Resistance to freezing temperatures in *Aedes (Ochlerotatus) albifasciatus* (Macquart) eggs (Diptera: Culicidae) from two different climatic regions of Argentina

M. J. Garzón[1,2✉], O. Jensen[3], and N. Schweigmann[1,2]

[1]*Grupo de Estudio de Mosquitos, Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. Ciudad Autónoma de Buenos Aires, Argentina, maxigarzon@ege.fcen.uba.ar*
[2]*CONICET, Departamento de Investigación en Salud, Secretaria de Salud, Chubut, Argentina*
[3]*Departamento de Investigación en Salud, Secretaria de Salud, Chubut, Argentina*

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ABSTRACT: *Aedes* (*Ochlerotatus*) *albifasciatus* (Macquart) has the capacity to proliferate in different kinds of climates within its distribution range in South America. With the aim of studying local thermal adaptations of eggs, we exposed egg stocks from two climatically different localities: temperate humid pampa (Buenos Aires) and cold arid Patagonian (Sarmiento), to freezing conditions and then evaluated the effect on some features at this level. First, we thermally described the substrate where this species lays its eggs in the arid region. A typical thermal condition during winter was 10 h at -12° C. Second, we evaluated the effect of freezing on primary hatching (vs total hatching) and embryo survival. We also compared the proportion of embryonated eggs from both populations. The proportions of embryonated eggs were not different between localities, with averages of 78% and 83% in Sarmiento and Buenos Aires, respectively. Survival was equally successful after freezing in the two localities with an average range between 94-99%. Whether or not the eggs from Buenos Aires and Sarmiento were under freezing conditions, hatching was more than 98% after the first flooding. The results suggest that eggs of *Ae. albifasciatus* from Sarmiento and Buenos Aires have the same ability to survive at extreme temperatures (<0° C), showing a regional thermal adaptation rather than a local one. ***Journal of Vector Ecology*** **38 (2): 339-344. 2013.**

Keyword Index: Mosquitoes, embryonic survival, egg hatching, thermal adaptation, winter temperature.

INTRODUCTION

Aedes (Ochlerotatus) albifasciatus (Macquart) is the southernmost floodwater mosquito of the American continent. The larval habitats of this species are temporary puddles, and females lay eggs on the muddy soil surrounding the puddles. The habitats of this species are present in different climatic regions of its distribution area and are situated on all types of grass fields with periods of flooding. In Argentina, *Ae. albifasciatus* has been detected in both humid temperate and cold arid areas, such as Buenos Aires city and Patagonia, respectively, and even in extreme microenvironments, such as the high altitudes of the Andean valleys (Duret 1954) and the salt ponds of Santiago del Estero (Bachmann and Casal 1962).

Sarmiento Valley, located in Chubut Province within the Patagonian steppe (Cabrera 1971), is characterized by hard winters. The mean air temperature in this area is 0.2° C, while the absolute minimum reaches -33° C (series 1931-1960) (Elissalde et al. 1998). In contrast in the temperate region where Buenos Aires is situated, winter air temperatures are higher, with an average of 11.5° C and a rare absolute minimum of -5° C in 1918 (Servicio Meteorológico Nacional 2013). In temperate regions, immature and adult stages have been observed throughout the year and are associated with rainfall patterns (Almiron and Brewer 1994, Fontanarrosa et al. 2000, Fava et al. 2001), whereas in the arid Patagonian region, these stages occur only during the summer period and are associated with artificial flooding (Burroni et al. 2007).

Therefore, if *Ae. albifasciatus* is observed annually in Sarmiento Valley, it would be interesting to know its origin and to find out whether its population is endemic or if adults come from surrounding areas due to wind drift effects (Bejaran et al. 2008) or active dispersion from nearby breeding sites (Gleiser and Gorla 1997). If populations are endemic, then eggs would overwinter and be able to survive extremely low temperatures during the Patagonian winter. This would be in agreement with that found for the Arctic Aedines (Strathdee and Bale 1998) that adopt either freeze tolerance or freeze avoidance as a strategy to survive the cold (Bale 2002). Otherwise, the eggs could be protected from extremely cold temperatures in a microclimate generated by decomposition of organic matter or litter (Campos and Sy 2006). We could ask if the substrate where the eggs lie gets cooled below the freezing point. If so, the question that emerges is whether the eggs are able to survive freezing temperatures.

