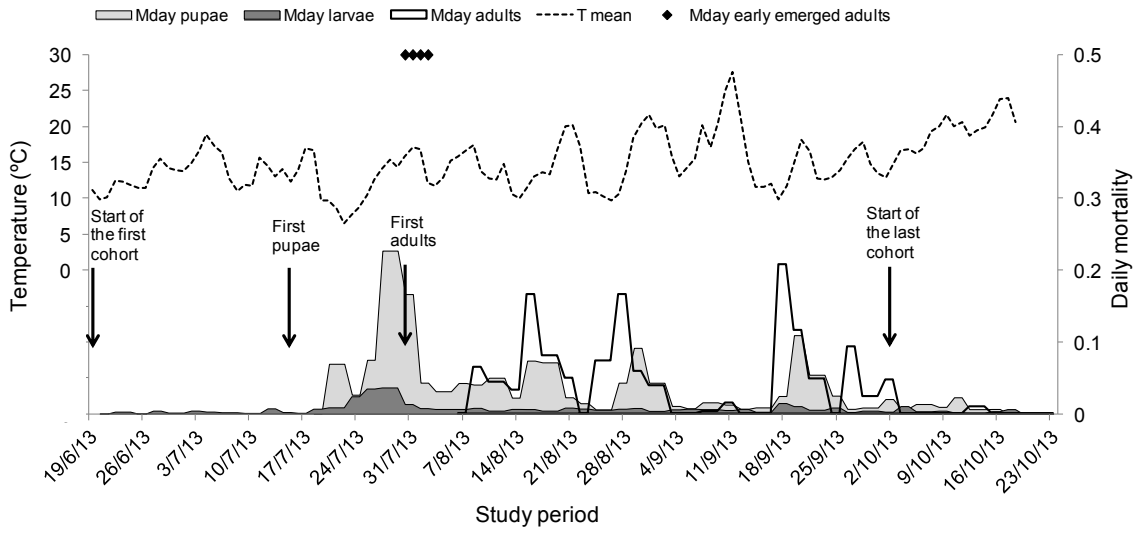

Figure 1. Variations in the daily mortality of *Ae. aegypti* larvae, pupae, and adults (Mday larvae, Mday pupae, Mday adults, respectively) during the winter in outdoor conditions, and daily mean temperatures (Tmean). Arrows indicate representative events within the experiment.

The daily mortality of larvae, pupae and adults showed a negative and significant relationship with temperatures, and in all cases delays were detected with regard to the occurrence of low temperature events, although some differences between stages were observed (Figure 2). The strongest relationship of mortality with previous temperatures was observed for adults, with the highest correlation associated with minimum and mean temperatures with a 2-day delay. For pupae, a strong relationship with previous temperatures was also observed, which was similar between minimum, maximum and mean temperatures, with a 5-6-day delay. For larvae, the correlation with previous temperatures was weaker than for pupae and adults, and the best correlations were observed for minimum and mean temperatures with a 4-day delay.

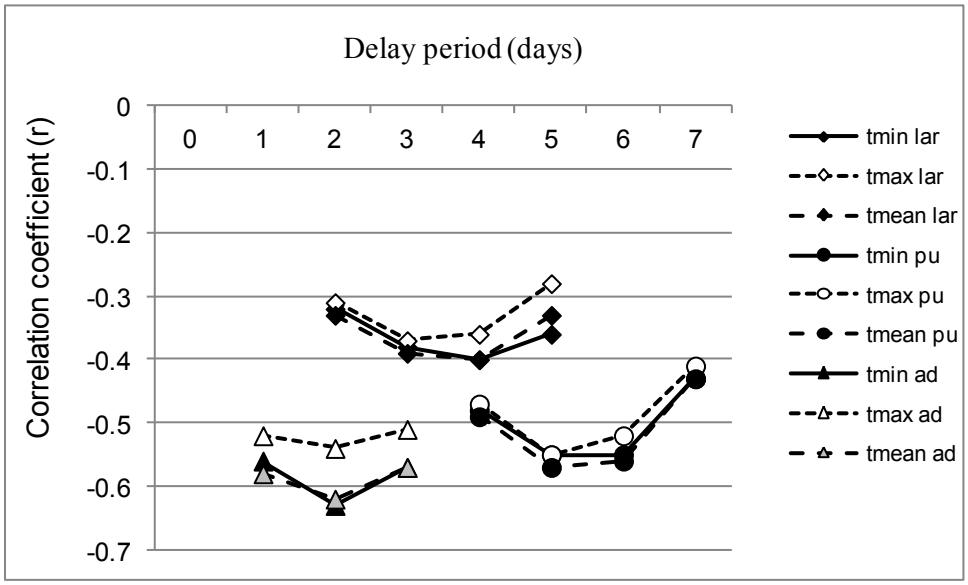

Figure 2. Correlation coefficients of the daily mortality of larvae, pupae and adults with minimum, maximum and mean temperatures considering different delay periods.

