Cold season mortality and hatching behavior of *Aedes aegypti* L. (Diptera: Culicidae) eggs in Buenos Aires City, Argentina

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ABSTRACT: In temperate regions, populations of *Aedes aegypti* survive the cold season in the egg stage. In the present work, we studied the cold-season mortality of *Ae. aegypti* eggs and their subsequent hatching pattern in Buenos Aires city. Eggs were exposed during the winter season (three months) in three neighborhoods located along a gradient of distance toward the Río de la Plata River, coincident with a gradient of activity of *Ae. aegypti.* Results showed mortalities lower (30.6%) than those from tropical regions during the dry season. Significant differences were detected among the egg mortalities of each site with a maximum value at the site nearest the Río de la Plata River (50%), and a minimum value at the most continental site (9%). Post-experimental hatching response of eggs differed between sites, with the highest proportion of hatched eggs during the first immersion in the site nearest to the river and the lowest proportion in the most continental site. The hatching proportion also differed between age classes, with older (early-laid) eggs hatching later than new (late-laid) ones. Our results provide the first information of *Ae. aegypti* egg mortality in temperate South America and support the hypothesis that differences in egg mortality are associated with abundance patterns of *Ae. aegypti* in Buenos Aires city. *Journal of Vector Ecology* **36 (1): 94-99. 2011.**

Keyword Index: Population dynamics, egg survival, hatching, winter diapauses, temperate climate.

INTRODUCTION

Aedes aegypti L. (Diptera: Culicidae) has a worldwide distribution, ranging from tropical to temperate regions. This mosquito species is the main vector of dengue in America, where the number of cases increases each year (Spiegel et al. 2005). Like other mosquito species with drought-resistant eggs, *Ae. aegypti* populations survive unfavorable periods in the egg stage (Sota and Mogi 1992). In temperate regions, such periods are determined mainly by the low winter temperatures, which may preclude the hatching of eggs and development of larvae (Christophers 1960). Therefore, survival of the eggs until the next favorable period is a key factor for the persistence of *Ae. aegypti* populations. In spite of this, to our knowledge, no studies have been conducted on the mortality of *Ae. aegypti* eggs under natural conditions in temperate regions, and only a few have been made in tropical conditions (e.g., Trpis 1972, Russell et al. 2001).

After survival through the unfavorable season, egg hatching is the next necessary step to ensure the continuity of the population cycle. It is known that the eggs of *Ae. aegypti*, like those of other species with drought-resistant eggs, do not necessarily hatch at the first stimulus (Gillett 1955, Vinogradova 2007). Although it has been demonstrated that the differences in hatching time of *Ae. aegypti* eggs are determined genetically, a certain variability can be observed among eggs of the same egg batch, even

among those subjected to similar environmental conditions (Gillett 1955). It has been suggested for *Aedes albopictus* that an increase in the risk of egg mortality would increase their sensitivity to a given hatch stimulus (Vitek and Livdahl 2006). The mortality risk may be higher when eggs are exposed to harsher environmental conditions, and under comparable environmental conditions this risk is expected to be higher for older eggs.

Buenos Aires is located near the southern limit of the worldwide distribution of *Ae. aegypti*. However, the abundance of adults of this mosquito species is high during the summer months (Schweigmann et al. 2002). Given the fact that the presence of immature stages and adult activity are not detected during the cold season (Vezzani et al. 2004), it can be assumed that the population survives this season in the egg stage and restarts the cycle in spring when the temperature rises above a threshold level (Schweigmann et al. 2002, Vezzani et al. 2004).

In Buenos Aires, *Ae. aegypti* has shown heterogeneous patterns in its distribution. *Ae. aegypti* populations present in neighborhoods located towards the east (i.e., nearer the Río de la Plata River) show lower oviposition activity only during the summer months, whereas those in neighborhoods located in northern, western, and southern areas of the city (i.e., the periphery) have higher infestation with oviposition activity from spring to fall (Carbajo et al. 2006). The heterogeneous distribution of *Ae. aegypti* has been related mainly to the urbanization gradient in Buenos

Aires (Carbajo et al. 2004, 2006). Nevertheless, because of the analysis scale used, it is most likely that these studies have overlooked some differences in oviposition activity between sites with similar urbanization. For example, residential neighborhoods near the Río de la Plata River show consistently lower oviposition activity than similar urbanized areas in the southwest side of the city. Such differences could be attributed to differential egg mortality during the cold season, taking into account that the increase in the adult population during the next reproductive season is initiated by the surviving eggs. Furthermore, a differential hatching pattern of eggs exposed to different environmental conditions could either increase or compensate for the effects of egg mortality on the population recovering during the spring months.