In temperate regions, egg hatching of *Ae. albifasciatus* in winter has been observed when the air temperature was -0.5 °C (Fava et al. 2001). However, egg diapause seems to be the general rule for winter survival in mosquitoes. The low temperatures and decreased photoperiod before winter triggers this physiological process as with *Ae. albopictus* (Focks et al. 1994). Campos and Sy (2006) found that diapause decreases when thermal conditions in the field or in the laboratory become favorable and it can be reflected in the hatching rate of *Ae. albifasciatus*. Campos and Sy (2006) also suggested that a litter layer creates a microclimate where the differences between maximum and minimum temperatures are less disproportional and may thus be favorable to the acclimatization of the eggs when air temperatures are extremely cold. However, there are no studies about egg freeze tolerance for this region. Eggs surviving freezing conditions would indicate the existence of cold adaptation. However, these extreme conditions are rare in Buenos Aires where hoar frost is most frequent. Otherwise, eggs dying under freezing conditions suggest that in nature they are placed in locations that provide shelter against extreme cold or where temperatures are less extreme (Campos and Sy 2006). The latter strategy has been observed in arthropods from artic regions (Danks et al. 1994).

Our second question is whether the eggs from Buenos Aires are able to survive freezing temperatures. Differential survival under freezing conditions could indicate local adaptations to regional thermal conditions for this population parameter. It has been found that regional thermal conditions induce local adaptations for some characters, such as survival of *Aedes albopictus* eggs in Indiana, U.S.A. (Hawley et al. 1989). Studies on the pitcher-plant mosquito, *Wyeomyia smithii,* from the temperate region of the U.S.A have shown that survivorship and fertility decline with increasing latitude. The decline in summer heat and increase in the duration and intensity of winter cold as one moves north also impose a latitudinal gradient in the length of the favorable growing season. (Bradshaw et al. 2000).

Another example occurs in insects of the order Thysanoptera from England, where local adaptation has been observed regarding the relation between degree days and minimum temperature for development (Stacey and Fellowes 2002). No differences in survival percentage between *Ae. albifasciatus* populations from Patagonia and those from Buenos Aires indicate that there is a minimum level of gene flow that preserves that character (cold hardiness), as indicated for central Argentina in trials with allozymes (De Souza et al. 1996).

The aim of this study was to evaluate the survival and hatching of eggs of *Ae. albifasciatus* populations from the cold-arid Patagonian region and from the temperate humid pampa, exposed experimentally to one event of subzero thermal conditions recreating the Patagonian winter. We describe the thermal dynamics of the soil of a temporary puddle where this species deposits eggs in the Patagonian region.

MATERIALS AND METHODS

Winter thermal characteristics

Daily absolute minimum air temperatures were plotted from May to September, 2011 for the two regions studied. Buenos Aires, temperate humid, temperature data sets were obtained from the Servicio Meteorológico Nacional (SMN), whereas those of Sarmiento Valley (Chubut), cold arid, were obtained from Intituto Nacional de Tecnologia Agropecuaria (INTA) of Sarmiento (no data were available for the period between 26 July and 16 September).

Ground level temperatures were recorded with HOBO® thermal sensors to describe the thermal characteristics of the wet soil substrate where *Ae. albifasciatus* winter eggs could be deposited. The site chosen for the recording was a representative temporary puddle in a farm of Sarmiento (45°35'29.51"S, 69°2'20.28"W) associated with a flood irrigation system. Puddles have historically been producers of mosquitoes. Records were made every half hour between May to September, 2009. Daily absolute minimum temperatures were plotted and thermal patterns analyzed.

Egg stocks

For both populations, *Ae. albifasciatus* eggs were obtained from females captured in the field and conditioned in breeding boxes in the laboratory at ≈24° C and 14:10 light-dark photoperiod. Breeding boxes (30x30x30 cm) consisted of a wood bottom and floor, plastic side walls, and tulle roof and front, with a cloth sleeve. To maintain humidity above 80%, a wet absorbent cloth was placed on the roof and floor of the boxes, which was wetted whenever necessary. Females were fed daily on a blood source to induce oviposition. For oviposition, they were offered a wet towel as a substrate. Towels with eggs were removed from the box and placed inside a nylon bag and this whole set was placed inside another nylon bag that was sealed. This was then placed at room temperature (≈24° C) for seven to ten days to ensure full development of the embryo (Fava et al 2001). After this period, the egg stock was stored in darkness at 5° C (Campos et al. 2007) between two and six months to induce diapause, or at least to reduce metabolic activity, until used in the assays.