Daily mortality showed positive correlations with the CCDH accumulated during the previous days, with the best correlations obtained for threshold temperatures of 7ºC with an eight-day accumulation period for larvae, of 9ºC with a four-day accumulation period for pupae, and of 16ºC with a three-day accumulation period for adults. A delay of one day in the response to cooling degree hours was observed for larvae and adults, whereas a delay of five days was observed for pupae (Figure 3).

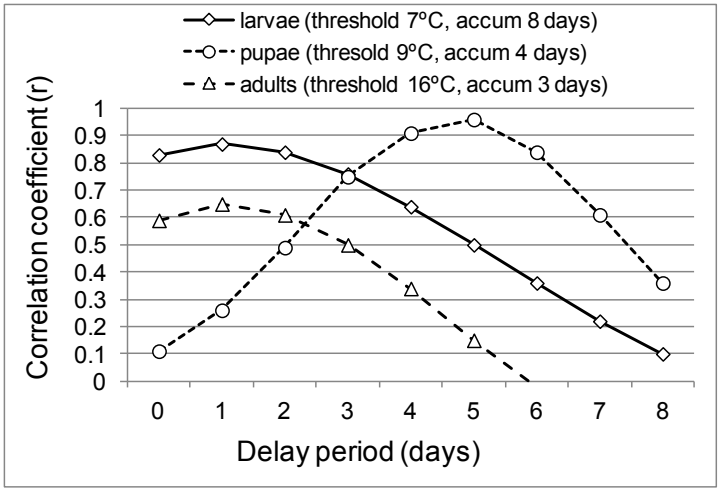

Figure 3. Best correlation coefficients of the daily mortality of larvae, pupae and adults with cooling degree hours for threshold temperatures.

Both for pupae and for larvae, the correlations were higher for the cooling degree hours than for the minimum, maximum or mean temperatures of one day, while for adults similar correlations were obtained for cooling degree hours and for minimum and mean temperatures.

Four mortality events caused by cold fronts were identified in late July, mid August, late August, and mid September respectively (Figure 1). In all of them, the mean temperature dropped more than 4ºC from one day to the next, and the mean temperatures remained below 12ºC for at least three consecutive days.

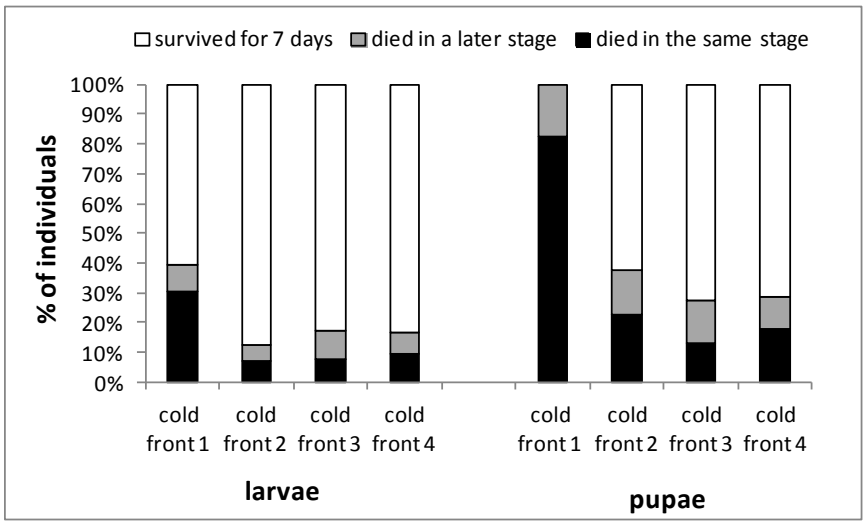

Figure 4. Percentages of larvae and pupae that died during the same stage, percentage of those that died in a later stage, and percentages of those that survived for seven days after the end of different cold fronts.

Individuals that were in the pupal stage at the beginning of the cold front had a higher mortality (average: 0.41) than those that were in the larval stage (average 0.20) (Figure 4), and the stage at the beginning of the cold front had a significant effect on mortality (GLMM Wald test: $F_{1,6} = 58.96$, $p < 0.001$). Some individuals died in a later stage than that in which they passed the cold front (i.e. larvae at the beginning of a cold front died as pupae or adults, and pupae at the beginning of a cold front died as adults). The proportion of larvae that molted to later stages and died (average: 0.40) was similar to that of pupae (average: 0.37), and no significant effect of the initial stage was detected (GLMM Wald test: $F_{1,6} = 0.35$, $p < 0.37$).

