Hatching Response of *Aedes aegypti* (Diptera: Culicidae) Eggs at Low Temperatures: Effects of Hatching Media and Storage Conditions

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ABSTRACT In temperate regions, *Aedes aegypti* (L.) (Diptera: Culicidae) populations remain in the egg stage during the cold season. To ensure the start of a new breeding season, eggs should hatch at the beginning of a favorable period. The aim of the current study was to investigate the hatching response of two *Ae. aegypti* egg batches collected and stored for 3 mo under different conditions, to different low immersion temperatures. Two different hatching media (water and yeast solution) were used for the first batch and only one (water) for the second egg batch. Eggs were immersed for 8 d, during which the number of hatched eggs was recorded daily. The proportion of hatched eggs, delay of the hatching response, proportion of dead larvae, and proportion of remaining eggs within the first egg batch were compared between the two hatching media at each temperature. These parameters also were compared between the two batches immersed in water. Hatching rates were higher and faster in the yeast solution. The hatching response was lower at lower immersion temperatures and among eggs stored under field conditions at colder temperatures (second batch). Among the eggs stored in the laboratory (first batch), older eggs exhibited lower hatching response. The proportion of dead larvae was higher in the yeast solution and in the eggs stored in the laboratory. The conditions that triggered a lower hatching response led to higher proportions of remaining eggs, allowing the population to maintain an egg bank for future favorable opportunities.

KEY WORDS temperate climate, hatching stimuli, unfavorable condition, Culicidae

Aedes aegypti (L.) (Diptera: Culicidae) is a mosquito species of tropical origin that, at present, has a worldwide distribution, ranging from tropical to temperate regions. In Argentina, this species can be found in northern and central provinces (Vezzani and Carbajo 2008). Buenos Aires city is located in the temperate region of the country, near the southern distribution limit of *Ae. aegypti.* The population dynamics of this species in the region is modulated by temperature (Otero et al. 2006), and high abundances are reached during the warm months (De Majo et al. 2013). These high abundances seem to be sufficient to allow for local transmission of dengue virus (family Flaviviridae, genus *Flavivirus*) as recorded during the summer of 2009 (Seijo et al. 2009). In contrast, during unfavorable thermal conditions, no adults or larvae are detected (Vezzani and Carbajo 2008), and thus the population persists in the egg stage. The breeding season begins in spring from eggs remaining from the previous warm season.

Egg hatch at the beginning of the favorable period is a critical event to ensure the population increase at the start of a new breeding season. Factors such as the immersion temperature, the hatching medium, and the physiological condition of eggs may determine the hatching response of *Ae. aegypti* eggs (Christophers 1960, Clements 1992, Vinogradova 2007).

Studies on the hatching response of *Ae. aegypti* eggs in relation to the immersion temperature are scarce. In the laboratory, an increase in hatching response has been observed at temperatures ranging from 15 to 27°C (Dickerson 2007, Farnesi et al. 2009). At temperatures lower than 15°C, the available information is largely anecdotal. Christophers (1960) noted massive egg hatch (and living larvae) at temperatures as low as 13°C, but only occasional hatch and no living larvae at lower temperatures. In contrast, under natural conditions, Bond et al. (1970) observed egg hatch at 4°C, but no living larvae after 24 h at temperatures lower than 10°C.

In temperate regions, spring is characterized by a gradual increase in temperature. Because eggs are subjected to daily mortality that seems to increase with time (Meola 1964, Juliano et al. 2002), initiating hatch immediately after favorable environmental conditions resume would be an adaptive response. Optimal temperature conditions for immature development of *Ae. aegypti* are attained above 20°C, although the lower thermal limit for which complete development (with 24% of survival) has been demonstrated is

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Table 1. Environmental conditions during collection and storage of *Ae. aegypti* eggs

Environment	First batch		Second batch	
	Collection (field)	Storage (laboratory)	Collection (field)	Storage (field)
Temperature (mean ± SD)	23.1 ± 3.2°C	21 ± 1°C	15 ± 2.4°C	12.2 ± 3.4°C
Humidity (mean ± SD)	71 ± 8%	≈80%	73 ± 10%	76 ± 10%
Photoperiod variation (hours:minutes)	Decreasing 14:12–12:34	Constant 12:00	Decreasing 11:34–11:12	Decreasing–increasing 11:12–10:46–11:17

14°C. At temperatures equal to or lower than 13°C, all individuals die during development (Bar Zeev 1958). Thus, it is expected that the hatching response begins at 14°C and increases with higher temperatures.