In this study, we tested the hypothesis that areas where oviposition activity starts later in the season and attains lower maximum values in the summer exhibit higher egg mortality during the cold season. In addition, we explored the hatching behavior of the eggs exposed to different environmental conditions.

MATERIALS AND METHODS

Study area

Buenos Aires (34° 36 S and 58° 26' W) lies on the western shore of the Río de La Plata River (Figure 1). The city has a temperate humid climate with seasonally varying temperatures. The mean temperature in the cold season is 11.5° C, with the mean humidity in the winter during the last ten years ranging between 74% and 79% (National Meteorological Service 2009). The city covers an area of 200 km^2, has a population of three million, and is surrounded by urban and suburban areas covering 3600 km^2 with nine million people (Atlas Ambiental de Buenos Aires 2009).

Study sites were selected to represent the gradient of oviposition activity described by Carbajo et al. (2004). We selected three residential neighborhoods with a similar construction type, predominantly single-family dwellings of low height with backyard gardens and tree-shaded streets. The sites were located in the northeast (site A, in the quarter of Belgrano), the west (site B, in the quarter of Villa Devoto), and the southwest (site C, in the quarter of Mataderos) of the city (Figure 1). A preliminary study with ovitraps during the high activity season of *Ae. aegypti* from late summer (February) to early fall (April) confirmed the expected differences in oviposition activity, with 26%, 42%, and 51% of infested ovitraps at sites A, B, and C, respectively (unpublished data).

Experimental design

Eggs obtained from a city-wide monitoring program with ovitraps were used to start a colony of *Aedes aegypti*. The breeding conditions in the laboratory were a 12:12 photoperiod, and a mean temperature of 22 $\pm$ 1° C. Larvae were fed with a solution of powdered baker's yeast *ad libitum*, and, once pupated, transferred to a breeding box where adults emerged. Once blood-fed, females were
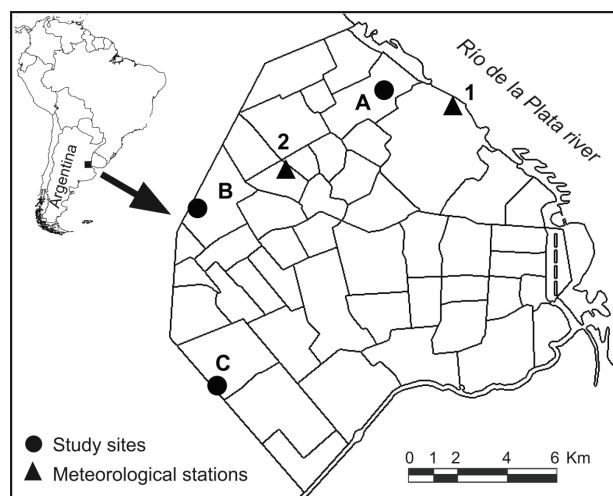

Figure 1. Location of the three study sites and two meteorological stations in Buenos Aires city, Argentina.

transferred to a separate box, where approximately 40 oviposition substrates (wooden paddles 9.5 x 1.8 cm, commercially available as tongue depressors) were placed in containers with water. The wooden paddles were collected once they accumulated at least 20 eggs and replaced by new ones. This procedure (collection and replacement of paddles) was repeated until a sufficient number of substrates was obtained. The week of collection was recorded for each paddle, and eggs used in the experiment were F1 eggs, laid during the first four consecutive weeks. Two age classes were distinguished: eggs from paddles collected during weeks 1-2 (early-laid eggs) and eggs from paddles collected during weeks 3-4 (late-laid eggs).