Assays

Egg stocks between two- and six-months-old after oviposition and stored in the cold were used for treatments. Preliminary analysis suggested no significant differences in age for hatching response, by the Fisher's exact test ($p>0.05$).

a) Freezing conditions (subzero temperature): Since it is known that a long storage period at cold temperatures and long acclimatization periods improve the hatching response (Campos et al. 2007), the eggs used in these experiments were stored at 5° C. For each treatment we used batches of eggs produced during the same period. When the batch size was low we performed more repetitions. Two or four stocks with 36-117 eggs stored at diapause were placed at -12° C for 10 h. This condition simulated a cold winter night in Patagonia (see results of winter thermal characteristics). Eggs were then placed again at 5° C for one week and then at laboratory temperature (24° C) for two weeks with a 14:10 (L:D) photoperiod to acclimatize and reverse the state of diapause (Campos and Sy 2006).

Eggs were flooded in distilled water with yeast solution for 12 h to stimulate hatching. After this time, hatched larvae were counted and unhatched eggs were extracted and stored at room temperature for one week and were flooded one more time (second flooding). Unhatched eggs were then bleached with 50% sodium hypochlorite solution (Campos and Sy 2006) to check the presence or absence of embryos.

b) Controls: The procedure was similar to that for freezing conditions, except that the eggs were not exposed to an extreme cold event (-12° C for 10 h) and were acclimated directly from cold storage (5° C) at room temperature.

Data analysis

Total egg data were initially used to calculate percentages of hatched eggs, embryonated eggs, and embryonated unhatched eggs. For the egg stock from each treatment, we analyzed primary hatching (Hatching 1st), calculated as the ratio between the number of eggs hatched after the first flooding and the total number of hatched eggs (after the first plus second floodings). The proportion of embryonated eggs was calculated as the ratio between the number of embryonated eggs (hatched and unhatched) and the total number of eggs (embryonated and non-embryonated). Finally, embryo survival was the the ratio between the number of hatched larvae for both the first and second flooding and the total number of embryonated eggs (hatched and unhatched).

The effect of freezing and the origin of the population on hatching and embryo survival were analyzed by a nonparametric analysis of variance (Kruskal Wallis), while the proportion of embryonated eggs was analyzed by analysis of variance ANOVA, data arcsine transformed previously (Zar 1996). The statistical software used was Infostat® (2008). The level of significance used for the statistical tests was 0.05. Each egg stock was treated as a replica.

RESULTS

Winter temperatures

In the Sarmiento Valley, the absolute minimum air temperatures ranged between -11.2 and 8.7° C, and thermal conditions below the freezing point were observed on 43% of the days (N = 100 days, no data for the period between 26 July and 16 September). On the other hand, the records obtained from SMN showed that in temperate and humid Buenos Aires, the values of the absolute minimum air temperatures ranged between 1.4 and 16° C, and there were no freezing temperatures (Figure 1).

The HOBO® thermal sensors showed that daily absolute minimum records at ground level in the puddle of Sarmiento Valley were between -12.4 and 11° C and 49% of the days recorded with freezing conditions (N = 145 days, no data for the period between 12 and 19 May) (Figure 2). The mean duration of a freezing event was approximately 10 h for the month of June, while the lowest range of absolute minimum temperatures was for the month of July (- 4.4 / -12.4° C) (Figure 2). These values (10 h/ -12° C) were used as a reference point for the subzero thermal conditions in Patagonia.

Hatched eggs, embryonated eggs, and embryo survival

The egg hatching percentage after the first flooding (N_{SAR} < 0° C = 201; N_{SAR} control = 310; N_{BsAs} <0° C = 231; N_{BsAs} control = 200) ranged from 66.8 to 84.6%. Hatching after the second flood did not exceed 0.5%. Furthermore, embryonated unhatched eggs were less than 6% and non-embryonated eggs ranged from 10 to 31.9% (Figure 3).

The proportion of embryonated eggs showed no significant differences between populations (ANOVA, p>0.05) and resulted in an average of 78% (arcsine average = 62.76) for eggs from Sarmiento Valley and 83% (arcsine average = 68.68) for eggs from Buenos Aires (Table 1). Both the freezing temperatures (<0° C) and the origin of the population did not significantly affect the proportion of hatched eggs (embryo survival) (Kruskal Wallis, p>0.05). The mean of each treatment ranged between 93.5 and 98.7% and no interaction was found between the factors analyzed (Table 1). For the total of hatching eggs, 98-100% hatched after the first flooding regardless of exposure to the freezing temperatures and population origin (Kruskal Wallis, p> 0.05) (Table 1).