To hatch, some species of Aedine mosquitoes also require exposure to favorable temperatures before immersion (Vinogradova 2007). This has been observed in *Aedes taeniorynchus* Wied (Moore and Bickley 1966), *Aedes sticticus* (Meigen) (Horsfall and Trpis 1967), and *Ochlerotatus albifasciatus* (Macquart) (Campos and Sy 2006).

The hatching response of *Ae. aegypti* eggs may also be influenced by the characteristics of the hatching medium. In particular, the presence of bacteria in the medium triggers the hatching response at 25–28°C (Gillett et al. 1977, Ponnusamy et al. 2011), although its effects at lower temperatures have not been established.

Most studies on egg hatch have summarized this event as cumulative egg hatch following a predetermined time period, but the temporal dynamics of egg hatch after immersion has not been established. Some well-known population dynamic models for *Ae. aegypti*, including CimSim (Focks et al. 1993), Skeeter Buster (Magori et al. 2009), and AedesBa (Otero et al. 2006), base daily egg hatching rates on results from a single study where hatching was studied under natural conditions in Bangkok, Thailand, where temperature averages 30°C throughout the year (Southwood et al. 1972). There is no information about variations in the temporal dynamics of hatching in relation to environmental conditions before and during immersion.

The aim of the current study was to quantify the hatching response of *Ae. aegypti* eggs previously stored for 3 mo to simulate postwinter conditions in a temperate region. We assessed the response of two egg batches, collected and stored under different conditions, to different low immersion temperatures.

Materials and Methods

The eggs used were collected with ovitraps placed in highly urbanized residential neighborhoods within the metropolitan Area of Buenos Aires, Argentina (Mataderos quarter and Olivos quarter), ≈15 km from downtown, in sites with high abundances of this mosquito species. All the eggs collected were assumed to correspond to *Ae. aegypti* because this is the only container breeding Aedine mosquito species in this region (Rubio et al. 2012). Ovitraps consisted of containers partially filled with tap water and oviposition substrates (wooden paddle sticks 9.5 by 1.8 cm, commercially available as tongue depressors) attached to the inner wall in the vertical position by a clip. Two egg batches were collected.

The first batch was collected over six consecutive weeks from midsummer to early fall (from the third week of February to the end of March 2011), when the environmental conditions for *Ae. aegypti* were favorable in this region. Meteorological data were provided by the Servicio Meteorológico Nacional, Argentina (Table 1). Ovitraps were reconditioned weekly and the water and paddle sticks replaced in situ. Recovered paddle sticks with attached eggs were stored for ≈3 mo in the laboratory, in closed containers under controlled conditions (Table 1).

The second batch was collected during mid-fall (May 2011), when the environmental conditions for *Ae. aegypti* were less favorable (Table 1). Most eggs were laid during the first two weeks of May. Paddle sticks with eggs were stored for ≈3 mo in the same container where oviposition had occurred. Containers were placed outdoors under natural winter conditions (Table 1) but protected from rainfall.

At the end of the storage period (3 mo), paddle sticks were inspected under a stereoscopic microscope, and intact eggs were counted and collapsed or hatched eggs were removed. Each batch then was subjected to the following treatments:

First batch: 90 paddle sticks in total, each containing an average (range) of 34.3 (18–110) eggs were immersed in one of five constant temperatures (12, 14, 16, 18, and 20°C) and one of two hatching media (weak and strong stimuli). The hatching medium representing a weak stimulus consisted of reverse osmosis filtered water, whereas that representing a strong stimulus consisted of a 0.25 g/liter yeast solution in mineral water.

Paddle sticks were stratified into three categories according to the oviposition period (midsummer, late summer, and early fall, each corresponding to 2 wk of sampling) and randomly assigned to treatments. Thus, nine paddle sticks containing ≈300 eggs in total were used for each combination of temperature and hatching medium.

Immersion temperatures corresponded to the temperature range characteristic of late winter and spring in Buenos Aires and were obtained with thermal baths regulated through programmable thermostats (Loetti et al. 2001). In each thermal bath, maximum and minimum daily temperatures were monitored to detect excessive variations in the programmed temperature. The maximum standard deviation observed in the temperature baths was T ± 0.9°C.