Discussion

The relatively low mortality of immature stages observed during the experiment suggests that, at least during most of the winter period, the conditions experienced in Buenos Aires City are not lethal for the aquatic stages of *Ae. aegypti*. The events of higher mortality occurred after cold fronts, showing that these represent the most harmful conditions for immature stages. Thus, the daily mortality was not constant along the winter period and the cumulative mortality of a cohort seems to be a function of two variables: the longer duration of development when temperatures are relatively low (as observed in previous studies) and the additional mortality that occurs as a consequence of extraordinary cold periods. The vulnerability to cold fronts has been previously mentioned by Chang *et al*. (2007) in an *Ae. aegypti* population of Taiwan, where the highest mortality affected the cohorts exposed to one or more cold fronts.

In the present study, the cold fronts that induced mortality events were characterized by a sharp decrease in temperature (over 4ºC average temperatures in 24 hours), together with a period of three or more days with temperatures below 12ºC. These conditions, very similar to the definition of a meteorological cold front for Buenos Aires according to the National Meteorological Service (2020), seem to be close to the thermal tolerance limit of this population. The sharp decrease in temperature might be an important contribution to the vulnerability of the individuals to low temperatures, since it has been shown that larvae and adults of *Ae. aegypti* acclimated to low temperatures are less vulnerable to low temperatures than not acclimated individuals (Jass *et al*., 2019).

In our study, pupae were substantially more affected by cold fronts than larvae. These results suggest that pupae might be the most vulnerable stage within the aquatic phase of the life cycle of *Ae. aegypti*, which coincides with the results of a previous study for pupae with more than three days of age (Bar Zeev, 1957). Adults were also very vulnerable to

low temperatures, which also coincides with previous studies (Bar Zeev, 1957). However, it has to be noted that adult mortality was recorded only immediately after emergence and thus may have been highly underestimated. Specific studies are necessary to assess the vulnerability of adult *Ae. aegypti* to low temperatures.

One of the questions we attempted to answer was the relationship of mortality with different temperature indices. The better correlation of daily mortality with the cooling degree hours accumulated for several days, as compared to the correlation with temperatures (mean, minimum or maximum) of one day, suggests that individuals are more affected by the accumulation of cold for several days than by the temperatures experienced during a short time interval. The study by Bar Zeev (1957) showed that exposure to 8ºC caused the mortality of 50% of individuals after 75 and 83 hours for pupae and larvae respectively. This temperature of 8ºC appears to be the upper limit of chill coma induction in some individuals (Jass *et al*., 2019), and is also near the lowest average temperature observed in our study.

With regard to the timing of the mortality events, the results of all our analyses showed a trend of mortality events to occur with a certain delay in relation to the lowest temperatures, both for larvae and for pupae. Delayed effects have been long recognized to occur and some examples have been previously documented in dipterans such as *Eurosta solidaginis* (Bale *et al*., 1989), *Sarcophaga crassipalpis* (Yocum *et al*., 1994), and *Drosophila melanogaster* (Kostal *et al*., 2019). These studies, and most of those assessing the cold hardiness of insects, have tested temperatures around or below the freezing point, which are far below those observed in our study. However, the patterns seem to be similar in all cases when temperature thresholds are passed, which might differ among species. The delay in the time of death of individuals exposed to a cold front can be explained by damages in the tissues of individuals due to low temperatures which, if not repaired, cause death a few days later regardless of favorable temperatures (Kostal *et al*., 2019). In the case of *Ae. aegypti*, physiological damages have been shown to affect immature individuals exposed to low temperatures through a loss in the balance of Na^+ and K^+ concentrations in the hemolymph (Jass *et al*., 2019).

In some individuals, the delay in the mortality events in relation to cold fronts was accompanied by a delay in the stage at which mortality occurred. This suggests that many studies in which mortality is assessed only a few hours after finishing the experimental conditions might highly underestimate the effects of low temperatures on individuals. In the present study on *Ae. aegypti*, effects of extreme temperatures on later stages of

development were observed both for larvae and for pupae, similar to previous observations in other dipteran species (Bale *et al*., 1989, Yocum *et al*., 1994, Kostal *et al*., 2019).

A gradient of responses to low temperature events (cold fronts) was observed. Some individuals died immediately, others died with a certain delay and even after molting to a later developmental stage, and others emerged alive. This suggests a range of variation in the vulnerability to cold temperatures within the *Ae. aegypti* population of Buenos Aires, where the most vulnerable individuals die immediately, while the most tolerant ones complete development and probably reproduce. This suggests that low winter temperatures may constitute a selective force, leading the population to a higher tolerance to low temperatures. In fact, the experimental selection for a higher cold tolerance has been previously demonstrated in *Ae. aegypti* after only four generations when larvae were exposed to low temperatures (Chang *et al*., 2007). If such processes occur under field conditions as well, a further expansion and increased success of *Ae. aegypti* in temperate regions should be expected in the near future.

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The authors declare no conflicts of interest.

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