DISCUSSION

We have shown that the eggs of *Aedes albifasciatus* have the ability to survive under freezing conditions and that this property is maintained even in populations where such conditions are rare. Thermal conditions at ground level in Sarmiento reach values below the freezing point, suggesting that eggs would naturally be exposed to extreme cold temperatures during the winter. Furthermore, regarding air temperature, we observed that days under freezing conditions were usually more frequent in Patagonia than in Buenos Aires.

The average time of exposure to freezing temperatures in the substrate frequently ranges from 25 to 42% of the day. In laboratory assays carried out with Agromyzidae (Diptera) at -10° C for 12 h without prior cold acclimation (5 or 10° C), 98% of pupae died (Chen and Kang 2004). For mosquito eggs, these freezing events in the substrate could imply death if they are not previously acclimated or in diapause. We considered that one period of up to six months at 5° C may have equalized the differences between the two climates. Given the climate scenario of each region and egg treatments

Table 1. Egg hatching succes in different temperature conditions.

	Proportion of embryonated eggs		Proportion of embryo survival	Hatching first time (%)	N_{stock}
Bs As <0ºC	52-99	(83)	88-99 (93.5)	100 (100)	114; 117
Bs As control	85-95		92-95 (93.5)	99-100 (99.5)	100; 100
Sarmiento <0ºC	83-88	(78)	97-100 (98.5)	99-100 (99.8)	36; 41; 42; 82
Sarmiento control	55-80		97-100 (98.7)	98-100 (99.3)	100; 100; 100

Range of embrionated eggs (embryonated / total eggs); embryo survival (hatching / embryonated) and hatching eggs after first flooding (hatching 1st / total hatching x100). Numbers in parentheses indicate the averages.

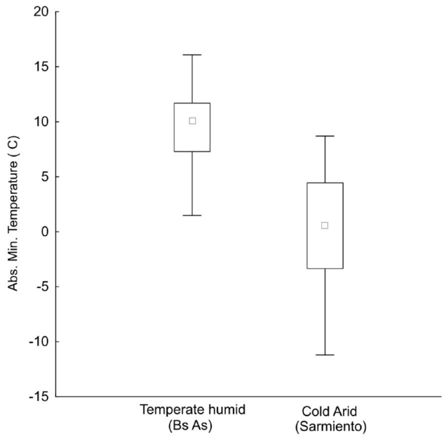

Figure 1. Fluctuations of the air absolute minimum temperature in the study areas.

of both populations, embryo survival suggests that this stage of the life cycle, independently of egg origin, is cold hardy and resistant to freezing. This has also been observed in insects from other cold regions (Bliss and Gill 1933, Bale 2002).

In Sarmiento, the cold hardiness of eggs plus the presence of immature and adult stages only during the summer season (when flood irrigation occurs), together with the lack of wind dispersion, would indicate that the first cohort of *Ae. albifasciatus* in the late spring comes from the hatching of local eggs, and therefore would be an endemic population. In Buenos Aires, resistance to freezing of the eggs is maintained, although not necessary.

Survival at freezing temperatures has been observed for *Aedes* species inhabiting Arctic regions and suggests that the strategies to prevent freezing mortality are either freeze tolerance or freeze avoidance (Danks et al. 1994, Strathdee and Bale 1998, Bale 2002). We have suggested freeze tolerance for *Aedes albifasciatus* because it is more cost-effective, stable, and common among insects (Strathdee and Bale 1998). In general, the minimum temperatures in Buenos Aires are less extreme than those in Patagonia, with some exceptions. However, regional thermal conditions have not caused a selective pressure on the character of resistance to freezing. Possible explanations for the latter could be: I) that there is a minimum gene flow between the two populations, II) that geographical isolation has not caused local thermal adaptation, and III) that there is a strong selection for character conservation and its genetic architecture (Gandon et al. 1998, Kawecki and Ebert 2004).

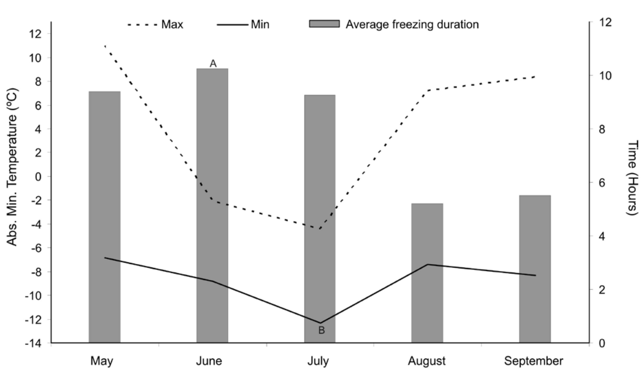

Figure 2. Monthly range of absolute minimum temperature at ground level during the winter period (2009) in a temporary puddle in Sarmiento, Chubut (lowest minimum: continuous line; highest minimum: dotted line). Monthly average freezing duration (bar). A and B indicate the values of time and temperature, respectively, chosen to represent a thermal condition of winter from an arid region.