Before applying the hatching stimulus, each paddle stick was acclimatized for 60 h in an individual container under dry conditions, at the same temperature at which the eggs were later immersed. Afterwards, each paddle stick was transferred to a hatching tube immersed in a thermal bath at the corresponding temperature. Hatching tubes consisted of plastic containers (50-ml Falcon tube) filled with 40 ml of hatching medium. To assess the temporal dynamics of the hatching response, each paddle stick was transferred to a new hatching tube, conditioned similarly to the original one, 6 h after the first immersion and every 24 h for eight consecutive days. On each occasion, the content of each recovered tube was observed under a stereoscopic microscope and the number of live and dead larvae counted, and then all larvae discarded. After 8 d of immersion, paddle sticks were dried and checked under a stereoscopic microscope, and hatched, intact, and dead (collapsed and semicollapsed) eggs were counted.

Second batch: 23 paddle sticks, in total, each containing an average (range) of 18.8 (8–34) eggs were immersed in hatching tubes at one of three constant temperatures (12, 14, 16°C) with a weak hatching stimulus (water). Paddle sticks were randomly assigned to treatments and 7–8 of them containing ≈140 eggs in total were used for each temperature. Because of the limited number of paddle sticks available for this batch, temperatures were selected to be representative of the range characteristic of late winter and early spring in Buenos Aires.

Paddle sticks were acclimatized and immersed with the same procedure as that described for the first batch and transferred to new hatching tubes every 24 h for eight consecutive days. On each occasion, the content of each recovered tube was observed under a stereoscopic microscope, the number of live and dead larvae was counted, and then all larvae were discarded. After 8 d of immersion, paddle sticks were dried and checked under a stereoscopic microscope, and hatched, intact, and dead (collapsed and semicollapsed) eggs were counted.

Data Analyses. Four indicators, three accounting for the hatching response and one accounting for survival after the hatching stimulus, were calculated.

P_{hatch} (proportion of hatched eggs): calculated for each paddle stick by dividing the number of observed larvae by the initial number of intact eggs (initially valid eggs).

H_{delay} (delay in hatching response): the value obtained is the weighted average of hatching time of larvae over the observation period (8 d), quantified with the formula $(\Sigma Ni*ti)/Ntot$, where Ni represents the number of observed larvae for the i count, ti the mean time for the interval between time ti–1 and ti is measured in days, and Ntot is the total number of larvae observed on each paddle stick.

P_{dead} (proportion of dead larvae): estimated as the number of dead larvae observed during counting divided by the total number of larvae observed.

P_{rem} (proportion of remaining eggs): estimated as the number of intact remaining eggs at the end of the experiment, divided by the number of initially valid eggs. Intact eggs were assumed to be alive.

Effects of Immersion Temperature and Hatching Medium. For this analysis, we used data corresponding to the first batch. The effects of the immersion temperature and hatching medium were analyzed for the four indicators, and the category of the oviposition period was included as an additional factor. Statistical analyses were performed using three-way analysis of variance (ANOVA). To satisfy assumptions of homogeneity of variances, H_{delay} was log-transformed, P_{dead} was transformed to root arcsine, and P_{rem} was square root–transformed. To assess significant differences, post hoc multiple comparisons were made with Tukey's HSD test for balanced data (temperature) or unequal data (laying period).

Effects of Immersion Temperature and Previous Storage Conditions. For this analysis, we used data of paddle sticks immersed in water at the lower temperature treatments (12, 14, and 16°C) from the first batch, and all data from the second batch. Differences in hatching response in function of the immersion temperature and storage conditions were analyzed for the four indicators by means of a two-way ANOVA. For H_{delay}, we used reciprocal transformation $(1/H_{delay})$, and for P_{rem} we used root arcsine transformation to satisfy the assumption of homogeneity of variances. Significant effects were compared with post hoc multiple comparisons with Tukey's HSD test for unequal data. Paddle sticks with no hatched eggs were deleted from the analyses of H_{delay} and P_{dead} because a minimum of one egg hatching event for each substrate is necessary to calculate these indicators.

Results

Effects of Immersion Temperature and Hatching Medium. Eggs hatched at all the temperatures assessed, including the lowest (12°C), in both hatching media and within the three oviposition period categories.

In general, the hatching response was lower at lower temperatures in filtered water and within the older eggs (midsummer), although a high variability was observed among paddle sticks within the same treatment (Table 2).