Before starting the experiment, each paddle was inspected under a stereoscopic microscope, intact eggs were counted, and all damaged or collapsed eggs were removed. All paddles with at least 12 eggs (mean 31, range 12-107) were used in the experiment, and paddles collected each week were evenly assigned to treatments. The experiment consisted of exposing a known number of eggs in each study site for a three-month period (July-September 2008). Paddles containing the previously counted eggs were placed in earthen pots (10 cm high x 10 cm diameter) with a plastic cover attached 0.8 cm over the top, covering the pot completely to prevent rain from falling into it and eggs from hatching (Figure 2). Each paddle was placed in a separate pot and fixed in a vertical position with a clip.

Two treatments were differentiated (Figure 2). One consisted of natural conditions as described above, and
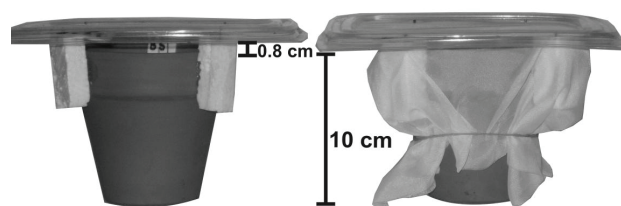

Figure 2. Experimental pots; left: natural conditions, right: protected conditions.

one of protected conditions, where each pot was covered with a fine mesh screen of nylon on top (mesh size of 0.3 mm) to prevent access of macroscopic organisms. Eighteen replicates were placed for each treatment at each site at the beginning of the winter season. Hourly temperatures at each site were recorded with a HOBO® Pendant Temperature Data Logger placed in a pot.

All paddles were removed after 90 days (at the beginning of spring), and transferred to the laboratory. The eggs remaining in each paddle were counted, and the number of completely collapsed, partially collapsed, or intact eggs recorded. All completely collapsed eggs were removed from the paddle and recorded as dead. To stimulate hatching, eggs were transferred into white styrofoam cups containing a solution of 40 mg of powdered baker's yeast in 168 ml dechlorinated tap water. Each paddle was immersed separately, and after two to four days the number of hatched larvae was counted. After four days, the paddles were removed from the water, and the immersion process was repeated the following weeks. A total of ten immersions were carried out, after which the number of remaining unhatched eggs was negligible. Corions of unhatched eggs remaining on the paddles were bleached with a commercial 50% sodium hypochlorite solution to allow direct observation of the embryos. Embryos that were creamy white with eye spots and presented a hatching spine and distinct abdominal segmentation were considered viable. Yellow-brown or red-brown embryos were considered non-viable (McHaffey and Harwood 1970). In this work, completely collapsed eggs and eggs with non-viable embryos were considered dead, whereas eggs that either hatched or contained viable embryos were considered alive.

Data analyses

Mean temperature and standard deviation were calculated based on the data measured. Temperature data were compared between pairs of sites using the paired t-test (Zar 1999). Humidity data were recorded in two meteorological stations of the National Meteorological Service of Argentina: one at the river side (station 1) and the other at a more continental location (station 2). Mean hourly percent humidity from both stations was compared using the paired t-test.

Egg mortality for each paddle was calculated by dividing the number of dead eggs by the total number of eggs recovered after the field exposure. Because on some paddles either no eggs were retrieved or the number of eggs was too low, we selected those paddles with more than ten recovered eggs for this analysis. In order to maintain a balanced design, randomly selected replicates were deleted from the treatments with exceeding paddles, following Underwood (1997). Effects of site and treatment were analyzed by means of two-way ANOVA on arcsine-transformed data. To assess significant differences, post-hoc comparisons were made with Tukey´s (HSD) test. Average and 95% confidence intervals were obtained for each site from the transformed data. To express these in terms of proportions, the inverse transformation was applied (Zar 1999).

The hatching response of eggs was determined analyzing the intensity of the response after each immersion and the cumulative response to the successive immersions. The intensity of the hatching response was assessed by calculating the conditional proportion of hatched eggs: the number of larvae observed on each occasion divided by the number of remaining living eggs before the respective immersion (eggs were considered alive either if they hatched after any stimulation or if they contained viable embryos after the bleaching). The proportions of hatched eggs at each immersion were compared between categories of exposure site and between age classes 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). To study the cumulative hatching response, the accumulated proportion of hatched eggs was calculated (sum of eggs hatched at the previous and present immersion divided by the total number of living eggs). Results were plotted for each site by age class combination.