Ae. albifasciatus eggs showed no evidence of an adaptation to thermal local conditions despite the climatic differences between these regions of Argentina. However it has been observed in the eggs of *Aedes albopictus,* which is an invasive species in the United States (Hawley et al. 1989). The exposure time and temperature may have been insufficient to see effects on egg survival from the cold arid region but not for the eggs of the humid temperate region. Furthermore, exposure time to similar temperatures have proved lethal to other *Aedes* species, as observed for *Ae. albopictus* of Europe (Thomas et al. 2012).

In organisms that resist temperatures below 0° C, dehydration occurs during the freezing process (Storey and Storey 1996). Therefore, it has been suggested that the adaptation to this latter phenomenon was evolutionarily secondary (Danks et al. 1994). In Buenos Aires, since temperatures below 0° C are rare, moisture would be the most influential environmental variable on egg survival, while the difficulties for Sarmiento egg populations would be freezing temperatures combined with a lack of moisture. In both cases, there is an adaptation of *Ae. albifasciatus* to adverse weather conditions that allows it to maintain and proliferate as an endemic population. In the case of Buenos Aires, freeze tolerance could become a useful character for extreme cold weather conditions.

In Patagonia and sometimes in the temperate region of Buenos Aires, winter temperatures become extremely low, so we might think that low temperatures are necessary to improve the hatching rate of *Ae. albifasciatus*, similar to some seeds from cold environments that require a period of low temperatures for germination (cold stratification) (Chien et

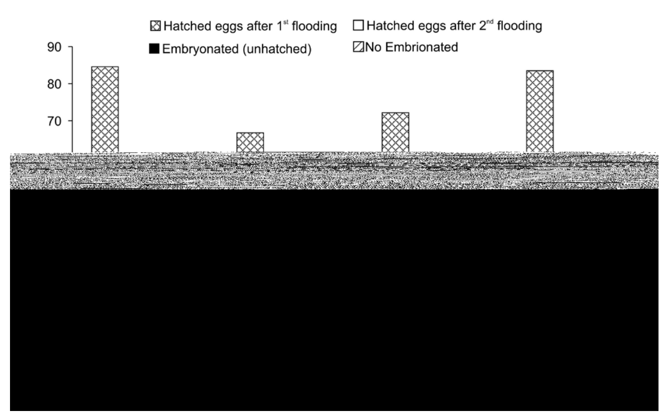

Figure 3. Hatching situation of eggs collected from two different areas (Bs As: Temperate humid , Buenos Aires; Sar: Arid cold, Sarmiento).

al. 1998, Walck et al. 2000, Penfield et al. 2005). However, the hatching percentage was high regardless of exposure to freezing temperatures, suggesting that an extreme thermal event does not influence egg hatching in either population. The highest percentage of egg hatching occurred after the first flooding, without previous freezing, as earlier noted by Campos (2008).

In La Plata (Buenos Aires province), Campos and Sy (2006) observed that 80.4% of 4,222 eggs hatched after the third flooding and 81.5% of the remaining 800 unhatched eggs were embryonated. The rest of them, aproximately 5%, had no signs of an embryo and were interpreted as unfertilized. In our study, proportions of embryonated eggs (78-83%) indicated a high percentage of larval embryogenesis. Moreover, the 93 to 98% rate of embryonic survival that we observed suggests a high larval birth rate independent of the freezing conditions and the latitude. This disagrees with that found for locusts and flies (Jing and Kang 2003, Chen and Kang 2004).

The ability of *Ae. albifasciatus* to survive one freezing event (and probably more than one in the Patagonian winters) explains its wide territorial distribution in the world's southernmost region. Naturally, abundance levels depend on the weather conditions (precipitation) in the Pampa region and on snowmelt flooding in Patagonia. However, in the southern valleys, farm activity promotes the proliferation of this species, because the irrigation system used for farming generates many breeding sites. Knowledge about the biology of *Ae. albifasciatus*, especially in the Patagonian valleys, is important for future environmental planning that can control and mitigate the inconvenience caused by the high abundance of these mosquitoes every summer. Future research could be directed toward other features of the egg biology, such as the minimum lethal temperatures, egg freeze tolerance without acclimatization, physiology of the strategy used for cold hardiness, and phylogeographic genetic studies in the populations studied.

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