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## Effects of continuous drought and immersion on hatchability of *Ochlerotatus albifasciatus* (Diptera: Culicidae) eggs stored at low temperature

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### Efecto de la sequía continua y la inmersión sobre la respuesta de eclosión de los huevos de *Ochlerotatus albifasciatus* (Diptera: Culicidae) almacenados a baja temperatura

■ **RESUMEN.** Se estudió el rol de los factores ambientales sobre la eclosión de los huevos de *O. albifasciatus* (Macquart), simulando condiciones de invierno. La hipótesis planteada fue que la respuesta de eclosión de los huevos depende de: (1) tiempo de permanencia a baja temperatura, (2) condiciones de almacenamiento, y (3) tiempo de aclimatación. Los huevos fueron almacenados a 5° C en condiciones de sequía y sumergidos durante 7, 21, 35, 90 y 146 días; luego fueron aclimatados a 22° C durante 24, 72, y 168 horas y posteriormente inundados para inducir la eclosión. El porcentaje de huevos eclosionados se analizó con un Modelo Lineal Generalizado con distribución binomial. Los resultados mostraron que: (1) largos períodos de almacenamiento en frío y largos tiempos de aclimatación mejoran la respuesta de eclosión, (2) la condición de almacenamiento tiene un efecto moderado en la eclosión, siendo más marcada en los huevos almacenados en seco; (3) tiempos prolongados de aclimatación mejoran aún más las eclosiones de los huevos sumergidos, que los almacenados en seco; (4) largo tiempo en frío mejora la eclosión de los huevos almacenados en seco, que los sumergidos; (5) los huevos que permanecieron más tiempo en frío respondieron mejor cuando el tiempo de aclimatación fue prolongado.

**PALABRAS CLAVES.** Mosquitos de inundación. Diapausa. Dormancia. Huevo. Factores de eclosión.

■ **ABSTRACT.** The role of environmental factors on the hatching response of *O. albifasciatus* (Macquart) eggs is studied by simulating winter conditions in the laboratory. The hypothesis tested was that hatching response is affected by: (1) time that eggs remain at low temperature, (2) storage conditions, and (3) acclimatization time. Eggs were stored at 5°C in drought and immersion conditions during 7, 21, 35, 90 and 146 days, immediately after, they were acclimatized at 22°C during 24, 72, and 168 hours, and then eggs were flooded to induce hatching. Percent of hatched eggs was analyzed with a Generalized Lineal Model with a binomial distribution. Results show that: (1) a long storage period at cold temperature and long acclimatization periods improve the hatching response; (2) storage conditions have a moderate effect on hatching, being more evident in eggs stored dry; (3) a long time of acclimatization favors the hatching response in submerged eggs more than in eggs stored dry; (4) long time at cold favors the

hatching response in eggs stored dry more than in submerged eggs; (5) the hatching response of eggs that remain for a long time at low temperature is higher when the time of acclimatization is long.

**KEY WORDS.** Floodwater mosquito. Diapause. Dormancy. Egg. Hatching factors.

## INTRODUCTION

*Ochlerotatus albifasciatus* (Macquart) is a multivoltine floodwater mosquito distributed from Brazil to southern Argentina (Forattini, 1965), and incriminated as vector of the western equine encephalitis virus in Argentina (Avilés *et al.*, 1992). Although this mosquito is present year-round in Buenos Aires Province (temperate Argentina), during winter and summer hatching numbers are lower than during spring and fall, showing a conspicuous seasonal pattern of abundance (Maciá *et al.*, 1995). Seasonality has been attributed to rainfall thresholds that vary throughout the year, and to air temperature, which affects evaporation rate and water permanence in pools (Fontanarrosa *et al.*, 2000).