The proportion of hatched eggs (P_{hatch}) was significantly affected by temperature ($F_{4,60} = 3.38$, $P < 0.05$), hatching medium ($F_{1,60} = 11.94$, $P < 0.005$), and oviposition period ($F_{2,6} = 18.43$, $P < 0.001$), whereas the interaction terms were not significant ($P > 0.05$). Post hoc comparisons detected significant differences between extreme temperatures, with a higher P_{hatch} at 20°C than at 12 and 14°C ($P < 0.05$). P_{hatch} at 16 and 18°C was intermediate and not significantly different from P_{hatch} at lower or higher temperatures. P_{hatch} was also significantly higher for eggs collected in early fall than for those collected during midsummer ($P < 0.05$), which, in turn, showed a higher P_{hatch} than eggs from late summer ($P < 0.05$; Table 2).

Table 2. Number of wooden paddle sticks (oviposition substrates) and eggs used and average proportion of hatched eggs (P_{hatch}) and remaining intact eggs (P_{rem}) for each temperature, hatching medium, and collection period corresponding to the first batch

Treatment	*N* (substrata)	Total no. of initial valid eggs	Avg proportion of hatched eggs (P_{hatch})	Avg proportion of remaining intact eggs (P_{rem})
Temperature (°C)				
12	18	632	0.43a	0.30a
14	18	610	0.53a	0.14b
16	18	613	0.60ab	0.03b
18	18	586	0.55ab	0.13b
20	18	648	0.72b	0.10b
Hatching medium				
Water	45	1561	0.48a	0.25a
Yeast solution	45	1528	0.65b	0.03b
Oviposition period				
Midsummer	18	487	0.38a	0.14a
Late summer	50	1843	0.55b	0.16a
Early fall	22	759	0.75c	0.09b

Averages with different letters are significantly different.

The delay in the hatching response (H_{delay}) was significantly affected by temperature ($F_{4,60} = 5.03$, $P < 0.005$) and the hatching medium ($F_{1,60} = 78.04$, $P < 0.001$) but not by the oviposition period or the interactions ($P > 0.05$). Post hoc comparisons showed a significantly longer H_{delay} for the eggs immersed at 12°C than for those immersed at the remaining temperatures ($P < 0.05$), among which no differences were detected. The hatching response was maintained along the 8 d of the experiment, although a large variability among paddle sticks was observed. H_{delay} was longer in water than in yeast solution. In the latter, >80% of the eggs hatched within the first 6 h at all temperatures except at 12°C, at which 80% of the eggs hatched after the first 24 h. In contrast, in water, 80% of the eggs hatched after 2 d of immersion, except at 12°C, at which this percentage was reached during the fourth day of immersion (Fig. 1).

Both dead and living larvae were recorded at all temperatures and hatching media. The proportion of dead larvae (P_{dead}) was significantly affected by the hatching medium ($F_{1,60} = 15.31$, $P < 0.001$), with higher mortality in yeast solution (0.27) than in water (0.11), but not by the temperature or the interaction ($P > 0.05$).

Furthermore, a marginally significant effect of the oviposition period was detected ($F_{2,60} = 2.99$, $P = 0.058$), with a trend toward higher P_{dead} in midsummer eggs (0.29), intermediate P_{dead} in late-summer eggs (0.17), and lower P_{dead} in early-fall eggs (0.14). The proportion of remaining eggs (P_{rem}) varied significantly in relation to temperature ($F_{4,60} = 6.43$, $P < 0.001$), hatching medium ($F_{1,60} = 36.58$, $P < 0.001$), and oviposition period ($F_{2,60} = 7.30$, $P < 0.005$), whereas the interaction terms were not significant ($P > 0.05$). Post hoc comparisons detected a larger P_{rem} within eggs immersed at 12°C than within eggs immersed at higher temperatures ($P < 0.05$). P_{rem} was also significantly higher in water than in the yeast solution and for eggs collected in mid and late summer than for those collected in early fall ($P < 0.05$; Table 2).

Effects of Immersion Temperature and Previous Storage Conditions. P_{hatch} was significantly affected by temperature ($F_{2,44} = 6.41$, $P < 0.005$) and storage conditions ($F_{1,44} = 11.88$, $P < 0.005$) but not by the interaction between both variables ($F_{2,44} = 0.64$, $P > 0.05$).