The proportion of eggs that could not be recovered after the field exposure (missing eggs) on each paddle was calculated as the difference between the number of eggs initially present on the paddle and the number of eggs recovered after the three-month exposure, divided by the number of eggs initially present on that paddle. For each treatment and site, the mean number of missing eggs was calculated from the proportions obtained for individual paddles. Because different transformations did not meet the necessary assumptions, a non-parametric two-way ANOVA was performed using the Sheirer-Ray-Hare method (Sokal and Rohlf 1995). The factors considered were: site (three levels) and treatment (two levels). All 18 replicates were included in the present analysis.

RESULTS

The mean temperature values were 13° C, slightly higher at site A (13.08 ± 2.87° C), intermediate at site B (13.05 ± 4.5° C), and lower at site C (12.96 ± 3.33° C). Statistical comparisons showed significant differences between site A and site C (t = 4.89, d.f. = 1,799, p < 0.001). The relative humidity was higher at the riverside station (76.73 ± 15.90%) than at the continental station (74.56 ± 16.54%), and differences were significant (t = 10.54, d.f. = 2,201, p < 0.001).

A total of 3,079 (87.3%) out of the initial 3,525 eggs was recovered at the end of the experiment. Among them, 943 (30.6%) did not survive the winter season. The proportion of egg mortality after three months was significantly higher at sites A and B than at site C (Figure 3). The statistical comparison detected significant differences in egg mortality between sites (F = 13.82, d.f. = 2, p < 0.001), while no differences were detected between exposure conditions, egg age, and interaction terms.
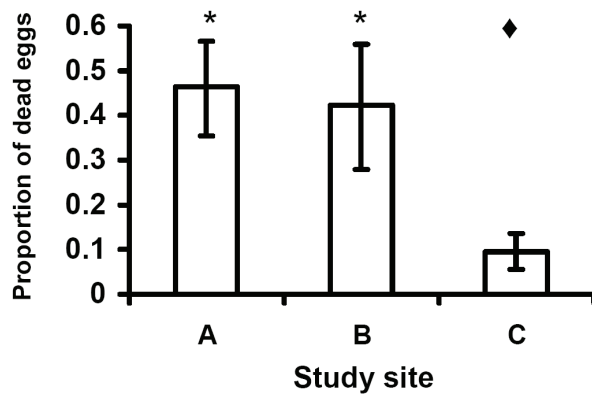
Figure 3. Mean and confidence intervals (95%) for the proportion of egg mortality of *Aedes aegypti* at three sites in Buenos Aires city. Different symbols indicate significant differences detected by Tukey test (p<0.05).

Of the 2,136 viable eggs, 2,127 hatched during the ten immersions and only nine remained unhatched. The overall proportion of hatched eggs was highest during the first (0.79) and second (0.65) immersions, and lowest during the fifth (0.10). The remaining immersions induced intermediate hatching rates.

The intensity of hatching was highest at the first immersion and decreased progressively through the fifth, after which a new increase was observed (Figures 4a,b). The age of eggs showed a significant relationship with the pattern of hatching (Figure 4a), with a significantly higher proportion of late laid eggs hatching during the first ($\chi^2 = 53.67$, df = 1, p<0.001), second ($\chi^2 = 13.73$, df = 1, p<0.001), and third ($\chi^2 = 12.27$, df = 1, p<0.001) immersions. A significantly higher proportion of early laid eggs hatched during the ninth immersion ($\chi^2 = 6.54$, df = 1, p<0.05). The eggs exposed at the different sites also showed a difference in the hatching pattern (Figure 4b). Differences in hatched eggs were significant between sites for the first ($\chi^2 = 31.64$, df = 2, p<0.001), the fifth ($\chi^2 = 6.07$, df = 2, p<0.05), and the seventh immersion ($\chi^2 = 8.54$, df = 2, p<0.05). The proportion of eggs hatched during the first immersion was high for eggs exposed at site A, intermediate for those at site B, and low for those at site C. The opposite was observed during the fifth immersion, when eggs exposed at sites A and B attained lower hatching proportions than those exposed at site C (Figure 4b).

The comparison of the cumulative hatching curves of the six combinations of site by age showed a consistent pattern of early laid eggs hatching in later immersions (Figure 5). Hatching showed the highest heterogeneity between site and age combinations during the first immersion. The cumulative proportions of hatched eggs increased faster for early laid eggs than for late laid eggs, with more than 97% of late laid eggs hatched after three immersions, and similar numbers of early laid eggs hatched after seven or more immersions.