The most frequent larval sites of *Ochlerotatus albifasciatus* in temperate Argentina are shallow, ephemeral and intermittent puddles, driven mainly by rainfall (e.g.: Campos *et al.*, 2004) or stream overflow (Maciá *et al.*, 1995). Larvae have also been collected from a wide variety of sites including temporary (Fischer *et al.*, 2000; Fontanarrosa *et al.*, 2000) and permanent pools with conspicuous variation in the water level (Micieli & García 1999), and with or without aquatic vegetation (García *et al.*, 1994). In this diversity of larval sites, eggs of *O. albifasciatus* are exposed to a variety of environmental situations from laying to hatching: whereas in ephemeral puddles eggs are exposed to short floodings and long drought periods, in temporary and permanent pools eggs remain submerged during longer periods.

Although favorable environmental conditions are required for hatching, physiologic factors determine both when the mosquito eggs enter dormancy and when they hatch. It has been observed that the

hatching mechanism and the survival of dormant eggs depend on egg maturity, dormancy degree (Horsfall & Trpis, 1967), hormonal control (Mitchell, 1988), resistance to unfavorable environment (Horsfall, 1972), and response to physical and chemical stimuli (e.g.: Horsfall *et al.*, 1958; Judson, 1960; Judson *et al.*, 1965).

The aim of this study is to evaluate the hatching capability of *Ochlerotatus albifasciatus* eggs laid at the end of the fall and exposed to cold temperature during the winter. The hypothesis tested is that the hatching response of *O. albifasciatus* occurs as a function of: (1) the time that eggs remain at low temperature before acclimatization, (2) the humidity conditions during that period (eggs immersed in water or resting over the soil), and (3) the acclimatization time at warm temperature before inundation of the larval site. From the result of a previous study (Campos & Sy 2006) and field observations (R. E. Campos & V. E. Sy, unpubl. obs.) on the variation of the hatching response of *O. albifasciatus* eggs, it was predicted that the hatching response of *O. albifasciatus* eggs exposed to cold temperature would: (1) increase as the storage time before acclimatization becomes longer, (2) be greater in eggs exposed to drought (resting over the soil) than in those immersed in water, and (3) increase as the acclimatization period becomes longer.

## MATERIAL AND METHODS

**Egg collection.** *Ochlerotatus albifasciatus* eggs were obtained from 260 females collected at Pereyra Iraola Provincial Park (34° 51'S; 58° 08'W), Buenos Aires Province, Argentina, from May 6th to 10th, 2004. Wild-caught females were kept in a cage at room temperature (≈22°C) and 12:12 hours light-

dark photoperiod. From May 10th to 14th, mosquitoes were blood fed on a guinea pig and stored individually in tubes (6 cm in height x 3 cm in diameter) with a moist piece of towel paper resting on a cotton pad at the bottom of the tube. To decrease the likelihood of microbial contamination, each female was transferred to a clean laying tube on the 4th day after feeding, once defecation had ceased (Gillett *et al.*, 1977). Egg batches laid before female transference were eliminated.

Eggs used in this study were laid from May 21st to 26th and kept in the tubes for over nine days to ensure complete maturity of embryos (Fava *et al.*, 2001). Because it was desirable to obtain eggs of similar age, two groups of batches were separated, which later were assigned to each storage condition. For each group egg-layings were pooled and then randomly assigned to a treatment to minimize the effects of factors affecting female fertility (e. g. physiological age, unmated females, etc) on the results of the experiment.

#### **Experimental design and procedure.**

Experiments were set up following a factorial design with three factors: storage conditions (drought and immersion), period of egg storage at low temperature (7, 21, 35, 90, 146 days) and period of egg acclimatization at 22°C (24, 72, 168 hours). These time periods, were chosen in order to simulate some of the natural conditions that may affect *Ochlerotatus albifasciatus* eggs. For storage, eggs were kept in a standard refrigerator at an average of 5.14°C (SD + 1.26, n = 217) without photophase. For acclimatization eggs were transferred to a room at 22°C and 12:12 hours light-dark photoperiod.