A trend toward a higher P_{hatch} was observed at higher temperatures (Table 3), and the differences between 12 and 16°C were significant ($P < 0.05$), while intermediate values were observed at 14°C, with no differences to higher and lower temperatures. Furthermore, P_{hatch} was higher for eggs of the first batch (laid during the warm season and stored in the laboratory at warm temperatures) than for eggs of the second batch (laid at the end of fall and stored under natural winter conditions (Table 3). The only treat-

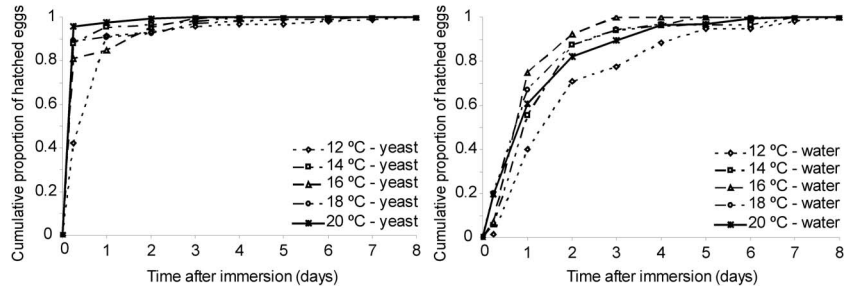

Fig. 1. Cumulative proportion of hatched larvae after immersion of *Aedes aegypti* eggs stored in laboratory conditions. Left graph corresponds to yeast medium and right graph to water.

Table 3. Number of paddle sticks and eggs used and average proportion of hatched eggs (P_{hatch}) and remaining intact eggs (P_{rem}) immersed in the weak stimulus (water) for each of the lower temperatures and storage conditions

Treatment	*N* (substrata)	Total no. of initial valid eggs	Avg proportion of hatched eggs (P_{hatch})	Avg proportion of remaining intact eggs (P_{rem})
Temperature (°C)				
12	17	445	0.25a	0.66a
14	17	453	0.41ab	0.48b
16	16	470	0.56b	0.32b
Storage condition				
Laboratory (first batch)	27	936	0.52a	0.28a
Field (second batch)	23	432	0.27b	0.73a

Averages with different letters are significantly different.

ment where some paddle sticks showed no hatching response was that of eggs stored under natural conditions and immersed at 12°C.

H_{delay} showed differences among temperatures ($F_{2,40} = 4.99$, $P < 0.05$), being significantly higher at 12°C than at the other temperatures ($P < 0.05$). Although a trend toward a delayed hatching was observed at 12°C for eggs stored under natural conditions as compared with those stored in the laboratory (Fig. 2), the statistical analysis showed no effects of the storage conditions ($F_{1,40} = 2.36$, $P > 0.05$) or the interaction among variables ($F_{2,40} = 2.01$, $P > 0.05$) on H_{delay}, which might be related to the high variability among paddle sticks within the same treatment.

P_{dead} was significantly affected by storage conditions ($F_{1,40} = 4.28$, $P < 0.05$), being higher among eggs of the first batch stored in the laboratory (0.15) than among those of the second batch stored under natural conditions (0.03), but not by temperature ($F_{2,40} = 0.75$, $P > 0.05$) or the interaction term ($F_{2,40} = 0.54$, $P > 0.05$).

P_{rem} was significantly affected by temperature ($F_{2,40} = 8.34$, $P < 0.001$) and storage conditions ($F_{1,40} = 50.98$, $P < 0.001$) but not by the interaction ($F_{2,40} = 1.93$, $P > 0.05$). P_{rem} was significantly higher at 12°C ($P < 0.05$) than at 14 and 16°C, which did not differ from each other. The eggs stored under natural conditions exhibited a higher P_{rem} than those stored in the laboratory (Table 3).

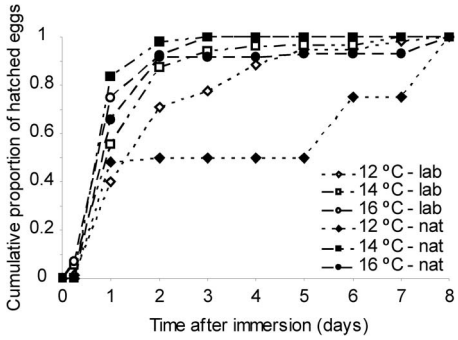

Fig. 2. *Aedes aegypti* eggs stored in different conditions. Filled symbols for eggs stored in natural conditions, empty symbols for eggs stored in laboratory conditions.