Egg loss was recorded in all treatments. Approximately 50% of the paddles from the treatments with protected conditions had missing eggs, while percentages of paddles
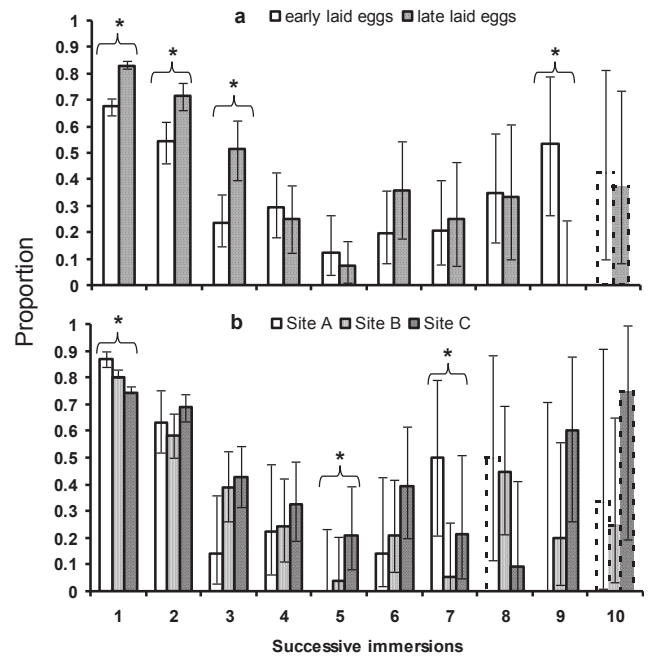

Figure 4. Conditional proportion of hatched eggs of *Aedes aegypti* after ten successive immersions for two egg classes (a) and three sites (b). Error bars indicate 95% confidence intervals. * indicates significant differences by the chi square test. Columns with dotted lines indicate proportions calculated from less than ten remaining living eggs at the respective immersion.

with missing eggs in the treatments with natural conditions were 67%, 72%, and 79% in sites A, C, and B respectively. The proportion of missing eggs showed significantly lower values in the treatments with protected conditions (H = 5.961, d.f. = 1, p < 0.02) (Figure 6). No significant effects of the study site or the interaction of site vs exclusion effect were detected.

DISCUSSION

Cold season egg mortality in Buenos Aires was lower than during the dry seasons in tropical regions. In Tanzania,
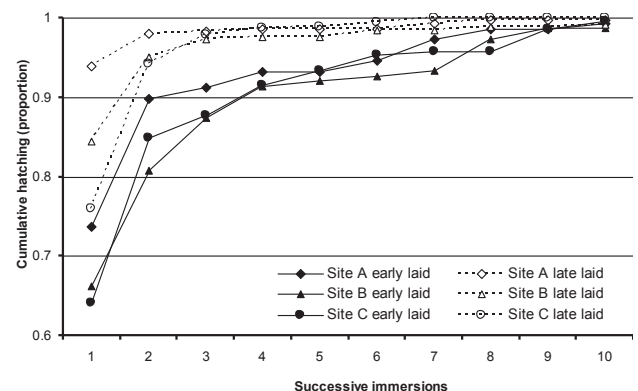

Figure 5. Cumulative proportion of hatched eggs of *Aedes aegypti* after ten successive immersions for each age class by site combination.
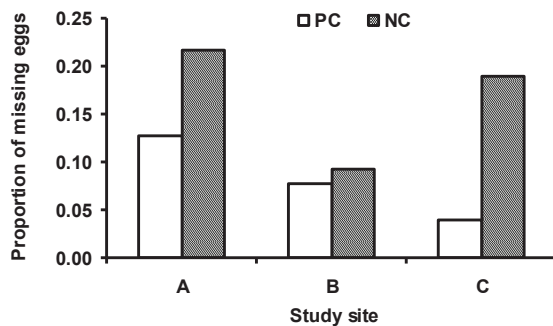
Figure 6. Mean proportions of missing eggs of *Aedes aegypti* under two experimental conditions (NC: natural conditions, PC: protected conditions) at three sites (A, B, C).