In the continuous drought treatments, groups of 25 egg were transferred to individual Petri dishes (50 x 9 mm style with tight lid) provided with a moist cotton pad and towel-paper. For each variable combination, 35 replicates were used. On the other hand, in the continuous immersion treatments, groups of 20 eggs were transferred to individual vials containing cold dechlorinated tap water. When storage time was reached, eggs were transferred to Petri dishes provided with moist cotton pad and towel paper, and kept for acclimatization. For

each variable combination, 25 replicates were used. The number of replicates and eggs per replicate differed between storage conditions, because eggs used for each treatment were laid at different times.

Immediately after acclimatization time, each group of eggs was transferred into Petri dishes (10 cm in diameter, 1.5 cm in height) containing 40 ml of dechlorinated tap water, and 10 mg of yeast to stimulate hatching. Temperature and photophase conditions were the same as during acclimatization. After 24 hours, hatched larvae were counted and chorions of unhatched eggs were bleached with 50% commercial sodium hypochlorite solution to check viability. Embryos that were creamy white with eyespots, hatching spine, and distinct abdominal segmentation, were assumed viable. Yellow-brown or red-brown embryos were assumed nonviable (McHaffey & Harwood, 1970) and were excluded from the analysis.

**Data analysis.** To model the relation between variables, a generalized lineal model (McCullagh & Nelder, 1989) was used, assuming a binomial distribution of the response variable (proportion of hatched eggs) and a logit link function. The numerical predictor variables were days of storage at low temperature and hours of acclimatization, whereas the categorical predictor where drought exposure and immersion condition. Analyses was performed using the glm function from R statistical software version 2.3.1 following Venables & Ripley (2002).

## **RESULTS**

As predicted, results of the test show that the three studied factors and the interaction between them affect the hatching response of *Ochlerotatus albifasciatus* eggs (Table I).

From the analysis of the effect of the acclimatization time it was observed that hatching response increases as period of acclimatization was longer. This increase was more evident in treatments stored at continuous immersion than in those under drought (Table I and Fig. 1).

**Table I.** Generalized linear model with binomial distribution (link logit function) of the effects of storage period, storage condition and acclimatization period of *Ochlerotatus albifasciatus* eggs exposed to 5°C.

Source	Parameter estimated	S. E.	z	Pr (> z )
Intercept	-0.701	0.063	-11.08	<2E-16
Storage period (SP)	0.076	0.001	10.16	<2E-16
Storage condition (SC)	0.237	0.074	3.20	0.001
Acclimatization period (AP)	0.014	0.001	20.96	<2E-16
SP x SC	0.003	0.001	3.58	0.000
SC x AP	-0.008	0.001	-11.88	<2E-16
SP x AP	3.0E-5	0.000	4.78	1.8E-06

As regards storage period, hatching response increased with increasing storage period, this trend being more evident on eggs stored dry than on those stored immersed. In addition, hatching response was higher on eggs exposed to a longer acclimatization time (Table I). From the interaction between these factors, three behaviors could be distinguished: (1) Before 35 days of storage, there is no defined pattern of hatching. (2) From 21 to 35 days of storage, a decrease in hatching response is observed in the short acclimatization time treatments. (3) From 35 to 146 days, there was a progressive increase in hatching response as storage period become longer (Fig. 1).

The storage conditions showed a moderate effect on hatching response. However, eggs under drought conditions showed a slightly higher hatching response than those immersed (Table I). This is a result of the differences found on mean percent hatching in the 24 h of acclimatization treatments (Fig. 1).

## DISCUSSION

In this study, hatching response in both groups of eggs, stored under drought and stored immersed, was strongly influenced by the interaction between storage and acclimatization periods. This is evident from the high variation in the hatching response during the first three months of storage. When eggs were stored for more than five months, hatching response became higher and tended to be independent of acclimatization time under both conditions.

The different behaviors on the hatching response observed in the present study as a result of the interaction between the acclimatization and storage periods can be explained by differences in diapause intensity (Danks, 1987). The heterogeneous hatching response observed in the 7 and 21 days treatments could be ascribed to the variable number of eggs entering diapause as a result of a short cold period. When the storage at low temperature was longer (35 days), more eggs entered diapause and the intensity of it was stronger. As a result, there was a decrease in the hatching response with short acclimatization times, but longer acclimatization allowed eggs to finish diapause and hatch. After three months of dormancy hatching response was high, even when acclimatization time was short. It is possible that the eggs were in post diapause quiescence. This interpretation is supported by the results of a previous study (Campos & Sy, 2006) which shows a different hatching response as a result of the diapause intensity.