Discussion

Eggs hatched in all experimental treatments, although the temperature, hatching medium, oviposition period, and storage conditions significantly affected the hatching dynamics. Our results suggest that the eggs analyzed were in a state of quiescence (Vinogradova 2007), and thus were able to hatch when environmental conditions turned favorable.

The negative effect of the immersion temperature on the proportion of hatched eggs, the delay in the response, and the proportion of remaining living eggs demonstrates the importance of this variable on the dynamics of the egg bank of *Ae. aegypti* in this region. The detection of larvae even at the lowest temperature shows that egg hatch is not completely inhibited within the thermal range analyzed. Our results do not allow us to differentiate whether this represents a major cause of larval mortality because of "inopportune" egg hatch at temperatures that are not favorable to complete development, like that observed under natural conditions by Bond et al. (1970), or an adaptive tolerance to lower temperatures in the temperate region, like that observed experimentally by Chang et al. (2007). Although larval mortality was not affected by low temperatures during the first 24 h in the current study, studies on the ability to complete immature development and on survival rates at low temperatures would be necessary to assess whether the observed hatching response is adaptive or not.

The higher proportion of remaining living eggs at lower temperatures suggests that when environmental conditions are less favorable, this species increases the spreading of the risk, allowing the populations to use the first opportunities to initiate the developmental cycle during spring but maintaining an egg bank for future opportunities in case thermal conditions are not sufficiently favorable to complete development.

The higher proportion and speed of egg hatch in the yeast solution is coincident with expectations based on results of previous studies because yeast simulates the bacterial bloom, which in turn triggers egg hatch in natural conditions (Christophers 1960, Vitek and Livdahl 2006, Ponnusamy et al. 2011). The hatching delay observed in filtered water could be related to the results of a previous study, where the density of bacteria (initially close to zero) increased by six orders of magnitude during the first 8 h, and by two additional

orders of magnitude within the next 8 h, when 70% of cumulative egg hatching was attained (Ponnusamy et al. 2011). Although we did not quantify microorganisms in the hatching media, it is likely that their abundance in the filtered water solution increased during the first hours of the study, stimulating egg hatch later than in the yeast solution. The higher proportion of dead larvae observed in the yeast solution suggests that this medium forced the hatching of eggs that were not physiologically ready to resume development.

Although no studies have assessed whether the two hatching media used in the current study are representative of natural conditions, studies performed on *Aedes albopictus* (Skuse) have shown that distilled water has an effect on egg hatch similar to that of stimuli in natural habitats like tires or tree holes, and that solutions with microorganisms represent a stronger stimulus (Vitek and Livdahl 2006). Thus, filtered water might be comparable with the content in containers that are initially dry and then filled with rain, which at first contain a low number of microorganisms that may increase in the subsequent hours by using the nutrients and organic matter present in those habitats, whereas the yeast solution might be equivalent to the situation in containers that previously hold water and then continue to be filled by rain, thus containing a large initial number of microorganisms. Future studies should assess egg hatch under a wide range of natural conditions to confirm the representativeness of our results.

The higher hatching proportion of early fall eggs in the first batch could be related to the time during which eggs were stored before immersion. These results are similar to those obtained in a previous study in Buenos Aires (Fischer et al. 2011), supporting the hypothesis that younger eggs have a higher hatching response than older eggs. This pattern is probably caused by the loss of water from the eggs as a result of transpiration (Meola 1964). Thus, to hatch, these eggs will need repeated stimuli to first compensate the loss of water. This explanation also is supported by the higher proportion of remaining living eggs among the eggs laid in the summer.

Regarding the differences between batches, our results show that *Ae. aegypti* eggs laid in late fall and stored under natural conditions in winter have a lower predisposition to hatch than eggs laid earlier in the season and stored at higher temperatures in the laboratory. Previous studies have shown that the hatching response of *Ae. aegypti* eggs stored at 10°C is much lower than that of eggs stored at higher temperatures (Meola 1964) and almost null when eggs are stored at 4°C for 50 d (Weisman Strum and Kindler 1962). These results suggest that this species, as other Aedine mosquitoes, has mechanisms to reduce "inopportune" hatching during short periods of higher temperatures during the adverse season and requires favorable thermal conditions prior to immersion to increase the hatching response (Vinogradova 2007). Nevertheless, it is not possible to rule out the existence of a seasonal effect on the hatching response of *Ae. aeygpti* eggs (i.e., a dependence on the period when eggs were laid), and complementary studies on this matter are needed.

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