East Africa, the mortality of eggs after three months was found to be 65% (Trpis 1972), while in Queensland, Australia, the mortality of eggs after the same period reached 86% (Russell et al. 2001).

The most likely explanation for the lower mortalities obtained in our study is that climatic and/or environmental conditions are significantly more favorable for *Ae. aegypti* eggs at the study area during the cold season than those observed at tropical regions during the dry season. The cold season in Buenos Aires city the year of our study was characterized by relatively high humidity (>70%) compared to that in other studies (30%), and lower temperatures. Laboratory studies have demonstrated that the survival of *Ae. aegypti* eggs is negatively affected by low environmental humidity (Juliano et al. 2002). On the other hand, a laboratory experiment under saturated humidity conditions showed a slight decline in the survival of eggs at 10° C as compared to eggs kept at 16° C (Meola 1964), indicating that cold season temperatures in Buenos Aires are almost optimal for the survival of *Aedes aegypti* eggs.

The results of the present study show a heterogeneous mortality of *Ae. aegypti* eggs among sites in Buenos Aires. Egg mortality was higher at site A, where lower oviposition activity occurred during the warmer months. These results support the hypothesis that the lower and delayed oviposition activity in some areas near the Río de la Plata River could be a consequence of a reduced egg bank at the end of the cold season, with fewer surviving individuals to start the population increase during the next favorable season. Since only slight differences in temperature and humidity were recorded between sites, there are probably other factors involved in the mortality differences.

The differences in hatching behavior related to the exposure site and egg age provided results that encourage further studies in this direction. The differences in hatching response between the eggs exposed at the different sites were consistent with differences in mortality. Site A with higher mortality showed a higher hatching proportion during the first immersion, whereas site C showed the opposite. One possible explanation for such results would be that eggs subjected to a higher mortality risk will hatch as soon as possible in order to avoid or reduce the risk of dying at the egg stage. Observations in this direction have been made

for different taxonomic groups such as some lizard, fish, spider, and amphibian species (Werner and Gilliam 1984, Gomez-Mestre and Warkentin 2007). Whether or not the positive relationship of both phenomena (egg mortality and hatching rates) is related to a third one (i.e., depth of diapause as defined by Gillett (1955) deserves further research. Studies on various other *Aedes (Stegomyia)* species have shown higher survival of diapause eggs than of non-diapause eggs (Sota and Mogi 1992), which might support this hypothesis.

The negative association of hatching with age of the eggs is consistent with early qualitative observations made by different authors (reviewed by Christophers 1960) who have suggested a delayed hatching in eggs stored for prolonged periods. Such behavior could be related to the water loss experienced, even when stored in saturated environments (Meola 1964). Nevertheless, opposite results were obtained for *Ae. albopictus*, which showed higher hatching rates for older eggs (Vitek and Livdahl 2006). Vitek and Livdahl (2006) also related these higher hatching rates of older eggs to a response to minimize the risk of death with increasing age. Since *Ae. aegypti* eggs exhibit higher survival rates than *Ae. albopictus* eggs (Juliano et al. 2002), older individuals of the former species may not necessarily be obliged to hatch at the first stimulus.

An unexpected result of the experiment was the loss of eggs in both treatments of protected and natural conditions. This suggests the possibility of a spontaneous detachment of the eggs from the substrate (ranging in values from 3% through 13%) during the cold months. Unfortunately, the experimental pots were not inspected to recover eggs that could have fallen to the bottom, and we do not know whether those eggs were living or dead. Whether the detached eggs finally die or are able to hatch without rainfall events when falling into the water requires further research. On the other hand, the number of missing eggs was consistently higher in the treatment with natural conditions than in that with protected conditions. These differences could be at least partially attributed to the action of predators, although no direct observations were made. Results in this direction were obtained in studies of other regions (i.e., Russell et al. 2001, Focks et al. 1993), where predator activity substantially increased the natural mortality of eggs of *Ae. aegypti*. Since ants (Lee et al. 1994), coleopterans (Yang 2006), cockroaches (Russell et al. 2001), and pillbugs (Focks et al. 1993) have been reported to prey on eggs of *Ae. aegypti* or related species, they constitute the first candidates to be evaluated in laboratory experiments.

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