Although the hatching pattern was similar for eggs stored immersed and under drought, some variations were found in different acclimatization periods. Eggs stored immersed showed a lower percentage of hatching than those stored in drought conditions when acclimatization period was short (24 h). This variation might be explained by need of eggs to be exposed to drought before hatching, which may have been insufficiently long for eggs under the immersion treatment. On the other hand, as acclimatization period increased, hatching response was higher in eggs stored under immersion than in those stored under drought

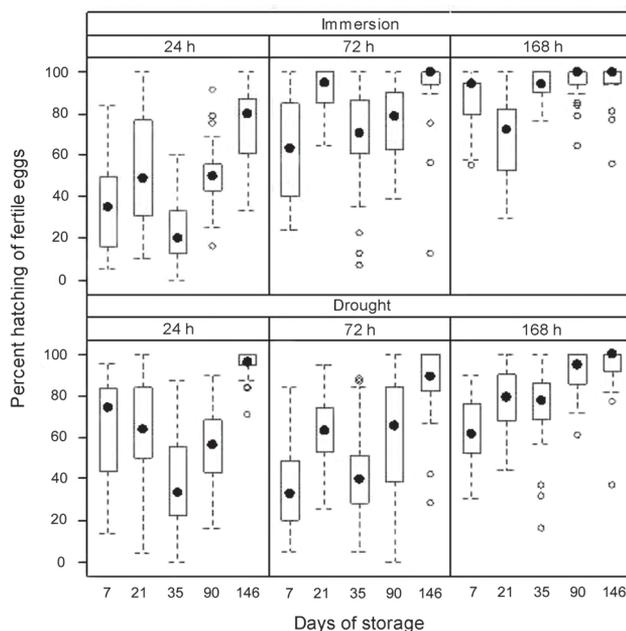


Fig. 1. Percent hatching response from *Ochlerotatus albifasciatus* eggs stored at 5°C in continuous immersion and drought, and flooded after acclimatization at three different time periods. Solid circle: median; box: 25 - 75% percentiles; vertical bar: data range excluding outliers; open circle: outliers.

conditions. In this situation the higher egg hydration may have favored hatching.

We have observed that *Ochlerotatus albifasciatus* eggs laid in pools that remain dry during winter are exposed to a progressive increment in temperature when spring starts (Campos & Sy, 2006). These conditions prepare the eggs to produce massive hatchings with the first rains of the spring. Results from the present study support this assumption, since both a long storage period and a period of acclimatization favor hatching response.

Taking into account these results and those from a previous study showing that eggs flooded at low temperatures after being stored at winter temperatures do not hatch (Campos & Sy, 2006), we suggest that *Ochlerotatus albifasciatus* eggs are able to hatch during winter only if they have finished the diapause period and have at least experimented, a short drought period at warm temperature. We think it is unlikely that egg could hatch at low temperatures (3.2°C, -0.5°C), as was suggested by García & Micieli (2000) and Fava *et al.* (2001), if they have not been previously acclimatized. To solve this issue, more specific laboratory experiments are

necessary, because none of these studies refers to the minimum temperatures necessary for eggs to hatch without an acclimatization period.

We conclude that the high and heterogeneous hatchability of *Ochlerotatus albifasciatus* eggs under drought and immersion allow larvae to inhabit a wide spectrum of larval sites. Thus, the use of permanent or semi permanent pools as oviposition sites confers low risk of mortality, even if the pools stay inundated for long periods. The use of these pools also is possible thanks to the capacity of eggs to survive for long periods at low temperature, and to the short acclimatization period required to hatch. These characteristics associated with erratic hatching (Campos & Sy, 2006) assure the survival of *O. albifasciatus* breeding in puddles exposed to intermittent floodings in the temperate area of Argentina.

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